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EFEITO NEUROPROTETOR DO EXERCÍCIO DE NATAÇÃO EM UM MODELO DE DOENÇA DE ALZHEIMER INDUZIDO PELO PEPTÍDEO BETA-AMILÓIDE (1-42) EM CAMUNDONGOS: RELEVÂNCIA DE CITOCINAS INFLAMATÓRIAS E ATIVAÇÃO DA INDOLEAMINA-2,3-DIOXIGENASE

TESE DE DOUTORADO

Leandro Cattelan Souza

Uruguaiana, RS, Brasil.
2017

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por

Leandro Cattelan Souza

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Elaborada por

Leandro Cattelan Souza

Como requisito parcial para obtenção do grau de **Doutor em Bioquímica**

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2017

DEDICO

Aos meus pais, Luis Carlos e Edna, que me deram
a vida e me ensinaram a vivê-la com dignidade.
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obscuros com afeto, amor e dedicação incondicionais,
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RESUMO

Tese de Doutorado

Programa de Pós-Graduação em Bioquímica

Universidade Federal do Pampa

EFEITO NEUROPROTETOR DO EXERCÍCIO DE NATAÇÃO EM UM MODELO DE DOENÇA DE ALZHEIMER INDUZIDO PELO PEPTÍDEO BETA-AMILÓIDE (1-42) EM CAMUNDONGOS: RELEVÂNCIA DE CITOCINAS INFLAMATÓRIAS E ATIVAÇÃO DA INDOLEAMINA-2,3-DIOXIGENASE

Autor: Leandro Cattelan Souza

Orientador: Cristiano Ricardo Jesse

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A doença de Alzheimer (DA) é uma desordem neurodegenerativa, sendo considerada a principal causa de demência. O envelhecimento é o principal fator de risco para a DA, cuja estimativa anual de incidência e prevalência aumenta dramaticamente com a idade. A principal manifestação clínica da DA é o progressivo declínio das funções cognitivas, com a perda da memória sendo um dos sintomas iniciais da doença. No entanto, os pacientes de DA frequentemente apresentam sintomas comportamentais e psicológicos da demência (BPSD), também conhecidos como sintomas neuropsiquiátricos da demência, e.g. depressão, ansiedade e psicose, piorando a inabilidade e o fardo da doença. Apesar de os exatos mecanismos não estarem totalmente entendidos, evidências emergentes indicam que a ativação da indoleamina-2,3-dioxigenase (IDO), a enzima limitante da via da quinurenina (KP), está envolvida na neurotoxicidade do peptídeo beta-amilóide $(A\beta)_{1-42}$ e na patogenia da DA. O exercício físico tem sido considerado uma intervenção efetiva na DA, atenuando ou limitando sua progressão. Não obstante, os mecanismos neurobiológicos subjacentes ao efeito neuroprotetor do exercício ainda não estão totalmente elucidados. Em vista disso, no presente estudo nós buscamos investigar três questões principais. Primeiro, os efeitos de uma infusão intracerebroventricular (i.c.v.) do peptídeo $A\beta_{1-42}$ (400 pmol/animal; 3 µl/sítio) sobre a regulação de biomarcadores da KP (atividade da IDO e níveis de triptofano (TRP) e quinurenina (KYN)) e o impacto deste peptídeo sobre os níveis de fatores neurotróficos foram investigados como potenciais mecanismos de ligação entre a neuroinflamação e os distúrbios cognitivos e emocionais em camundongos. Segundo, nós examinamos se o envelhecimento está associado a uma maior sensibilidade às alterações comportamentais e neuroquímicas provocadas pela infusão de $A\beta_{1-42}$, e se a KP está envolvida nesses efeitos. Por fim, nós investigamos o efeito protetor de um treinamento de natação (ST) de 8 semanas sobre as funções cognitivas e não-cognitivas e seu papel em modular os biomarcadores da KP após a administração do peptídeo $A\beta_{1-42}$ dentro do cérebro dos camundongos. Os nossos resultados mostraram níveis elevados de citocinas pró-inflamatórias nos camundongos tratados com $A\beta_{1-42}$, os quais levaram a um

aumento da atividade da IDO no córtex pré-frontal (PFC) e hipocampo (HC). A ativação da IDO subsequentemente aumentou a produção de KYN e a razão KYN:TRP, assim como diminuiu os níveis de fatores neurotróficos no PFC e HC, contribuindo para os seguintes distúrbios comportamentais Aβ-associados: prejuízos na memória no teste de reconhecimento de objetos (ORT), comportamento tipo-depressivo e tipo-ansiedade no teste de suspensão de cauda (TST) e no labirinto em cruz elevado (EPMT), respectivamente. Além disso, nós confirmamos que os camundongos idosos, quando comparados aos animais jovens, apresentaram maior déficit cognitivo no ORT e maiores níveis de comportamento tipo-ansiedade no EPMT, após a administração de $A\beta_{1-42}$. Os camundongos idosos também responderam ao $A\beta_{1-42}$ com uma maior deficiência do fator neurotrófico derivado do cérebro, menores níveis de glutationa e menor capacidade antioxidante total, assim como responderam com uma maior atividade da IDO e maiores níveis de KYN e razão KYN:TRP no PFC e HC. Estes efeitos do peptídeo $A\beta_{1-42}$ estavam associados a um maior estado pró-inflamatório, como indicado pelos maiores níveis de interleucina-6 e menores níveis de interleucina-10 nestas estruturas cerebrais. Por outro lado, os nossos resultados demonstraram que o ST foi efetivo em prevenir a perda de memória no ORT e os comportamentos tipo-depressivo e tipo-ansiedade, no TST e EPMT, respectivamente. O ST aboliu a resposta neuroinflamatória e a deficiência neurotrófica no PFC e HC induzidos por $A\beta_{1-42}$. Ademais, o peptídeo $A\beta_{1-42}$ aumentou a atividade da IDO, os níveis de KYN e TRP e a razão KYN:TRP no PFC e HC – alterações que foram bloqueadas pelo ST. Portanto, o presente estudo forneceu 3 principais achados: (I) a ativação da IDO no cérebro desempenha um papel-chave na mediação dos distúrbios da memória e emocionais em um modelo experimental baseado na neuroinflamação Aβ-induzida; (II) o estado inflamatório associado ao envelhecimento e a infra-regulação de substâncias neuroprotetoras endógenas torna os camundongos idosos mais vulneráveis à perda de memória, aos sintomas de ansiedade e à desregulação da KP induzidos pelo peptídeo $A\beta_{1-42}$; e (III) o ST é eficaz em prevenir os déficits comportamentais e neurobiológicos induzidos pelo peptídeo $A\beta_{1-42}$ e estes efeitos neuroprotetores estão provavelmente envolvidos com a inibição da ativação da inflamação/IDO e com a supra-regulação de fatores neurotróficos no cérebro de camundongos. Em conclusão, nós sugerimos que o exercício físico pode ser usado como uma intervenção não-farmacológica para aliviar tanto os sintomas cognitivos quanto não-cognitivos associados ao envelhecimento e à DA.

Palavras-chave: Doença de Alzheimer, envelhecimento, neuroinflamação, indoleamina-2,3-dioxigenase, via da quinurenina, fatores neurotróficos, memória, depressão, ansiedade, exercício físico

ABSTRACT

Doctoral Thesis

Program of Post-Graduation in Biochemistry

Federal University of Pampa

NEUROPROTECTIVE EFFECT OF SWIMMING EXERCISE IN A MOUSE MODEL OF ALZHEIMER'S DISEASE INDUCED BY AMYLOID-BETA (1-42) PEPTIDE IN MICE: RELEVANT TO INFLAMMATORY CYTOKINES AND INDOLEAMINE-2,3-DYOXIGENASE ACTIVATION

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Advisor: Cristiano Ricardo Jesse

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Alzheimer´s disease (AD) is the most common neurodegenerative form of dementia. Aging is the most important risk factor for AD with the estimated annual incidence and prevalence rising dramatically with age. The primary presentation of AD is progressive cognitive decline, with memory loss being a relatively early sign of the disease. Nevertheless, AD patients also frequently exhibit behavioral and psychological symptoms of dementia (BPSD), also known as neuropsychiatric symptoms of dementia, including depression, anxiety and psychosis, worsening the disability and caregiver burden. Despite de exact mechanisms are not completely understood, emerging evidence indicates that the activation of indoleamine-2,3-dyoxigenase (IDO), a first and rate-limiting enzyme in the kynurenine (KYN) pathway, is involved in amyloid-beta ($A\beta_{1-42}$)-neurotoxicity and AD pathogenesis. Physical exercise has been considered an effective intervention in AD, attenuating or limiting their progression. Nevertheless, the neurobiological mechanisms underlying the neuroprotective effects of exercise have not yet been fully elucidated. In view of this, in the current study we sought to investigate three main issues. First, the effects of an intracerebroventricular (i.c.v.) injection of $A\beta_{1-42}$ peptide (400 pmol/mice; 3 µl/site) on the regulation of KP biomarkers (IDO activity, tryptophan (TRP) and kynurenine (KYN) levels) and the impact of $A\beta_{1-42}$ on neurotrophic factors levels were investigated as potential mechanisms linking neuroinflammation to cognitive/emotional disturbances in mice. Second, we examined whether aging is associated with a higher sensitivity to behavioural and neurochemical alterations elicited by $A\beta_{1-42}$ injection, and whether KYN pathway is involved in these effects. Ultimately, we investigated the protective effect of an 8-week swimming training (ST) exercise on cognitive and non-cognitive functions and its role in modulating biomarkers of KYN pathway post $A\beta_{1-42}$ administration into the mouse brain. Our results showed increased levels of proinflammatory cytokines in the $A\beta_{1-42}$-treated mice, which led to an increase in IDO activity in the prefrontal cortex (PFC) and the hippocampus (HC). The IDO activation subsequently increased KYN production and the KYN/TRP ratio and decreased the levels of neurotrophic factors in the PFC and HC, contributing for the following Aβ-associated behavioral disturbances: memory impairment in the object recognition test (ORT), depressive-like and anxiety-like behaviour in the tail suspension test (TST) and elevated plus-maze test (EPMT), respectively. In addition, we confirmed that aged mice, when compared to young mice, displayed higher cognitive deficit in the ORT and higher anxiety-like behaviour in

the EPMT after the $A\beta_{1-42}$ administration. Aged mice also responded to $A\beta_{1-42}$ with a higher deficiency of brain-derived neurotrophic factor, glutathione levels and total radical-trapping antioxidant capacity, a higher IDO activity and a higher KYN and KYN/tryptophan ratio in the PFC and HC. These effects of $A\beta_{1-42}$ were associated with a higher proinflammatory status, as reflected by elevated interleukin-6 levels and reduced interleukin-10 levels in these brain structures. On the other hand, our results demonstrated that ST was effective in preventing the memory impairment in the ORT and depressive/anxiety-like behaviour in the TST and EPMT, respectively. ST abrogated the neuroinflammatory response and neurotrophic deficiency in the PFC and HC induced by $A\beta_{1-42}$. Also, $A\beta_{1-42}$ increased IDO activity, KYN and TRP levels and KYN:TRP ratio in the PFC and HC – alterations that were blocked by ST. Therefore, the present study provides three main findings: (I) brain IDO activation plays a key role in mediating the memory and emotional disturbances in an experimental model based on Aβ-induced neuroinflammation; (II) the age-associated inflammatory signature and down-regulation of endogenous neuroprotectants in the brain render aged mice more vulnerable to $A\beta_{1-42}$-induced memory loss, anxiety symptoms and KYN pathway dysregulation; and (III) ST is effective in preventing behavioural and neurobiological deficits induced by $A\beta_{1-42}$, and these neuroprotective effects are likely to involve the inhibition of inflammation/IDO activation and up-regulation of neurotrophic factors in brain of mice. In conclusion, we suggest that physical exercise can be used as a non-pharmacological approach to alleviates both cognitive and non-cognitive symptoms associated with aging and AD.

Keywords: Alzheimer's disease, aging, neuroinflammation, indoleamine-2,3-dyoxigenase, kynurenine pathway, neurotrophic factors, memory, depression, anxiety, physical exercise

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ARTIGO

MANUSCRITO II

LISTA DE ABREVIATURAS

DA = doença de Alzheimer

HD = *Huntington's disease* = doença de Huntington

PK = *Parkinson's disease* = doença de Parkinson

APP = *amyloid precursor protein* = proteína precursora amilóide

I.c.v. = intracerebroventricular

Aβ = *amyloid-beta* = beta-amilóide

$A\beta_{1-40}$ = isoforma do peptídeo beta-amilóide com 40 aminoácidos

$A\beta_{1-42}$ = isoforma do peptídeo beta-amilóide com 42 aminoácidos

BACE 1 = beta secretase 1

NFT = *neurofibrilary tangles* = emaranhados neurofibrilares

ST = *swimming training* = treinamento de natação

ORT = *object recognition test* = teste de reconhecimento de objetos

OFT = *open-field test* = teste de campo aberto

TST = *tail suspension test* = teste de suspensão de cauda

EPMT = *elevated plus-maze test* = teste de labirinto em cruz elevado

BPSD = *behavioral and psychological symptoms of dementia* = sintomas comportamentais e psicológicos da demência

NPSD = *neuropsychiatric symptoms of dementia* = sintomas neuropsiquiátricos da demência

SNC = sistema nervoso central

BBB = *blood brain barrier* = barreira hematoencefálica

HC = hipocampo

PFC = *prefrontal cortex* = córtex pré-frontal

TNF-α = *tumor necrosis factor-alpha* = fator de necrose tumoral-alfa

IFN-γ = interferon-gama

IL-1β = interleucina 1-beta

IL-4 = interleucina 4

IL-6 = interleucina 6

IL-10 = interleucina 10

IL-1ra = *IL-1 receptor antagonista* = antagonista do receptor de interleucina-1

sTNF-α = *soluble tumor necrosis factor-α receptor* = receptor solúvel do fator de necrose tumoral-alfa

TLR = *toll-like receptor* = receptores tipo-toll

NFκB = *nuclear factor-kappaB* = fator nuclear kappa-B

VEGF = *vascular endothelial growth factor = fator de crescimento endotelial vascular*

IGF-1 = *insulin growth factor 1* = fator de crescimento semelhante à insulina tipo1

BDNF= *brain-derived neurotrophic factor* = fator neurotrófico derivado do cérebro

TrkB = *Tropomyosin receptor kinase B* = receptor de tropomiosina relacionado à quinase B

GDNF = *glial cell line-derived neurotrophic factor* = fator neurotrófico derivado de células gliais

NGF = *nerve growth factor* = fator de crescimento neural

NT3 = neurotrofina 3

MAPK = *mitogen-activated protein kinase* = proteína quinase ativada por mitógenos

ERK = *extracellular signal regulated kinase* = quinase regulada por sinal extracelular

PI3K = *phosphatidylinositol 3-kinase*= fosfatidilinositol 3-quinase

AKT = serina treonina quinase (proteína quinase B)

PLC γ = *phospholipase C gamma* = fosfolipase C gama

CAMPK = *calcium/calmodulin-stimulated protein kinase* = proteína quinase dependente de cálcio/calmodulina

CREB = *cAMP response element-binding protein* = *proteína* ligante do elemento de resposta ao AMPc

ROS = *reactive oxygen species* = espécies reativas de oxigênio

TRAP = *Total radical-trapping antioxidant potential* = potencial antioxidante total

GSH = glutationa

PGC-1α = *peroxisome proliferator-activated receptor gamma coactivator 1-alpha* = coativador do receptor gama ativado por proliferador de peroxissoma

KP = *kynurenine pathway* = via da quinurenina

KYN = *kynurenine* = quinurenina

TRP = tryptophan = triptofano

IDO = indoleamina-2,3-dioxigenase

TDO = triptofano-2,3-dioxigenase

KAT = kynurenine aminotransferase = quinurenina aminotransferase

KYNA = *kynurenic acid* = ácido quinurênico

KMO = *kynurenine monooxigenase* = quinurenina monooxigenase

KYNU = *kynureninase* = quinureninase

QUIN = *quinolinic acid* = ácido quinolínico

3-HAAO = *3-hydroxyanthranilate 3,4-dioxygenase* = 3-hidroxiantranilato 3,4-dioxigenase

3-HK = *3-hydroxykynurenine* = 3-hidroxiquinurenina

1-MT = 1-metiltriptofano

NMDA = N-metil-D-aspartato

LPS = lipopolissacarídeo

APRESENTAÇÃO

A presente tese foi dividida em três partes principais. Na **parte I** encontram-se a **INTRODUÇÃO** e **OBJETIVOS**. Os resultados que fazem parte desta tese estão apresentados sob a forma de 1 artigo publicado em periódico científico e dois manuscritos, os quais se encontram nos itens **ARTIGO, MANUSCRITO I e MANUSCRITO II, parte II** deste trabalho. As seções materiais e métodos, resultados, discussão dos resultados e referências bibliográficas, encontram-se nos próprios artigo e manuscritos e representam a íntegra deste estudo. Os itens **DISCUSSÃO** e **CONCLUSÃO**, encontrados na **parte III** desta tese, apresentam interpretações e comentários gerais sobre os resultados apresentados na parte II deste trabalho. O item **REFERÊNCIAS** refere-se somente às citações que aparecem nos itens introdução, discussão e conclusão desta tese. No ítem **PERSPECTIVAS**, estão expostos os possíveis estudos para dar continuidade a este trabalho.

INTRODUÇÃO

1. Doença de Alzheimer

A demência é um dos mais importantes desafios de saúde pública relacionados ao envelhecimento. Dados recentes da Organização Mundial da Saúde (2016) sobre a prevalência de demência no mundo apontam que atualmente cerca de 47.47 milhões de pessoas vivem com esta doença, e estima-se que estes números alcancem 75.63 milhões em 2030 e 135.46 milhões em 2050. A doença de Alzheimer (DA), uma desordem neurodegenerativa, é a principal forma de demência, sendo caracterizada clinicamente por uma inexorável e progressiva deterioração das funções cognitivas e das habilidades para uma vida independente, causando grande sofrimento para os pacientes, familiares e seus cuidadores (Bateman et al., 2012). O envelhecimento é considerado o fator de risco mais importante para a DA com a incidência e a prevalência aumentando dramaticamente com a idade. Por exemplo, a prevalência da DA é de aproximadamente 15% nas pessoas entre 65-74 anos, passando de 44% na idade de 80 anos (Alzheimer´s Association, 2016).

Além do declínio cognitivo, os pacientes de DA frequentemente apresentam sintomas não-cognitivos, conhecidos como sintomas comportamentais e psicológicos da demência (BPSD), também denominados como sintomas neuropsiquiátricos da demência (NPSD). Os BPSD compreendem um espectro de distúrbios comportamentais, psicológicos e emocionais afetando até 90% de todos os pacientes com demência (Cerejeira et al., 2012). Estes sintomas incluem depressão, ansiedade, agressividade, psicose, entre outros, constituindo a maior carga da doença (Rosenberg et al., 2015), estando associados a uma progressão mais rápida para quadros graves de demência e a morte (Peters et al., 2015). Nesse sentido, a DA vem demonstrando impactos socioeconômicos importantes. Em 2010, o custo econômico com demência no mundo foi estimado em cerca de US$ 604 bilhões (Wimo et al., 2013). Somado a isso, em vista de sua etiologia multifatorial, uma cura efetiva para a DA permanece elusiva, sendo que os tratamentos farmacológicos atuais aprovados tratam temporariamente os sintomas

cognitivos, assim como o alívio dos sintomas neuropsiquiátricos requer tratamentos adicionais (Holmgren et al., 2014; Rosenberg et al., 2015). Portanto, a identificação de alvos moleculares potenciais para o desenvolvimento de estratégicas profiláticas e terapêuticas contra o amplo espectro sintomático da DA é uma necessidade urgente e também um dos maiores desafios das ciências biológicas e médicas do século XXI.

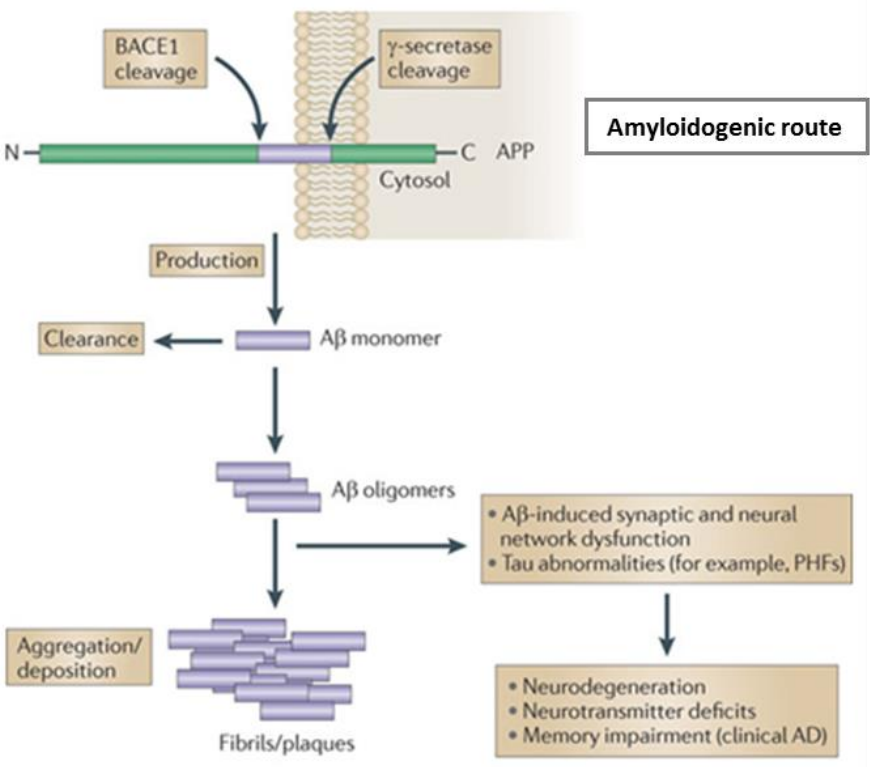
2. Teoria da cascata amilóide e o modelo experimental de DA induzido pela infusão intracerebroventricular do peptídeo $A\beta_{1-42}$

Os marcadores neuropatológicos da DA são as placas senis e os emaranhados neurofibrilares (NFT), estes últimos contendo principalmente formas hiperfosforiladas da proteína tau (Ballard et al., 2011). As placas senis são principalmente compostas pelo peptídeo beta-amilóide (Aβ), o qual é um peptídeo contendo 39-43 resíduos de aminoácidos, sendo derivado da ação enzimática sequencial da β-secretase (também conhecida como BACE 1) e γ-secretase sobre a proteína precursora amiloide (APP), uma glicoproteína transmembranar altamente expressa nas membranas dos neurônios (Di Paolo & Kim, 2011; **Figura 1**). A isoforma $A\beta_{1-40}$ é a mais predominante, seguida pela isoforma $A\beta_{1-42}$, a qual é de natureza hidrofóbica e sofre processo de agregação e deposição em taxas mais rápidas (Barage & Sonawane, 2015).

Embora os mecanismos fisiopatológicos subjacentes à DA não estejam totalmente elucidados, a “hipótese da cascata amilóide” tem sido suportada como a principal teoria no campo da pesquisa da DA nas últimas décadas. Ela afirma que o acúmulo do peptídeo Aβ no cérebro, resultante de alterações no seu processamento (produção aberrante e degradação disfuncional), é o evento inicial da DA (Laferla et al., 2007). A teoria original sustenta que a deposição amilóide em placas senis deflagra uma cascata de processos patogênicos que causam disfunção sináptica e uma gradual morte neuronal em regiões cerebrais como córtex frontal e hipocampo (Ballard et al., 2011). Apesar dos cérebros com DA tipicamente abrigarem placas senis que consistem de agregados insolúveis do peptídeo Aβ, as diferentes formas de oligomerização, incluindo formas solúveis como fibrilas, dímeros, trímeros e dodecâmeros, podem diferentemente contribuir para a patogênese da DA em

vários estágios da doença (Karran et al., 2011). Importantemente, as elevações dos oligômeros solúveis do peptídeo Aβ estão relacionadas à disfunção sináptica e anormalidades da proteína tau, deflagrando processos de neurodenegeração, déficits de neurotransmissores e, por último, culminando nos sintomas clínicos, como os prejuízos na memória (Di Paolo & Kim, 2011; **Figura 1**). Nesse sentido, tem sido proposto um refinamento da teoria original, focando-se na primazia do papel causal de formas oligoméricas do peptídeo Aβ na etiologia da DA (Hayden and Teplow, 2013). Corroborando esta idéia, estudos prévios demonstraram que formas oligoméricas do peptídeo Aβ possuem maior potencial neurotóxico do que formas fibrilares (Chambon et al., 2011, Hayden and Teplow, 2013). Ademais, tem sido reconhecido que os déficits cognitivos em pacientes e animais transgênicos da DA estão mais correlacionados com os níveis de formas amilóide solúveis do que com o aparecimento de placas (Chambon et al., 2011; Morley e Farr, 2014).

Figura 1: Processamento proteolítico amiloidogênico e o papel das formas oligoméricas Aβ na fisiopatologia da DA.



Fonte: Adaptado de Di Paolo & Kim, 2011.

Há um crescente corpo de evidências demonstrando que a administração intracerebroventricular (i.c.v.) de formas sintéticas dos peptídeos $A\beta_{1-40}$ e $A\beta_{1-42}$, análogas às encontradas em placas senis em pacientes de DA, é um modelo experimental válido para a caracterização da toxicidade do peptídeo Aβ quando as placas amilóides não estão presentes (Prediger et al., 2007; Piermartini et al. 2010; Chambon et al., 2011; Souza et al., 2013; Fukomoto et al., 2014; Chen et al., 2015). Este paradigma suporta a hipótese do papel patogênico chave das formas oligoméricas Aβ e permite a identificação de mecanismos neurobiológicos relacionados a fases iniciais da DA (Chambon et al., 2011).

Tem sido documentado que a infusão do peptídeo Aβ dentro do cérebro de animais de laboratório induz respostas de estresse oxidativo, neuroinflamação, redução nos níveis de fatores neurotróficos e neurodegeneração, concomitantemente a prejuízos no aprendizado e memória (Piermartini et al., 2010, Dao et al., 2013; Souza et al., 2013; Chen et al., 2015). Além dos distúrbios cognitivos, foi demonstrado que este modelo é capaz de causar comportamentos tipo-depressivo e tipo-ansioso em roedores, mimetizando os sintomas neuropsiquiátricos associados à DA (Pamplona et al., 2010; dos Santos et al. 2013; Dao et al. 2014,). Não obstante, os mecanismos da neurotoxicidade Aβ-mediada e, sobretudo, seus efeitos na indução de distúrbios comportamentais não-cognitivos ainda necessitam ser mais bem compreendidos. Até agora, não foram encontrados estudos usando o modelo de infusão i.c.v. de $A\beta_{1-42}$ em camundongos idosos e, em nosso ponto de vista, é de suma importância a investigação do fator envelhecimento neste paradigma, visto que a idade é o principal fator de risco para a DA.

Em última análise, a identificação dos efeitos celulares e comportamentais associados à desregulação inicial do metabolismo do peptídeo Aβ, os quais podem ser mimetizados através deste modelo, permitem o desenvolvimento de contramedidas que atuem a este nível e, consequentemente, interfiram com os processos degenerativos subsequentes.

3. Papel da neuroinflamação e da ativação da via da quinurenina na fisiopatologia da DA

A neuroinflamação pode ser definida como uma ativação do sistema imunológico inato no cérebro e sua principal função é proteger o sistema nervoso central (CNS) contra infecções, injúria e doenças (Zhang e Jiang, 2015). Essa resposta envolve uma série de alterações celulares e moleculares, com a participação de vias de sinalização intracelular e a liberação de mediadores inflamatórios no cérebro (Heppner et al., 2015).

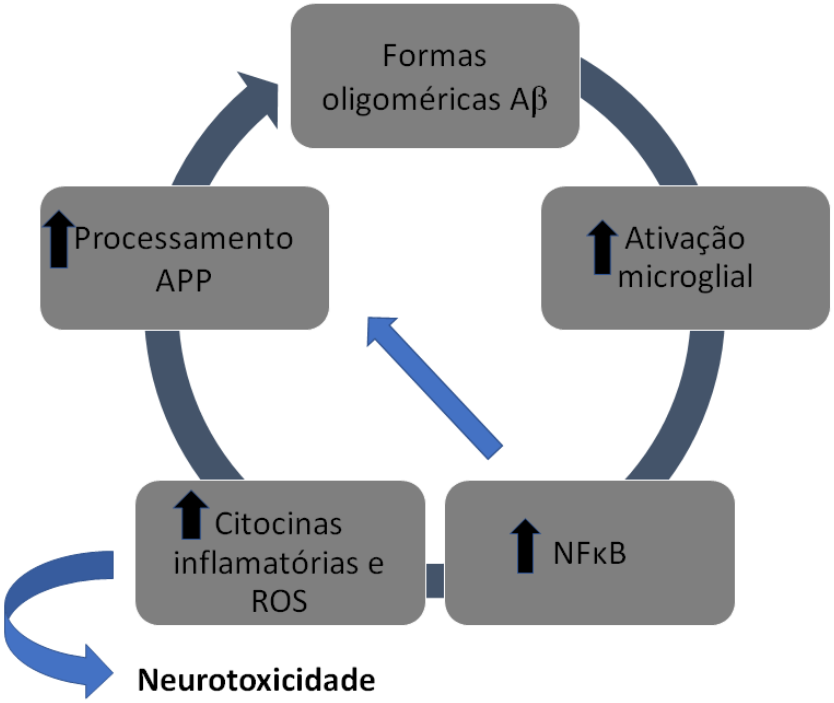
Evidências emergentes têm apontado que a neuroinflamação desempenha um papel crucial na fisiopatologia da DA (Heneka et al., 2015; Heppner et al., 2015; Wang et al., 2015). Tem sido proposto que o estado de inflamação crônica induzida por uma série de citocinas pró-inflamatórias, como a interleucina-1beta (IL-1β), interleucina-6 (IL-6) e o fator de necrose tumoral-alfa (TNF-α), liberadas por micróglias e astrócitos em estado ativado, está presente tanto em estágios iniciais como finais da DA (Heppner et al., 2015).

Foi reportado que o peptídeo Aβ é capaz de estimular a produção de citocinas inflamatórias através do acoplamento com receptores de superfície em micróglias, disparando a ativação de vias de sinalização dependentes do fator nuclear kappa B (NFκB), um fator de transcrição que induz o aumento da expressão gênica pró-inflamatória (Zhang e Jiang, 2015). Reciprocamente, os processos inflamatórios mediados pelas citocinas inflamatórias podem provocar um aumento nos níveis de Aβ, através da ativação da via amiloidogênica, culminando num ciclo patológico vicioso (Griffin et al., 1998). Especificamente, as micróglias podem interagir com o peptídeo Aβ, aumentando a expressão e liberação de citocinas inflamatórias, as quais podem ativar a β-secretase e a γ-secretase e reduzir a degradação amilóide. Consequentemente, ocorre um aumento da produção do peptídeo $A\beta_{1-42}$, da proliferação e ativação microglial e a morte neuronal, resultando num ciclo inflamatório autossustentado. Estas vias de sinalização intracelular são NFκB-dependentes, onde o NFκB também é reportado aumentar a produção de APP e o processo proteolítico de APP que resulta na produção do peptídeo Aβ (**Figura 2**).

Portanto, o peptídeo Aβ é considerado tanto causa como consequência da neuroinflamação na DA (Wang et al., 2015). Além disso, tem sido reportado

que as citocinas pró-inflamatórias podem modular os níveis de neurotransmissores cerebrais e fatores de crescimento, assim como são capazes de aumentar a produção de espécies reativas de oxigênio, afetando o funcionamento neuronal (Wuwongse et al., 2010; **Figura 2**). Todos estes fatores podem contribuir para a disfunção e morte de neurônios na DA, sozinhos ou em combinação (Zhang e Jiang, 2015).

Figura 2. Ciclo inflamatório vicioso na DA.



APP: proteína precursora amilóide; ROS: espécies reativas de oxigênio.

Fonte: Adaptado de Wang et al., 2015.

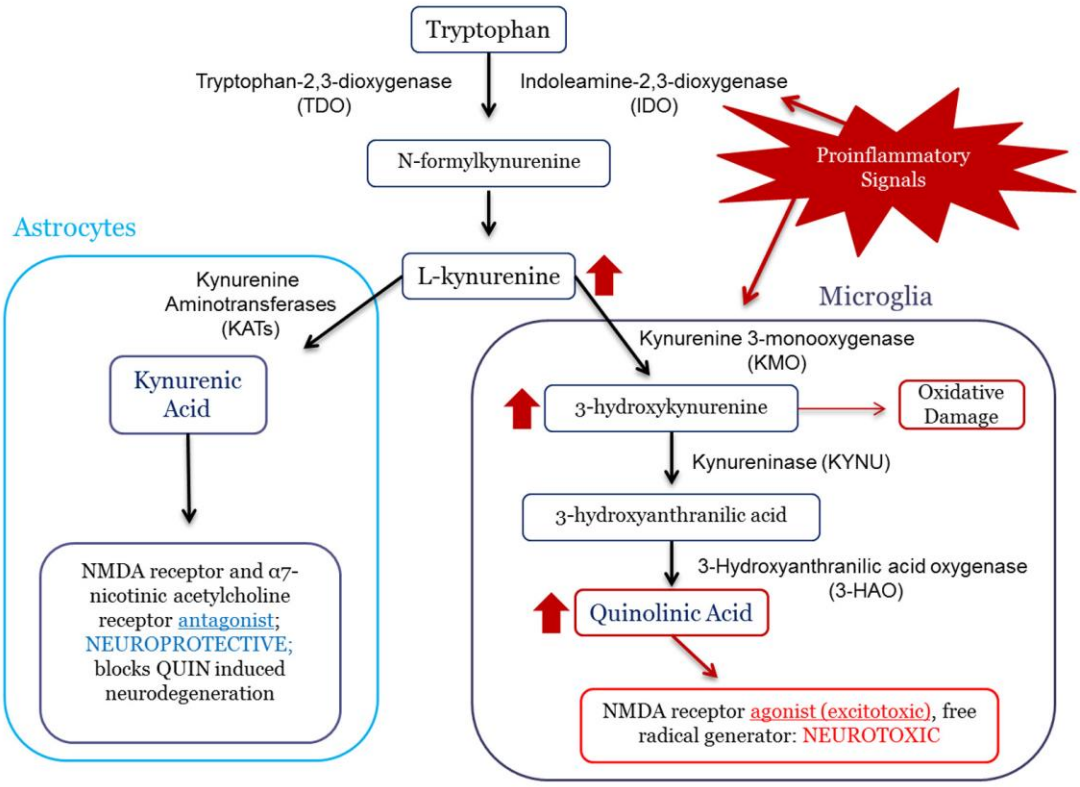
Estudos recentes têm demonstrado que a infusão i.c.v. do peptídeo $A\beta_{1-42}$ provoca um aumento dos níveis de citocinas pró-inflamatórias no hipocampo de roedores em associação com prejuízos na memória (Fukomoto et al., 2014; Liang et al., 2015). Utilizando o paradigma $A\beta_{1-40}$, um estudo prévio do nosso laboratório demonstrou que o aumento nos níveis de TNF-α e IL-1β e a diminuição dos níveis da citocina anti-inflamatória IL-10 no hipocampo e córtex pré-frontal estão relacionados com o déficit no reconhecimento de objetos em

camundongos (Souza et al., 2013). Este resultado corroborou a hipótese de que uma desregulação do estado inflamatório em regiões cerebrais implicadas com a memória pode ser um importante mediador neurobiológico do prejuízo cognitivo Aβ-induzido. Apesar de o aumento dos níveis de citocinas pró-inflamatórias também estar associado a alterações emocionais, incluindo os comportamento tipo-ansiedade e tipo-depressivo em modelos animais de depressão (O'connor et al., 2009; Salazar et al., 2012), há uma escassez de estudos investigando esta relação em modelos animais de DA. Em contrapartida, evidências clínicas tem reportado que o aumento de citocinas inflamatórias no soro estão associadas a distúrbios neuropsiquiátricos em pacientes de DA (Takeda et al., 2014). Em acordo com as evidências supracitadas, há ainda estudos que convergem no sentido de indicar uma "potencial ligação neurodegenerativa" entre a neuroinflamação e os sintomas cognitivos e neurocomportamentais na DA (Wuwongse et al., 2010; Holmgren et al., 2014).

Os mecanismos pelos quais a neuroinflamação está envolvida com a fisiopatologia da DA não estão totalmente compreendidos; no entanto, a ativação da via da quinurenina (KP) tem sido implicada (Tan et al., 2012; Maddison and Giogini, 2015). A KP é a principal rota do catabolismo do aminoácido essencial triptofano em mamíferos (Stone and Darlington, 2002). A maioria do triptofano da dieta é metabolizado através da KP. No entanto, a ativação desta via pode levar à formação de alguns metabólitos neuroativos, os quais foi mostrado que podem ter ação neuroprotetora ou neurotóxica, e no CNS são gerados principalmente pelas células gliais (Dantzer et al., 2011). Em condições fisiológicas, a enzima triptofano-2,3-dioxigenase (TDO), uma enzima predominantemente hepática, é responsável pelo passo inicial na via da quinurenina, convertendo o triptofano para N-formilquinurenina o qual é subsequentemente metabolizado para quinurenina (KYN). No entanto, em condições inflamatórias, a enzima extra-hepática indoleamina-2,3-dioxigenase (IDO) sofre uma supra-regulação tanto na periferia quanto no cérebro, aumentando a produção de KYN. A KYN pode ser subsequentemente metabolizada através de duas rotas distintas: (1) a via das quinureninas aminotransferases (KAT) formando o ácido quinurênico (KYNA), sendo

considerada o braço neuroprotetor da KP; (2) via da quinurenina monooxigenase (KMO), quinureninase (KYNU) e 3-hidroxiantranilato 3,4-dioxigenase (3-HAAO) produzindo o ácido quinolínico (QUIN), a qual é indicada como o braço neurotóxico da KP. O metabólito QUIN é considerado uma excitotoxina que age como um agonista dos receptores N-metil-D-aspartato (NMDA) no cérebro, sendo que a atividade da KMO nas micróglias é a etapa limitante para a sua produção. Por outro lado, o metabólito KYNA, é um antagonista de receptores NMDA e dos receptores alfa7 nicotínicos (α7nAChR), tendo papel neuroprotetor (Heisler & O'connor, 2015; **Figura 3**).

Figura 3. Via da quinurenina do metabolismo do triptofano.



Fonte: Adaptado de Heisler & O'Connor, 2015.

Recentemente, uma série de estudos demonstrou que uma superativação da KP ocorre em resposta a desafios inflamatórios periféricos e centrais induzidos por lipopolissacarídeo (LPS), uma endotoxina bacteriana (O'Connor et al., 2009; Salazar et al., 2012; Gibney et al., 2013; Lawson et al., 2013). Esta superativação pode resultar tanto na diminuição da

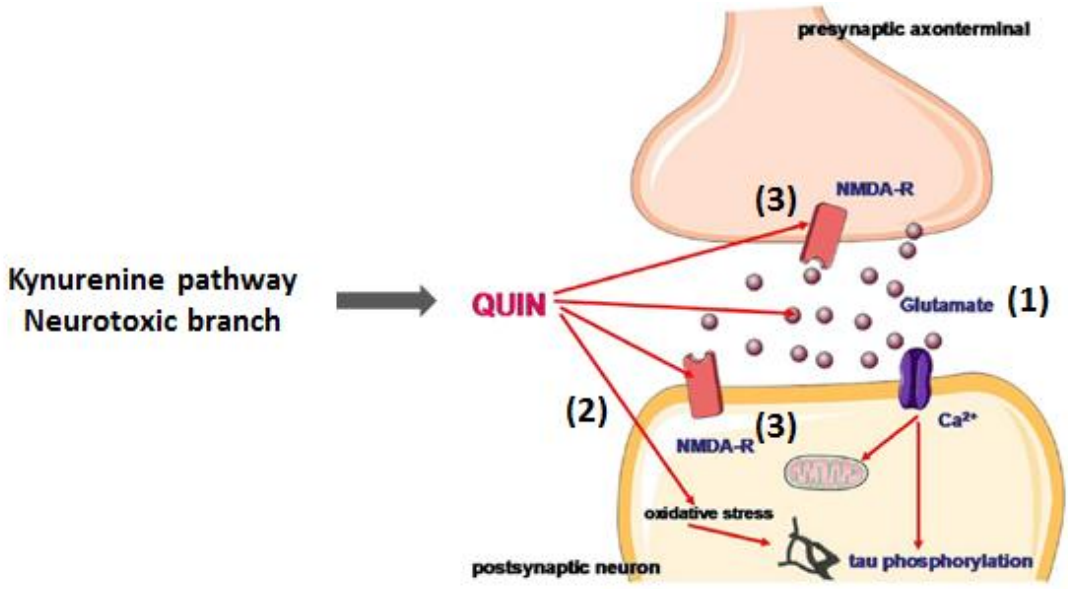
biodisponibilidade do triptofano para a síntese de serotonina (podendo causar uma disfunção serotoninérgica), quanto no aumento da produção de KYN e seus metabólitos neurotóxicos (Dantzer et al., 2011), os quais têm sido fortemente implicados com a patogenia da DA (Kincses et al., 2010). Este fenômeno tem sido descrito como o “desvio para o braço neurotóxico da KP”, o que significa em termos fisiológicos um desequilíbrio da homeostase desta via (Myint e Kim, 2003).

Estudos anteriores reportaram que a indução da indoleamina-2,3-dyoxigenase (IDO), a enzima limitante da KP em tecidos extra-hepáticos, é modulada pela inflamação (Stone and Darlington, 2002; Maes et al., 2011). Tem sido bem demonstrado que diversas citocinas pró-inflamatórias podem causar uma supra-regulação da IDO, como a IL-1β, a IL-6 e, principalmente, as citocinas interferon-gama (IFN-γ) e o TNF-α, que são consideradas suas principais citocinas indutoras (Guillemin e Brew, 2002). Interessantemente, diversas linhas de evidência mostraram que a IDO está altamente expressa em tecidos cerebrais, especialmente em conjunto com marcadores patológicos da DA, como as placas amilóides e os NFT (Guillemin et al., 2005; Bonda et al., 2010; Wu et al., 2013). É importante ressaltar que, no cérebro, a IDO está expressa principalmente em micróglias ativadas, as quais são observadas circundando as placas amilóides, o que indica que a estimulação da KP induzida por inflamação pode estar envolvida com vias neurodegenerativas da DA (Maddison & Giordini, 2015).

Nesse sentido, o aumento da ativação da IDO pode causar o desvio da KP para o braço neurotóxico, resultando na produção dos catabólitos 3-hidroxiquinurenina (3-HK) e QUIN, os quais são geradores de radicais livres (Gulaj et al., 2010). O estresse oxidativo gerado por estas neurotoxinas causa danos aos tecidos neuronais e podem potencialmente contribuir para a neurodegeneração, aumentando o acúmulo amilóide, a ativação glial e a supra-regulação da KP (Tan et al., 2012). Em particular importância, estudos em culturas de neurônios demonstraram que níveis aumentados do peptídeo $A\beta_{1-42}$ estão relacionados com o aumento da expressão da IDO e do metabólito QUIN, levando a morte neuronal (Guillemin e Brew, 2002; Guillemin et al., 2003

e 2005). Um resumo dos principais mecanismos neurotóxicos do metabólito QUIN na DA estão ilustrados na **Figura 4**.

Figura 4. Mecanismos neurotóxicos induzidos pelo QUIN na DA



(1): inibição da síntese de glutamina, levando a um aumento do acúmulo de glutamato no ambiente extracelular no CNS; (2): indução de estresse oxidativo levando a fosforilação da proteína tau; (3): ação agonista NMDA, provocando morte neuronal pela citotoxicidade.

Fonte: Adaptado de Tan et al., 2012.

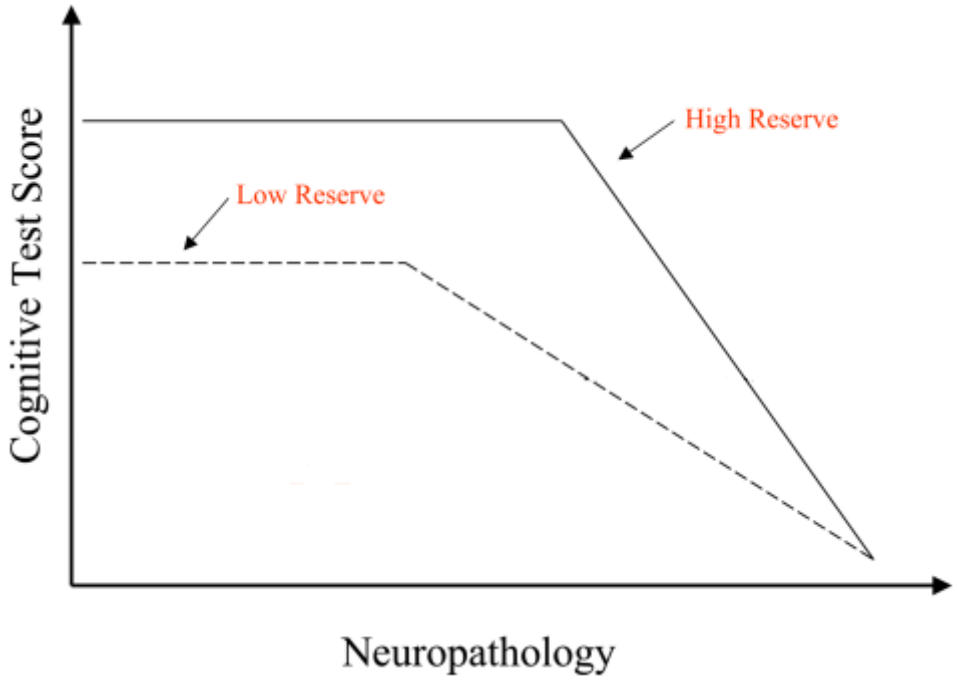
Embora recentes pesquisas em modelos animais de depressão tenham reportado que a ativação da IDO no cérebro está envolvida com os comportamentos tipo-ansiedade e tipo-depressivo e com o prejuízo na memória de reconhecimento de objetos (Salazar et al., 2012, Gibney et al., 2013; Heisler e O'Connor, 2015; Souza et al., 2017), esta relação ainda não foi investigada em modelos animais de DA. Portanto, novos estudos devem ser conduzidos a fim de prover novas evidências acerca da implicação da KP na fisiopatologia da DA, assim como estratégias de intervenção visando a modulação dos componentes desta via devem ser testadas.

4. Efeito neuroprotetor do Exercício Físico na DA

O interesse na pesquisa sobre os efeitos do exercício físico na saúde do CNS e em desordens neurológicas e neurodegenerativas tem ganhado crescente interesse nas últimas décadas (Svensson et al., 2015). Nesse sentido, o exercício físico é apontado como um dos principais fatores de proteção para a DA, junto com outros fatores como os nutricionais, ocupacionais e educacionais (Ballard et al., 2011). Evidências convincentes indicam claramente que o exercício físico feito de forma regular pode melhorar funções cognitivas e prevenir a demência em idosos (Kramer et al., 2006; Lautenschlager et al., 2008; Lista e Sorrentino, 2010; Paillard et al., 2015). Estudos clínicos mostram que o exercício físico tem uma importante ação preventiva na demência, desacelerando a sua progressão e mitigando a inabilidade (Erickson et al., 2012; Brett et al., 2016). Os dados clínicos também enfatizam que o exercício físico regular pode diminuir o declínio cognitivo, a atrofia cerebral de regiões como o hipocampo e aliviar os sintomas neuropsiquiátricos em pacientes de DA (Paillard et al., 2015; Matura et al., 2016).

Uma importante teoria formulada para explicar os efeitos protetores e preventivos do exercício na DA é a “hipótese da reserva cognitiva”, que foi originada inicialmente de observações epidemiológicas. Esta hipótese postula que o exercício é capaz de aumentar a capacidade de reserva do cérebro para reagir a insultos patológicos. Consequentemente, indivíduos com maior reserva cognitiva promovida por um estilo de vida ativo tolerariam e suportariam o desenvolvimento de patologias cerebrais predispostas por fatores genéticos ou desencadeadas por fatores ambientais (Fratiglioni et al., 2004). Em outras palavras, indivíduos com uma reserva cognitiva maior podem tolerar mais as alterações neuropatológicas (e.g. acúmulo de placas amilóides e a perda neuronal), de modo que o ponto no qual as funções cognitivas começam a ser afetadas ocorrerá mais tarde do que naquelas com uma reserva cognitiva mais baixa (Stern, 2012). A figura 5 ilustra as explicações teóricas destes achados.

Figura 5. Teoria da reserva cognitiva.



Fonte: Adaptado de Stern, 2012.

Nesse contexto, Davenport et al. (2012) descreveram a importância da aptidão física para prevenir ou atrasar o declínio cognitivo relacionado ao envelhecimento ou à demência através de mecanismos vasculares exercício-induzidos. Estes autores sugeriram que a associação entre altos níveis de condicionamento aeróbio e a melhoria da cognição em idosos é mediada, em partes, por processos que envolvem a melhoria da perfusão cerebral. Ou seja, o exercício pode conferir um aumento da "reserva cerebrovascular", i.e., a habilidade dos vasos sanguíneos cerebrais em responder a estímulos. Estas respostas de melhora nas funções cerebrovasculares estão relacionadas à diminuição da pressão arterial e do estresse oxidativo e ao aumento dos níveis de óxido nítrico e do fator neurotrófico derivado do cérebro (BDNF). Estes efeitos em conjunto resultam na promoção da neurogênese, angiogênese e sinaptogênese proporcionando, deste modo, substratos para preservar ou até mesmo melhorar a cognição (Davenport et al., 2012). Portanto, é razoável assumir que o exercício aeróbio pode melhorar a reserva cerebral, ao aumentar a plasticidade através de uma regulação positiva do BDNF.

Apesar do conhecimento dos efeitos do exercício em proteger o cérebro contra os processos neurodegenerativos em idosos e pacientes com DA, existem limitações éticas inerentes ao estudo desta intervenção no cérebro de humanos, que ficam restritos a exames de imagens e marcadores circulantes periféricos. Por esta razão, os modelos de experimentação animal são importantes ferramentas de pesquisa para o avanço do entendimento das bases neurobiológicas do efeito neuroprotetor do exercício físico em doenças neurodegenerativas, uma vez que permitem o estudo direto de estruturas cerebrais de interesse. Nesse contexto, os principais focos de pesquisas recentes tem sido a investigação dos efeitos positivos do exercício físico em áreas como a neuroinflamação, neuroplasticidade e neurodegeneração, assim como em domínios comportamentais, incluindo as habilidades motoras e cognitivas (Ryan & Kelly, 2016). Estudos utilizando modelos animais demonstraram que o exercício regular é capaz de alterar diversas características fenotípicas da DA, como redução do acúmulo amilóide e redução da morte neuronal assim como melhoria da memória, em diferentes modelos de camundongos transgênicos para DA (García-Mesa et al., 2011; Liu et al., 2011; Moore et al., 2016). Recentemente, nós demonstramos que o exercício de natação protege contra o estresse oxidativo, a neuroinflamação e o prejuízo na memória de camundongos que receberam infusão i.c.v. do peptídeo $A\beta_{1-40}$. Ainda neste paradigma, foi demonstrado que o exercício em esteira rolante é eficaz em proteger tanto funções cognitivas quanto não-cognitivas em ratos infundidos com o peptídeo $A\beta_{1-42}$, atuando principalmente através da modulação do BDNF (Dao et al., 2013, 2014).

Em resumo, um crescente corpo de estudos em animais e humanos sugere que os principais mecanismos neuroprotetores induzidos pelo exercício físico estão relacionados aos seus efeitos antioxidante, angiogênico, anti-inflamatório e pró-neurogênico em áreas cerebrais como o córtex frontal e o hipocampo, as quais estão particularmente envolvidas com a memória e a regulação emocional. No entanto, estudos recentes têm expandido essas linhas de evidência, aprofundando as investigações sobre os potenciais efeitos do exercício físico sobre a modulação de fatores neurotróficos e de citocinas no cérebro de animais de laboratório, uma vez que estes marcadores vêm sendo

considerados importantes elos neurobiológicos entre a demência e os sintomas neuropsiquiátricos relacionados à DA.

5. O Exercício Físico como modulador de fatores neurotróficos

Os fatores neurotróficos BDNF, fator de crescimento neural (NGF), fator de crescimento derivado de células gliais (GDNF) e a neurotrofina-3 (NT-3) são importantes para a sobrevivência, manutenção e regeneração de populações específicas de neurônios no cérebro adulto (Poo et al., 2013). Allen et al. (2013), assim como outros grupos de pesquisa (Sopova et al., 2014; Budni et al., 2016), compartilham a opinião de que a depleção destes fatores neurotróficos estão relacionados com a fisiopatologia e os sintomas da DA e, portanto, estratégias que visem a reposição destas substâncias são consideradas terapias potenciais para a DA.

Estudos prévios reportaram efeitos pró-neurogênicos do exercício físico em modelos animais da DA. A plasticidade sináptica (Zhao et al., 2015), a arborização dentrítica (Lin et al., 2015) e a proliferação e diferenciação neuronal foram aumentadas (Tapia-Rojas et al., 2016) em camundongos com dupla transgenia para a DA (APPswe/PS1ΔE9) que foram submetidos a treinamento físico. Também foi verificado que o exercício em rodas de correr aumentou a neurogênese no hipocampo de camundongos 3xTgAD, com transgenia tripla para a DA (Rodrigues et al., 2011). Ademais, vários estudos reportaram que um aumento da expressão de fatores neurotróficos estão associados com os efeitos exercício-induzidos na neurogênese hipocampal, incluindo o fator de crescimento tipo-insulina I (IGF-1) (Lista & Sorrentino, 2010), o fator de crescimento endotelial vascular (VEGF) (Davenport et al., 2012) e as neurotrofinas BDNF (Um et al., 2011; Cho et al., 2015), GDNF (Revilla et al., 2014), NGF (Um et al., 2011) e a neurotrofina-3 (NT-3) (Koo et al., 2013).

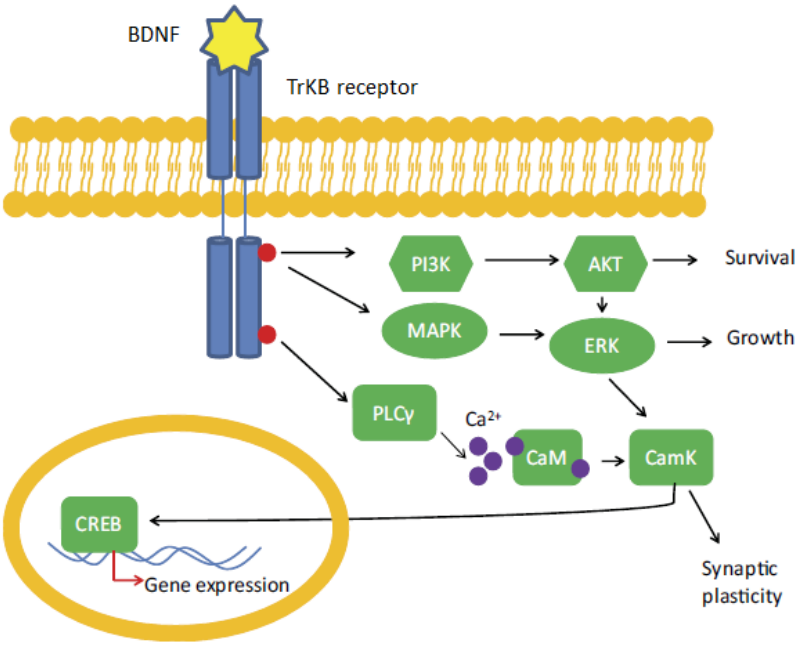
Diversas linhas de evidência convergem para a idéia de que o BDNF é o modulador crítico da neuroplasticidade induzida pelo exercício (Cotman et al., 2007; Lista & Sorrentino, 2010; Um et al., 2011; Stern, 2012; Dao et al.,2014). De fato, o BDNF é o fator de crescimento mais abundante no CNS e devido as suas funções biológicas está amplamente implicado com a fisiopatologia de

doenças neurodegenerativas e doenças neuropsiquiátricas (Autry & Monteggia, 2012; Allen et al, 2013). A liberação de BDNF evocada pelo exercício tem sido repetidamente postulada como subjacente aos efeitos pró-cognitivos, antidepressivos e ansiolíticos do exercício físico (Pietropaolo et al., 2008). Foi demonstrado que tanto os níveis quanto a expressão de BDNF em diferentes regiões cerebrais sofrem aumento em resposta ao exercício, sobretudo no hipocampo (Neeper et al., 1995; Berchtold et al., 2005). O exercício, particularmente o aeróbio, é capaz de modular os níveis periféricos de BDNF em humanos, aumentando os níveis séricos desta neurotrofina. O BDNF pode atravessar a barreira hemato-encefálica (BBB) nas duas direções e, em vista disso, seus níveis periféricos podem representar uma importante reserva cerebral (Coelho et al., 2012). Em roedores, foi demonstrado que o aumento basal dos níveis de BDNF no hipocampo ocorre em poucos dias de exercício voluntário em rodas de correr (Johnson e Mitchell, 2003), com este efeito persistindo por até 2 semanas após sua interrupção (Bertchold et al., 2010). Uma supra-regulação da expressão e dos níveis de BDNF induzida pelo exercício também foi reportada como um importante fator para a proteção contra a esquizofrenia e doenças neurodegenerativas, como a doença de Huntington (HD), a doença de Parkinson (PK) e a DA (Paillard et al., 2015; Spielman et al., 2016).

De acordo com Autry e Monteggia (2012), o BDNF é sintetizado como uma proteína precursora chamada pré-pró-BDNF que é clivada em pró-BDNF (34 kDa), a qual pode posteriormente ser clivada ao BDNF maduro (14 kDa). Recentemente, foi sugerido que o pró-BDNF e o BDNF maduro ativam diferentes vias de sinalização intracelular. O sinal da isoforma pró-BDNF ocorre através de uma baixa afinidade pelo receptor de neurotrofina p75. Em contrapartida, a sinalização do BDNF maduro ocorre através de sua alta afinidade pelo seu receptor de tropomiosina relacionado à quinase B (TrkB). Quando o BDNF se liga ao receptor TrkB, ele induz sua dimerização e a autofosforilação do receptor tirosina quinase, levando a ativação de cascatas de sinalização intracelular. Existem ao menos três vias de transdução de sinal ativadas pelo BDNF-TrkB. A via da fosfolipase C γ (PLC γ), a qual leva a ativação da proteína quinase C; a via do fosfatidilinositol 3-quinase (PI3K), que

ativa a serina/treonina quinase (AKT); e por último, a via das proteínas quinase ativadas por mitógenos (MAPKs)/quinase regulada por sinal extracelular (ERK), que por sua vez ativa diversos efetores à jusante. Cada uma dessas vias de sinalização pode conferir uma função única do BDNF nas células (para uma revisão detalha, veja Mattson, 2008). Resumidamente, rápidos efeitos sobre canais iônicos são dependentes da liberação dos estoques intracelulares de cálcio, eventos que são mediados pela via da PLC γ, a qual aumenta a atividade da proteína quinase dependente de cálcio/calmodulina (CAMK), levando à plasticidade sináptica. Não obstante, acredita-se que os efeitos mais duradouros são mediados pelas vias do PI3K e das MAPKs. A via do PI3K ativa a AKT, ativando a sobrevivência celular. A via das MAPKs/ERK ativa a diferenciação e o crescimento celular (**Figura 6**). Todas essas três vias convergem para ativar o fator de transcrição CREB (*cAMP response elemento binding protein),* uma molécula chave para a potenciação de longo prazo (LTP) no hipocampo – um fenômemo importante na formação da memória de longo prazo.

Figura 6. Visão geral da sinalização do BDNF através dos receptores TrkB.



Fonte: Adaptado de Autry & Monteggia, 2012.

Com efeito, estudos prévios demonstraram que o exercício pode aumentar tanto o BDNF quanto os componentes de suas vias de sinalização para a neurogênese e sinaptogênese (Lista & Sorrentino. 2010; Svensson et al., 2015). Através do bloqueio de algumas proteínas mediadoras da via neurotrófica, foi constatado que o exercício pode ativar as vias CAMPK e MAPK que conduzem à ativação do CREB e da sinapsina 1, que são cruciais para os processos de consolidação da memória. O BDNF pode inclusive, ativar indiretamente a neurogênese através do aumento dos níveis de neurotransmissores, uma vez que o BDNF é vital para o desenvolvimento e sobrevivência de neurônios gabaérgicos, dopaminérgicos, colinérgicos e serotoninérgicos (Martinowich et al, 2007; Autry & Monteggia, 2012). Portanto, estes achados sustentam a hipótese de que o exercício físico através da regulação da síntese de fatores neurotróficos, sobretudo o BDNF, pode prevenir o desenvolvimento de sintomas cognitivos e emocionais associados à DA.

6. Efeito anti-inflamatório do Exercício Físico

A inflamação é uma resposta para eliminar tanto a causa inicial de uma lesão celular, bem como para eliminar as células necróticas e tecidos resultantes do insulto original. Se a saúde do tecido não for restaurada, a inflamação se torna uma condição crônica (Rubio-Perez & Morillas-Ruiz, 2012). O termo neuroinflamação vem sendo utilizado para designar uma inflamação específica no CNS, que não reproduz as características da inflamação periférica, como inchaço, calor e dor. No entanto, a inflamação no cérebro induz eventos neurodegenerativos, como o aumento da deposição amilóide, neurite distrófica e fosforilação da proteína tau (Streit et al., 2004). Infecções, traumas, toxinas e outros estímulos são capazes de ativar o sistema imune inato no CNS. Esta resposta neuroinflamatória ativa as micróglias, que liberam mediadores inflamatórios, como citocinas e quimiocinas. Além disso, estes mediadores podem produzir estresse oxidativo, levando a um estado neuroinflamatório crônico, perpetuando o ciclo inflamatório (Frank-Cannon et al., 2009). Este estado crônico de inflamação no cérebro está relacionado a várias patologias neurodegenerativas, como a DP, HD, esclerose lateral amiotrófica e a DA (Frank-Cannon et al., 2009). No que se refere à DA, as

placas amilóides causam a ativação das micróglias, que liberam citocinas pró-inflamatórias, como o TNF-α e a IL-1β. Estas citocinas estão relacionadas à tauopatias e à indução de apoptose (Streit et al., 2004).

Tem sido sugerido que a inatividade física leva a um acúmulo de gordura visceral e, consequentemente, a ativação de uma rede de vias inflamatórias, as quais promovem o desenvolvimento de desordens crônico-degenerativas, dentre elas a resistência insulínica, a aterosclerose, o crescimento de tumores, a depressão e a demência, que fazem parte de uma rede ou conjunto de doenças identificadas como “doenças da inatividade física” (do inglês *diseasome of physical inactivity;* Pedersen, 2009). Estas desordens representam doenças cuja apresentação fenotípica é bastante diferente; no entanto, elas parecem compartilhar importantes mecanismos patogênicos. Dentre estes mecanismos, encontra-se a inflamação sistêmica de baixo grau, sendo definida por uma elevação dos níveis circulantes de mediadores inflamatórios, como as citocinas pró-inflamatórias (Pedersen, 2011). A inflamação crônica é apontada por influenciar diretamente a patogênese destas doenças (Brandt & Pedersen, 2010; Gleeson et al., 2011). Não obstante, é bem estabelecido que independentemente do índice de massa corporal (IMC), a inatividade física é um fator de risco para a mortalidade por todas as causas (Pedersen, 2007) e que a inflamação sistêmica crônica está associada com a inatividade física, mesmo sem a presença de obesidade (Fischer et al., 2007). Além disso, a inflamação crônica gerada pela inatividade física pode ser agravada pela idade avançada. É reconhecido pela literatura predominante que a inflamação crônica é uma característica difusa dos tecidos senescentes e da maioria das doenças relacionadas ao envelhecimento (Franceschi & Campisi, 2014). O termo “inflammaging” (inflamação associada ao envelhecimento) é utilizado para descrever a inflamação crônica de baixo grau no envelhecimento, na ausência de infecção implícita, sendo um fator altamente significativo para morbimortalidade em idosos (Franceschi et al., 2000). Evidências epidemiológicas têm reportado que este estado de inflamação leve, revelado pelo aumento de biomarcadores inflamatórios como a proteína C reativa e a IL-6, estão associados a muitos fenótipos do envelhecimento, como mudanças na

composição corporal, homeostase metabólica, senescência imune e saúde neuronal (Franceschi, et al., 2000; Franceschi & Campisi, 2014).

Outrossim, é importante ressaltar que o envelhecimento do cérebro é marcado por uma senescência microglial, o qual é acreditado estar relacionado com um aumento da ativação detrimental das micróglias (Wang et al. 2015). Por outra forma, a micróglia no cérebro envelhecido tem suas funções normais reduzidas, como o *clearance*, a migração e a capacidade de mudar de um estado pró-inflamatório para um estado anti-inflamatório, tendo suas funções de regular a plasticidade e os seus mecanismos de reparação prejudicados (Rubio-Perez & Morillas-Ruiz, 2012; Wang et al., 2015). Portanto, baseando-se nas informações apresentadas, é possível sugerir que a combinação inatividade física e envelhecimento são dois fatores que podem ter efeitos detrimentais aditivos, contribuindo para o aumento do risco de desenvolver doenças neurodegenerativas, como a DA.

Uma série de estudos recentes tem apontado que o exercício físico também pode atrasar o início ou a progressão de doenças neurodegenerativas através de mecanismos imunomodulatórios (Pedersen, 2009 e 2011; Svensson et al., 2015; Spielman et al., 2016; Ryan & Kelly, 2016). O efeito protetor do exercício contra as doenças associadas à inflamação crônica pode, até certo ponto, ser atribuído ao seu efeito anti-inflamatório, sugerindo que o exercício regular, por si, pode suprimir a inflamação sistêmica de baixo grau, assim como a inflamação no cérebro (Pedersen, 2011; Spielman et al, 2016). Em consonância com a aceitação do tecido adiposo ser um órgão endócrino, tem sido apontado que o músculo esquelético também deve ser visto desta forma. Nessa perspectiva, citocinas e outros peptídeos que são produzidos, expressos e liberados pelas fibras musculares podem exercer efeitos parácrinos e endócrinos, sendo classificados como miocinas (Brandt & Pedersen, 2010). Esse paradigma fornece uma base conceitual explicando as múltiplas consequências de um estilo de vida fisicamente inativo. Ou seja, se as funções endócrinas e parácrinas do músculo esquelético não forem devidamente estimuladas através das contrações, isso pode resultar em disfunção de vários órgãos e tecidos do corpo, aumentando o risco de doenças crônico-degenerativas (Pedersen, 2011). Assim sendo, o exercício pode regular as

funções imunes através do aumento das miocinas, cuja secreção é induzida pela contração muscular. Algumas destas miocinas incluem a interleucina-4 (IL-4), o BDNF e, mais notavelmente a IL-6 (Stranska & Svacina, 2015). Foi demonstrado que os níveis circulantes de IL-6 aumentam dramaticamente em resposta ao exercício. Durante e após o exercício agudo, é observado uma grande e transitória elevação da secreção de IL-6 pelas fibras musculares em contração, induzindo uma amplo espectro de respostas anti-inflamatórias na periferia: (1) diminuição da gordura visceral e da infiltração de macrófagos inflamatórios levando à redução da produção de citocinas e adipocinas inflamatórias e, (2) diminuição da expressão de receptores tipo-toll (TLR) em monócitos, os quais estão relacionados com cascatas de sinalização inflamatória, sendo este um importante mecanismo da modulação da inflamação sistêmica (Pedersen, 2011; Gleeson et al., 2011).

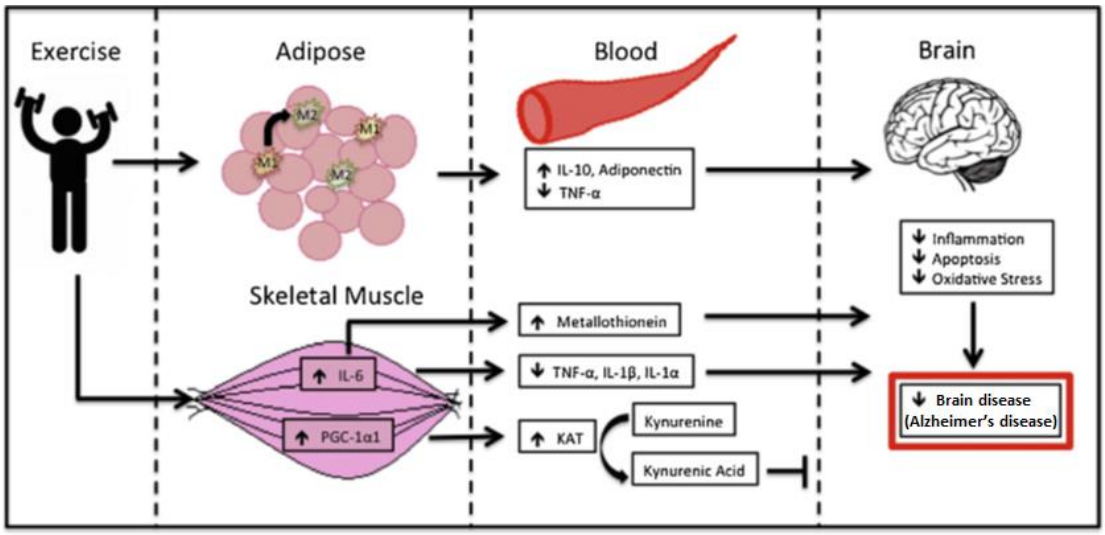
Estudos prévios indicam que a IL-6 é o principal modulador dos sistemas de sinalização recíproca entre o músculo e o SNC (Brandt & Pedersen, 2010; Spielman et al., 2016). A esse respeito, a IL-6 pode cruzar a BBB, promovendo uma ação de comunicação direta entre os músculos em contração e o sistema imune no CNS. Apesar da IL-6 ser uma citocina com dupla função, dependendo das circunstâncias, podendo exercer atividade neurotóxica ou neuroprotetora no CNS, tem sido sugerido que esta citocina age de uma forma anti-inflamatória no cérebro após o exercício (Pedersen, 2011). Por exemplo, a IL-6 aumenta a expressão da metalotioneína, uma proteína presente no complexo de Golgi, com fortes propriedades anti-inflamatória, antioxidante e anti-apoptótica. Desta forma, a produção de IL-6 induzida pelo exercício pode atenuar a morte neuronal, a neuroinflamação e a produção de espécies reativas de forma metalotioneína-dependente (Spielman et al., 2016). De fato, a IL-6 é a primeira citocina a aparecer na circulação durante o exercício, sendo esta resposta mais evidente e precedente ao aparecimento de outras citocinas (Petersen & Pedersen, 2005).

Em razão de as citocinas pró-inflamatórias, como o TNF-α e a IL-1β, geralmente não aumentam em resposta ao exercício e que, por outro lado, a IL-6 pode desencadear o aumento dos níveis plasmáticos de moléculas anti-inflamatórias como o antagonista dos receptores de interleucina-1 (IL-1ra), do

receptor solúvel de TNF-α (sTNF-α) e da citocina IL-10, tem sido proposto que o exercício induz um ambiente de citocinas anti-inflamatórias (Petersen & Pedersen, 2005). A IL-10 é secretada principalmente por macrófagos e linfócitos auxiliares T-CD4+ e inibe a produção de TNF-α, IL-1β e de proteínas quimiotáxicas, limitando as respostas inflamatórias agudas (Gleeson et al., 2011). Uma vez que todas estas citocinas anti-inflamatórias são capazes de cruzar a BBB, a regulação positiva da IL-6 após o exercício pode reduzir os níveis de citocinas pró-inflamatórias tanto a nível sistêmico, como a nível central, representando um importante mecanismo pelo qual o exercício pode reduzir a neuroinflamação crônica observada em doenças psiquiátricas, como a esquizofrenia e a depressão, assim como nas doenças neurodegenerativas, como a PD e DA (Spielman et al., 2016). Aqui, é importante ressaltar que as vias sinalização da IL-6 são marcadamente diferentes entre os miócitos e os macrófagos. Foi reportado que ao contrário dos macrófagos, onde a IL-6 é produzida por vias dependentes do NFκB, a expressão intramuscular de IL-6 é regulada por outras redes de cascatas de sinalização, incluindo a Ca^{2+}/fator nuclear de células T ativadas (NFAT) e a glicogênio/p38 MAPK. Portanto, a sinalização da IL-6 em células do sistema imune inato, criam uma resposta pró-inflamatória, ao passo que a ativação da sinalização da IL-6 no músculo é totalmente independente de uma resposta inflamatória ativada pelo NFκB (Pedersen, 2011).

Alternativamente, foi reportado que os benefícios neuroimunológicos do exercício podem ocorrer devido a modulação da KP. Foi postulado que o exercício pode aumentar a conversão intramuscular da KYN (a qual é capaz de atravessar a BBB), em KYNA (que é incapaz de cruzar a BBB), através da ativação do co-ativador de transcrição gênica PGC-1α (Agudelo et al., 2014). Uma vez que a diminuição dos níveis de KYN no CNS pode reduzir a inflamação e a morte neuronal em doenças neurológicas, este pode ser um dos efeitos imunomodulatórios mediados pelo exercício na proteção contra doenças neurológicas e neurodegenerativas. Os mecanismos anti-inflamatórios induzidos pelo exercício estão ilustrados pela figura 7.

Figura 7. Possíveis mecanismos subjacentes ao efeito anti-inflamatório do exercício físico.



Fonte: Adaptado de Spielman et al. (2016).

Em síntese, os efeitos benéficos do exercício físico realizado regularmente podem ocorrer devido ao efeito anti-inflamatório das contrações musculares. Desta forma, consecutivos episódios de exercício podem atuar de forma imunorregulatória, reduzindo os níveis de neuroinflamação. Tais regulações exercício-induzidas podem ocorrer através de efeitos a longo prazo, por meio da redução da adiposidade visceral/abdominal ou devido ao ambiente anti-inflamatório criado por cada sessão aguda de exercício. Este efeito anti-inflamatório foi confirmado em modelos de camundongos transgênicos para DA (Leem et al., 2011; Parachikova et al., 2008; Nichol et al., 2008), onde o exercício realizado em esteira e rodas de correr foi eficaz em reduzir os níveis de marcadores inflamatórios no hipocampo, com estes resultados ocorrendo em conjunto com a melhoria da função cognitiva. No estudo prévio do nosso laboratório, nós demonstramos que o exercício de natação foi capaz de reduzir os níveis de TNF-α e IL1-β e aumentar os níveis de IL-10 no cérebro de camundongos que receberam infusão i.c.v. do peptídeo $A\beta_{1-40}$, criando um microambiente anti-inflamatório no cérebro, o qual ocorreu em paralelo com a melhoria na capacidade de reconhecimento de objetos.

Apesar destas evidências, os mecanismos neurobiológicos subjacentes ao efeito neuroprotetor do exercício físico ainda precisam ser mais bem

elucidados, sobretudo no que se refere aos seus efeitos pró-neurogênicos e anti-inflamatórios; assim como o seu papel em proteger contra alterações emocionais relacionadas à demência é escasso. Na literatura pode-se verificar que foram testados diferentes protocolos de exercício em esteira e rodas de correr em modelos animais de DA. No entanto, poucos estudos se propuseram a investigar os efeitos do exercício de natação nesses modelos experimentais. Finalmente, a capacidade do exercício físico em modular marcadores da KP em um modelo de DA ainda não foi estudado. Até onde sabemos, o único estudo referente a este assunto é o de Liu et al. (2014), no qual o exercício de natação aliviou o comportamento tipo-depressivo através do seu efeito anti-inflamatório e ao bloquear a ativação da IDO no cérebro de ratos cronicamente estressados. Portanto, este é um mecanismo de atuação que necessita ser investigado a fim de fornecer novas evidências acerca do papel neuroprotetor do exercício físico.

OBJETIVOS

Objetivo Geral

Investigar as alterações comportamentais e neuroquímicas associadas à neurotoxicidade induzida pelo peptídeo $A\beta_{1-42}$ e de que forma o exercício de natação pode atuar na prevenção destas modificações.

Objetivos Específicos

Examinar o envolvimento da via da quinurenina nos distúrbios cognitivos e emocionais induzidos pela infusão i.c.v. do peptídeo $A\beta_{1-42}$ em camundongos jovens e idosos.

Caracterizar as alterações neuroquímicas associadas à neurotoxicidade induzida pelo peptídeo $A\beta_{1-42}$, no córtex pré-frontal e hipocampo de camundongos jovens e idosos.

Comparar as alterações comportamentais e neuroquímicas mediadas pelo peptídeo $A\beta_{1-42}$ entre animais jovens e idosos.

Avaliar o efeito protetor do exercício físico de natação contra as alterações comportamentais e neuroquímicas induzidas pelo peptídeo $A\beta_{1-42}$ em animais jovens.

PARTE II

ARTIGO

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Full-length Article

Indoleamine-2,3-dioxygenase mediates neurobehavioral alterations induced by an intracerebroventricular injection of amyloid-β_{1-42} peptide in mice

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder that is characterized by a progressive cognitive decline along with various neuropsychiatric symptoms, including depression and anxiety. Increasing evidence has been proposed the activation of the tryptophan-degrading indoleamine-2,3-dyoxigenase (IDO), the rate-limiting enzyme of kynurenine pathway (KP), as a pathogenic factor of amyloid-beta (Aβ)-related inflammation in AD. In the current study, the effects of an intracerebroventricular (i.c.v.) injection of $A\beta_{1-42}$ peptide (400 pmol/mice; 3µl/site) on the regulation of KP biomarkers (IDO activity, tryptophan and kynurenine levels) and the impact of $A\beta_{1-42}$ on neurotrophic factors levels were investigated as potential mechanisms linking neuroinflammation to cognitive/emotional disturbances in mice. Our results demonstrated that $A\beta_{1-42}$ induced memory impairment in the object recognition test. $A\beta_{1-42}$ also induced emotional alterations, such as depressive and anxiety-like behaviors, as evaluated in the tail suspension and elevated-plus maze tests, respectively. We observed an increase in levels of proinflammatory cytokines in the $A\beta_{1-42}$-treated mice, which led to an increase in IDO activity in the prefrontal cortex (PFC) and the hippocampus (HC). The IDO activation subsequently increased kynurenine production and the kynurenine/tryptophan ratio and decreased the levels of neurotrophic factors in the PFC and HC, which contributed to Aβ-associated behavioral disturbances. The inhibition of IDO activation by IDO inhibitor 1-methyltryptophan (1-MT), prevented the development of behavioral and neurochemical alterations. These data demonstrate that brain IDO activation plays a key role in mediating the memory and emotional disturbances in an experimental model based on Aβ-induced neuroinflammation.

Keywords: Alzheimer's disease, neuroinflammation, indoleamine-2,3-dyoxigenase, tryptophan, kynurenine, neurotrophic factor, memory, depression, anxiety.

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder that is characterized by a progressive loss of memory and mental function, leading to an inability to care for oneself (Bateman et al., 2012). In addition to cognitive deficits, AD patients frequently exhibit a number of non-cognitive symptoms, namely "neuropsychiatric symptoms of dementia" (NPSD), such as depression and anxiety (Cerejeira et al., 2012; Rosenberg et al., 2015). NPSD comprise the major component of the disease burden for patient and caregivers and are associated with a more rapid disease progression to severe dementia and death (Peters et al., 2015). The amyloid-beta (Aβ) cascade hypothesis of AD pathogenesis postulates that the Aβ accumulation in the brain triggers a cascade of pathological processes that causes a progressive synaptic dysfunction and neuronal loss; ultimately leading to cognitive decline (Ballard et al., 2011).

Emerging evidence suggests that neuroinflammation plays a causal role in AD pathogenesis (Heneka et al., 2015; Heppner et al., 2015). It has been widely recognized that chronic neuroinflammation induced by a number of proinflammatory cytokines, such as interleukin-1beta (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) released from activated microglia and astrocytes, is an early and continuous feature of the AD brain (Heppner et al., 2015; Wang et al., 2015). This inflammatory response may be responsible for the development of behavioral disturbances in AD, such as cognitive impairment (Passos et al., 2010; Souza et al., 2013; Zhang et al., 2012) and neuropsychiatric symptoms (dos Santos et al., 2013; Holmgren et al., 2014; Takeda et al., 2014).

While the mechanism underlying neuroinflammation-induced AD pathology remains not fully elucidated, increasing evidence suggests that the kynurenine pathway (KP) may play an important role (Bonda et al., 2010; Maddison and Giorgini, 2015; Tan et al., 2012; Wu et al., 2013). The KP is the major metabolic route for tryptophan catabolism in the mammalian tissues, which leads to the production of kynurenine and downstream metabolites that have been closely related to the pathogenesis of AD (Gulaj et al., 2010; Kincses et al., 2010). It has been shown that the induction of the first and rate-limiting

enzyme of KP, indoleamine-2,3-dyoxigenase (IDO), which is primarily generated by glial cells in the brain, is driven by proinflammatory cytokines (Maes et al., 2011; Stone and Darlington, 2002). Importantly, IDO was found to be highly expressed in the hippocampus of AD mice and of AD patients, specifically co-localized with pathological hallmarks of AD, such as senile plaques and neurofibrillary tangles (Wu et al., 2013). Accordingly, it has been proposed that the stimulation of the KP by proinflammatory cytokines could be involved with the neurodegenerative pathways in AD (Kincses et al., 2010; Tan et al., 2012).

A recent study shows that in a mouse model of inflammation-associated depression, the induction of IDO is related to the down-regulation of brain-derived neurotrophic factor (BNDF) in the prefrontal cortex and hippocampus of mice (Gibney et al., 2013), two brain regions that are critical components of the cortical-hippocampal circuitry for declarative memory (Eichenbaum, 2000). In fact, the expression of many neurotrophic factors was markedly reduced in the brain of AD patients (Allen et al., 2011; Holsinger et al., 2000) and AD mouse models (Fukomoto et al., 2014; Shin et al., 2014). Neurotrophic factors are important for driving neurogenesis, synaptic plasticity and neuronal survival (Park and Poo, 2013), which are all processes affected by the pathogenesis of AD (Sopova et al., 2014). Therefore, a neurotrophic deficiency could be an additional mechanism implicated with IDO activation by pro-inflammatory cytokines in AD brain.

Several previous studies have described that a single intracerebroventricular (i.c.v.) injection of synthetic Aβ peptides $A\beta_{1-40}$ and $A\beta_{1-42}$, is a useful rodent model for the characterization of Aβ-induced inflammatory and neurotoxic responses, because it mimics the early behavioral alterations observed in AD (Chambon et al., 2011; Chen et al., 2015; Fukomoto et al., 2014; Passos et al., 2010; Piermartini et al. 2010; Rammes et al., 2015; Szczepanik and Ringheim, 2003; Souza et al., 2013). Although the model is unable to induce all of the pathological AD hallmarks, such as amyloid plaque and phospho-tau positive cells, the central injection of Aβ peptides is a paradigm that supports the hypothesis of a key pathogenic role of oligomeric Aβ (Chambon et al., 2011) and enables preclinical evaluation of drugs targeting Aβ

(Van Dam and De Deyn, 2006). Despite extensive studies demonstrating cognitive deficits related to this paradigm, the mechanisms of Aβ-induced neurotoxicity and its effects on non-cognitive disturbances remains not completely understood. In particular, the connections between Aβ-mediated inflammation and activation of IDO in the brain, and how this contributes to behavioral alterations are unknown.

Thus, the objective of this study was to characterize the mechanisms by which the i.c.v. injection of $A\beta_{1-42}$ peptide induces cognitive impairment and emotional alterations, such as depressive and anxiety-like behaviors in mice, secondary to its neuroinflammatory actions. Moreover, neurochemical parameters related to inflammatory status, neurotrophic factors and KP activation were evaluated in the prefrontal cortex and hippocampus in an attempt to elucidate the potential mechanisms that link neuroinflammation to behavioral disturbances. A competitive inhibitor of IDO, 1-methyl-tryptophan (1-MT), was used to investigate the involvement of IDO in the mechanisms of Aβ toxicity.

2. Methods

2.1 Animals

Experiments were performed using male Swiss Albino mice (30-40 g, 90 days old). Animals were maintained at 22-25ºC with free access to water and food, under a 12:12 h light/dark cycle, with lights on at 7:00 a.m. All manipulations were carried out during light phase on the day. All efforts were made to minimize animal suffering and to reduce the number of animals used. The procedures of this study were conducted according to the guidelines of the Committee on Care and Use of Experimental Animals Resources and with the approval of Ethical Committee for Animal Use (CEUA protocol # 012/2012) of Federal University of Pampa, Brazil.

2.2 Experimental design

Two experiments were conducted. In the first experiment, mice (n=8 animals per group) received an i.c.v. injection of $A\beta_{1-42}$ to investigate the

behavioral disturbances and neurochemical parameters in a time-course curve (times: 6 h, 1, 3 and 7 days) (**Fig. 1**).

In the second experiment, mice were divided into four groups (n=8 animals per group): vehicle + PBS (sham-operated); 1-MT + PBS; Aβ + vehicle; and Aβ + 1MT. Seven days after the i.c.v. injection of $A\beta_{1-42}$ (peak effect), mice were subjected to behavioral tests. Afterwards, they were euthanized and the prefrontal cortex and hippocampus were removed for neurochemical assays (**Fig. 1**).

2.3 Drug treatment protocol

$A\beta_{1-42}$ (Sigma-Aldrich) was prepared as stock solution at a concentration of 1 mg/ml in sterile 0.1 M phosphate-buffered saline (PBS) (pH 7.4), and aliquots were stored at -20º C. $A\beta_{1-42}$ was aggregated by incubation at 37ºC for 4 days before use, as described previously (Souza et al., 2013). The aggregated form of $A\beta_{1-42}$ (400pmol/mouse) was administered by i.c.v. injection, using a microsyringe with a 28-gauge stainless-steel needle 3.0 mm long (Hamilton), as described previously (Cioanca et al., 2014; Piermartini et al., 2010; Yan et al. 2001). Briefly, mice were anesthetized with intraperitoneal (i.p.) injection of sodium pentobarbital (0.067 mg/g) and placed in a stereotaxic apparatus (Insight, Brazil). Under light anesthesia (i.e. just that necessary for loss of the postural reflex), the needle was inserted unilaterally 1 mm to the right of the midline equidistant from each eye and 1 mm posterior to a line drawn through the anterior base of the eyes (used as external reference). $A\beta_{1–42}$ was administered right-unilaterally into the lateral ventricle. The injection volume of 3µl of $A\beta_{1–42}$ or PBS was delivered gradually (1µl/min) using the following coordinates from bregma: anteroposterior (AP) = −0.1 mm, mediolateral (ML) = 1 mm, and dorsoventral (DV) = −3 mm. The sham-operated mice were injected with PBS (3µl/site; i.c.v.). The advantage of this i.c.v. route of administration is the rapid distribution of the peptide throughout the brain (Chambon et al., 2011). In order to confirm the accurate placement of the injection site (needle track) at the time of brain dissection, two mice in each group were submitted to dye injection (Evans blue dye, 0,5µl) into the ventricles (Davisson et al., 1998).

A non-selective racemic mixture of 1-MT (1-Methyl-D,L-tryptophan; Sigma-Aldrich) was administered through subcutaneous (s.c.) injection at a dose of 50 mg/kg in a volume of 5 ml/kg, as described earlier (Xie et al., 2014). The injections were administered twice daily at 12-hour intervals for 7 days before Aβ injection. According to Xie et al. (2014), this drug schedule was in accordance with the methods of Professor Keith W. Kelley, who administered 50 mg/kg of 1-MT to mice twice a day with an equal effect to that observed in studies using 5 mg/day pellets (e.g., the study of O'Connor et al., 2009). We prepared 1-MT using 0.1 M NaOH and adjusted the pH to 9.0 using 1 M HCl. The sham-operated and Aβ + vehicle groups were injected with vehicle (normal saline solution) at a volume of 5 ml/kg (s.c.).

2.4 Behavioral assessment

During a period of 2 days after i.c.v. injection of $A\beta_{1-42}$ (starting 1, 3 and 7 days after $A\beta_{1-42}$ injection in experiment 1, and starting 7 days after $A\beta_{1-42}$ injection in experiment 2), the animals were subjected to a battery of behavioral paradigms that included the open-field test and the object recognition test (day 1), and the tail suspension test and the elevated plus-maze task (day 2). In order to minimize the potentially negative effects of stress on cognitive performance of the animals, we separated the emotional tests from the object recognition test. Moreover, it is noteworthy that on day 1, the open-field test was also used as the habituation phase for object recognition test. All tests were carried out between 9:00 and 16:00 h and they were scored by the same trained raters (who were blind to the experimental treatments) in an observation room where the mice had been habituated for at least 1 h before the beginning of the tests.

2.4.1 Open-field test (OFT)

The OFT was carried out to evaluate whether the drugs affected locomotor activity. The animals were subjected to an OFT apparatus (Insight Ltd., Ribeirao Preto, SP, Brazil) individually for a period of 5 minutes. The total distance (unit: mm) was computed and used for analysis (Goes et al., 2014; Prut and Belzung, 2003).

2.4.2 Object recognition test (ORT)

After the OFT, the memory function of mice was assessed with the ORT. The task is based on the spontaneous tendency of rodents towards novelty. Specifically, when they are first exposed to two identical objects, rodents will then explore one of the objects (preference of a novel object) for a longer time than they explore the familiar object. This test requires no external motivation, reward, or punishment and very little training or habituation is required (Winters et al., 2008). The ORT was performed as described by Ennaceur and Delacour (1988) with some modifications. Mice were placed in an open box (similar to OFT), were allowed to explore two identical objects (sample phase) for 5 min, and were subsequently returned to their home cage. To evaluate short-term memory, mice were returned to the open box, after a delay of 90 min (intertrial interval), where they were exposed to two different objects (test phase), one was identical to the one previously encountered in the sample phase, therefore now familiar, and the other was novel. The animals were allowed to explore both objects for more 5 min. After each trial, the box and objects were cleaned with 70 % ethanol. The positions of the objects in the test and the objects used as novel or familiar were counterbalanced between the animals. Exploratory behavior was defined as sniffing or touching the object with the nose and/or forepaws. Any other behavior, such as sitting on or turning around the object was not considered as exploration. The amount of time each animal spent actively investigating the objects was manually scored and the recognition index was calculated as the time exploring novel or familiar object divided by the total time spent exploring both objects.

2.4.3 Tail suspension test (TST)

The second day of behavioral assessments started with the TST. Mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. The immobility time was recorded for 6 min. The immobility behavior was defined according to the method described by Steru et al. (1985).

2.4.4 Elevated plus-maze test (EPMT)

Three hours after the TST, the EPMT was used to evaluate anxiety-like behavior in the mice, in accordance with the method of Pellow et al. (1985). The experimental apparatus (Insight Ltd., Ribeirao Preto, SP, Brazil) was shaped as a plus sign and consisted of two open arms (30 × 5 cm) and two equal-sized closed arms (30 × 5 × 15 cm) extending from a common central platform (5 × 5 cm). The maze was made of opaque grey PVC and was kept elevated at a height of 50 cm above the floor. The experiments were conducted in a sound-attenuated room under low intensity light (12 lx). The animals were individually placed in the central area of the maze facing an enclosed arm and were observed for 5 min. The apparatus was cleaned with an ethanol solution (10% v/v) and dried with paper towels after each trial in order to prevent odors from carrying-over between trials. During a 5 min test period, the number of entries either the open or enclosed arms, plus the time spent in the open arms we recorded. An entry was defined as placing all four paws within the boundaries of the arm. The following measures were obtained from the test: (a) the time spent in the open arms compared to the total time spent in the plus-maze (300 s); (b) the number of entries into the open arms; (c) the number of entries into the closed arms. In this test, the anxiolytic effect of a drug is defined by a significant increase in the entries/or time spent in the open arms (Clénet et al., 2006).

2.5 Tissue Preparation for neurochemical determinations

After behavioral tests, mice were euthanized with barbiturate overdose (pentobarbital sodium 150 mg/kg; i.p. route) and transcardially perfused with 10 ml ice-cold saline via the aorta. The brain dissection was performed according to the method of Spijker (2011), a method to dissect multiple brain regions from a single brain based on existing atlases (Williams, 1999). Prefrontal cortex and hippocampus were bilaterally removed and rapidly homogenized in 50 mM Tris–Cl, pH 7.4. The homogenate was centrifuged at 2,400×*g* for 15 min at 4ºC and a low-speed supernatant fraction (S1) was used for assays.

2.6 Neurochemical assays

2.6.1 Cytokine levels

Levels of interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin 1-beta (IL-1β) and interleukin-10 (IL-10) in the prefrontal cortex and hippocampus were measured using sample aliquots of 100 µL and mouse cytokine ELISA DuoSet Kits from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's instructions (protein range of 31.25–2,000 pg). The level of cytokine was estimated by interpolation from a standard curve by colorimetric measurements at 450 nm (correction wavelength 540 nm) on an ELISA plate reader (Berthold Technologies-Apollo 8-LB 912, KG, Germany). Results are shown as pg/mg of tissue.

2.6.2 Neurotrophic factors levels

Protein levels of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) were measured using a commercially available sandwich enzyme-linked immune sorbent assay (ELISA) kit (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions. BDNF and NGF levels were evaluated in S1 of prefrontal cortex and hippocampus. The BDNF, NGF and GDNF levels were expressed as pg/mg wet weight of tissue.

2.6.3 Tryptophan (TRP) and kynurenine (KYN) levels

The levels of TRP and its metabolite KYN in the prefrontal cortex and hippocampus were performed in a Shimadzu LC-10A liquid chromatograph, according to Silva et al. (2002). The chromatographic separation was achieved using a 250- by 4.6-mm (inner diameter) C_{18} reverse-phase column (particle size, 4 µm; Aquapore RP-300 C-18). For TRP measurement, the column was eluted isocratically at flow rate of 1.0 ml/min with 0.015 M sodium acetate (pH 4.5) containing 15% methanol. For KYN determination, the column was eluted with acetonitrile at a 1:47 dilution in 0.1 M acetic acid–0.1 M ammonium acetate (pH 4.65). The absorbance of the column effluent was monitored at 280 and 365 nm for TRP and KYN respectively. The peaks of TRP or KYN were identified by comparison with the retention times of standard compounds

(Sigma), and quantification was based on the ratios of the peak areas of compound to the internal standard. The tissue levels were expressed in pg/mg tissue.

2.6.4 Indoleamine-2,3-dyoxigenase (IDO) activity

IDO activity in the prefrontal cortex and hippocampus was determined as previously described (Lestage et al., 2002). The supernatants (0.2 ml) were added to 0.8 ml of the reaction mixture containing 400 µM L-tryptophan, 20 mM ascorbate, 10 µM methylene blue, and 100 µg catalase in 50 mM potassium phosphate buffer pH 6.5. The reaction was carried out at 37ºC under agitation for 60 min. Then, it was blocked by adding 0.2 ml of 30% trichloroacetic acid and further incubated at 50ºC for 30 min to convert the N-formylkynurenine to L-kynurenine. Samples were centrifuged at 13,000 *g* for 10 min at 4ºC. The supernatants were filtered through microspin ultrafiltrates with a cut-off of 10,000 M_r before being taken for measurement of IDO.

The amount of L-kynurenine formed from TRP was determined by reversed phase high pressure liquid chromatography (HPLC). One hundred µl of the reaction product was injected onto a Merck LiChrospher column (150 mm long, 4.6 mm diameter, packed with 5 lm silica beads holding 18C long carbon chains). A cartridge guard column containing the same material as the analytical column was used. The mobile phase consisted of 0.1 M ammonium acetate buffer (pH 4.65) with 5% acetonitrile. Flow rate was 1 ml/min. KYN was detected using a spectrometer measuring absorbency at a wavelength of 365 nm and was quantified using known amounts of L-kynurenine. The retention time of KYN was around 5.35 min. All determinations were performed in duplicate. One unit of the activity was defined as 1 nmol KYN/h/mg protein at 37ºC.

2.7 Protein determination

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as the standard.

2.8 Statistical analysis

The data distribution was verified by applying the Kolmogorov-Smirnov test. The results are presented as means ± standard error medium (SEM). Comparisons between the experimental and the control groups were performed by one-way analysis of variance (ANOVA), followed by Tukey post hoc test or two-way ANOVA, followed by Bonferroni post hoc test, when appropriate. A value of $P < 0.05$ was considered to be statistically significant. All tests were carried out using the GraphPad software 5.0 (San Diego, California, USA).

3. Results

3.1 The i.c.v. injection of $A\beta_{1-42}$ affected cognitive and emotional behaviors

The injection of $A\beta_{1-42}$, at 3 and 7 days before the ORT, decreased the recognition index compared to the PBS treated control (sham-operated) group, thereby inducing a memory impairment ($A\beta_{1-42}$ × time interaction, $F_{1,24} = 6.23$, $p < 0.01$; $A\beta_{1-42}$, $F_{1,24} = 56.04$, $p < 0.001$). Post hoc comparisons demonstrated that the peak effect occurred at 7 days after Aβ injection ($p < 0.001$; **Fig. 2**A). Moreover, it is important to emphasize that the deficit in the ORT observed in Aβ-treated mice cannot be explained by a general reduced interest for novelty, as there were no differences observed among animal groups in the total investigation time (i.e., the sum of the time that animals spent investigating the two objects) during both the training and the test phases (data not shown).

Injection of $A\beta_{1-42}$ before TST significantly increased the immobility time when compared to PBS treated controls, which is interpreted as a depressive-like effect ($A\beta_{1-42}$ × time interaction, $F_{1,24} = 9.33$, $p < 0.01$; $A\beta_{1-42}$, $F_{1,24} = 26.90$, $p < 0.001$; time, $F_{1,24} = 5.95$, $p < 0.01$). Post hoc comparisons demonstrated that injection of $A\beta_{1-42}$ produced a depressive-like effect at 7 days ($p < 0.001$; **Fig. 2**B).

When exposed to the EPMT, Aβ-treated mice showed a significant reduction in the time spent on open arms, indicating anxiety-like behavior ($A\beta_{1-42}$ × time interaction, $F_{1,24} = 5.55$, $p < 0.01$). Post hoc comparisons demonstrated that anxiety-like behavior was more evident 7 days after $A\beta_{1-42}$ injection ($p < 0.001$; **Fig. 2**C). No significant differences among groups were observed in the

number of open-arms and enclosed-arm entries (data not shown). Moreover, the $A\beta_{1-42}$ injection did not cause significant alterations in the distance traveled in the OFT ($A\beta_{1-42}$ × time interaction, $F_{1,24} = 0.04$, $p = 0.96$; $A\beta_{1-42}$, $F_{1,24} = 0.02$, $p = 0.88$; time, $F_{1,24} = 0.29$, $p = 0.75$).

3.2 The i.c.v. injection of $A\beta_{1-42}$ induced neuroinflammation in the prefrontal cortex and hippocampus

The neuroinflammation induced by $A\beta_{1-42}$ was characterized by increased levels of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-1β and decreased levels of the anti-inflammatory cytokine IL-10.

In response to $A\beta_{1-42}$ injection, a significant increase in IFN-γ levels was found in the prefrontal cortex compared to PBS treated controls ($F_{4,35} = 97.02$, $p < 0.001$). Post hoc comparisons showed that the injection of $A\beta_{1-42}$ increased IFN-γ levels at 1, 3 and 7 days. The peak effect occurred at the 1 day time point ($p < 0.001$; **Fig. 3**A). IFN-γ levels also increased in the hippocampus compared to PBS treated controls ($F_{4,35} = 29.51$, $p < 0.001$). Post hoc comparisons revealed that the maximal effect occurred 7 days after $A\beta_{1-42}$ injection ($p < 0.001$; **Fig. 3**B).

Aβ-treated mice showed a significant increase in TNF-α levels in the prefrontal cortex ($F_{4,35} = 49.24$, $p < 0.001$) compared to PBS treated controls. Post hoc comparisons showed that the injection of $A\beta_{1-42}$ increased TNF-α levels at 1, 3 and 7 days. The peak effect occurred at 7 days ($p < 0.05$; **Fig. 3**C). A similar pattern of TNF-α levels was found in the hippocampus with the most significant increase occurring at 7 days post administration ($p < 0.001$) ($F_{4,35} = 15.87$, $p < 0.001$; **Fig. 3**D).

A significant increase in IL-1β levels was also observed in the prefrontal cortex ($F_{4,35} = 26.81$, $p < 0.001$) and hippocampus ($F_{4,35} = 40.73$, $p < 0.001$) in Aβ-treated mice compared to PBS treated controls. Post hoc comparisons showed that the levels of IL-1β increased significantly 1, 3 and 7 days after $A\beta_{1-42}$ injection in both brain regions, with a peak effect at 7 days ($p < 0.001$; **Fig. 3**E and **Fig. 3**F, respectively).

$A\beta_{1-42}$ injections caused a significant decrease in IL-10 levels in the prefrontal cortex compared to PBS treated controls ($F_{4,35} = 24,61$, $p < 0.001$).

Post hoc comparisons showed that the injection of $A\beta_{1-42}$ decreased IL-10 levels at 1, 3 and 7 days. The peak effect occurred at 7 day time point ($p < 0.001$; **Fig. 3**G). A significant decrease in IL-10 levels was observed in the hippocampus of Aβ-treated mice compared to PBS treated controls at 1 day, 3 days and 7 day, again with maximal effect at 7 days post $A\beta_{1-42}$ injection ($p < 0.01$) ($F_{4,35} = 8.393$, $p < 0.001$; **Fig. 3**H).

3.3 The i.c.v. injection of $A\beta_{1-42}$ caused a down-regulation of neurotrophic factors in the prefrontal cortex and hippocampus

Aβ-treated mice showed a significant decrease in BDNF levels in the prefrontal cortex compared to PBS treated controls ($F_{4,35} = 13.47$, $p < 0.001$). Post hoc comparisons showed that the injection of $A\beta_{1-42}$ decreased BDNF levels at 1, 3 and 7 days of the time points. The peak effect occurred at 7 days ($p < 0.001$; **Fig. 4**A). A similar pattern of BDNF levels was found in the hippocampus with the most significant increase occurring at 7 days post-injection ($p < 0.001$) ($F_{4,35} = 18.00$, $p < 0.001$; **Fig. 4**B).

In response to $A\beta_{1-42}$ injection, NGF levels were significantly reduced in the prefrontal cortex at 1, 3 and 7 days ($p < 0.05$, $p < 0.05$ and $p < 0.001$, respectively) ($F_{4,35} = 10.51$; $p < 0.001$; **Fig. 4**C). In the hippocampus, reduced NGF levels were found at 7 days post administration ($p < 0.01$) ($F_{4,35} = 10.69$, $p < 0.001$; **Fig. 4**D).

Decreased levels of GDNF were observed in prefrontal cortex at 7 days post $A\beta_{1-42}$ administration ($p < 0.05$) ($F_{4,35} = 5.339$, $p < 0.01$; **Fig. 4**E). A decrease in GDNF levels was also detected in the hippocampus in response to $A\beta_{1-42}$ 7 days post injection ($p < 0.05$) ($F_{4,35} = 4.713$, $p < 0.01$; **Fig. 4**F).

3.4 The i.c.v. injection of $A\beta_{1-42}$ caused IDO activation coupled with an increase of TRP levels and KYN production in the prefrontal cortex and hippocampus

A significant increase in IDO activity was found in the prefrontal cortex of Aβ-treated mice when compared to control animals ($F_{4,35} = 48.83$, $p < 0.001$). Post hoc comparisons showed that the injection of $A\beta_{1-42}$ increased IDO activity in a time-dependent manner. The peak effect occurred at 7 days ($p < 0.001$; **Fig. 5**A). In a similar fashion, an increase in IDO activity was found in

hippocampus when compared to PBS treated controls ($F_{4,35}$ = 32.24, p < 0.001). Post hoc comparisons revealed that the maximal effect occurred 7 days after $A\beta_{1-42}$ injection (p < 0.001; **Fig. 5**B).

Increased levels of TRP were observed in prefrontal cortex at 7 days post $A\beta_{1-42}$ administration (p < 0.05) ($F_{4,35}$ = 5.108, p < 0.01; **Fig. 5**C). In the hippocampus, increased TRP levels was found at 3 and 7 days post administration, again with peak effect at 7 days (p < 0.001) ($F_{4,35}$ = 12.78, p < 0.001; **Fig. 5**D).

When compared to PBS treated controls, mice exposed to $A\beta_{1-42}$ showed a significant increase in the levels of KYN in the prefrontal cortex at 1, 3 and 7 days post administration (p < 0.001) ($F_{4,35}$ = 47.21, p < 0.001; **Fig. 5**E). This translated to an increased KYN/TRP ratio at 1, 3 and 7 days post-Aβ administration (p < 0.001) ($F_{4,35}$ = 16.67, p < 0.001; **Fig. 5**F). Similarly, the levels of KYN produced in the hippocampus was significantly higher at 1, 3 and 7 days post $A\beta_{1-42}$ injection (p <0.01, p < 0.001, p < 0.001, respectively) ($F_{4,35}$ = 41.76, p < 0.001; **Fig. 5**G). Once again, this increase in KYN translated to an increase in the KYN/TRP ratio at 1, 3 and 7 days (p < 0.001) ($F_{4,35}$ = 26.34, p < 0.001; **Fig. 5**H).

3.5 $A\beta_{1-42}$-induced behavioral disturbances is blocked by 1-MT pretreatment

In experiment 2, mice were pretreated with 1-MT during 7 days before i.c.v. injection of $A\beta_{1-42}$. Memory function, non-cognitive behaviors and locomotor activity were assessed starting at 7 days (peak effect) post-Aβ injection.

Two-way ANOVA of recognition index in ORT demonstrated a significant effect of $A\beta_{1-42}$ ($F_{1,24}$ = 31.61, p < 0.001), 1-MT ($F_{1,24}$ = 26.66, p < 0.001) and $A\beta_{1-42}$ × 1-MT interaction ($F_{1,24}$ = 37.68, p < 0.001). Post hoc comparisons revealed that the 1-MT pretreatment significantly blocked the decrease in recognition index induced by $A\beta_{1-42}$ (p < 0.001; **Fig. 6**A). The total time exploration in ORT was not changed significantly by $A\beta_{1-42}$ ($F_{1,24}$ = 0.01, p = 0.9379), 1-MT ($F_{1,24}$ = 0.85, p = 0.3698) or their interaction ($F_{1,24}$ = 0.03, p = 0.8559; **Fig. 6**B), ensuring that the performance in the tests was not influenced by motivational interferences or locomotor deficits.

Statistical analysis of immobility time in TST demonstrated a significant effect of $A\beta_{1-42}$ ($F_{1,24}$ = 16.31, p < 0.001), 1-MT ($F_{1,24}$ = 85.35, p < 0.001) and $A\beta_{1-42}$× 1-MT interaction ($F_{1,24}$ = 67.11, p < 0.001). Post hoc comparisons revealed that the 1-MT pretreatment significantly protected against the increase in immobility time caused by $A\beta_{1-42}$ (p < 0.001; **Fig. 6**C).

Two-way ANOVA of time spent in open arms in EPMT yielded a significant $A\beta_{1-42}$ × 1-MT interaction ($F_{1,24}$ = 4.62, p < 0.05). Post hoc comparisons revealed that the 1-MT pretreatment significantly protected against the decrease in the time spent in open arms caused by $A\beta_{1-42}$ (p < 0.01; **Fig. 6**D). The number of open-arms entries in EPMT was not changed significantly by $A\beta_{1-42}$ injection ($F_{1,24}$ = 0.11, p = 0.7417), 1-MT pretreatment ($F_{1,24}$ = 0.37, p = 0.5550) or their interaction ($F_{1,24}$ = 0.02, p = 0.9174; **Table 1**). No significant differences among groups were observed in the number of entries in enclosed-arms ($A\beta_{1-42}$: $F_{1,24}$ = 0.01, p = 0.9294); (1-MT: $F_{1,24}$ =0.03, p = 0.8594); ($A\beta_{1-42}$ × 1-MT interaction: $F_{1,24}$ = 0.13, p = 0.7237) and open-arms ($A\beta_{1-42}$: $F_{1,24}$ = 0.11, p = 0.7417); (1-MT: $F_{1,24}$ = 0.11, p = 0.7417); ($A\beta_{1-42}$ × 1-MT interaction: $F_{1,24}$ = 0.37, p = 0.5550) in the EPMT and distance travelled in the OFT ($A\beta_{1-42}$: $F_{1,24}$ = 0.02, p = 8958); (1-MT: $F_{1,24}$ = 0.18, p = 0.6772); ($A\beta_{1-42}$ × 1-MT interaction: $F_{1,24}$ = 0.04, p = 0.8443), reinforcing that locomotor activity was not significantly affected by treatments (**Table 1**).

3.6 $A\beta_{1-42}$-induced neuroinflammation is attenuated by 1-MT

Two-way ANOVA of IFN-γ levels in the prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × 1-MT interaction ($F_{1,24}$ = 69.02, p < 0.001). Post hoc comparisons revealed that 1-MT pretreatment attenuated the increase of IFN-γ levels caused by $A\beta_{1-42}$ (p < 0.001; **Fig. 7**A). Similarly, two-way ANOVA of IFN-γ levels in the hippocampus yielded a significant $A\beta_{1-42}$ × 1-MT interaction ($F_{1,24}$ = 56.30, p < 0.001). Post hoc comparisons showed that the increased IFN-γ levels observed in the hippocampus of Aβ-treated mice was mitigated by 1-MT pretreatment (p < 0.001; **Fig. 7**B).

Statistical analysis of TNF-α in the prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × 1-MT interaction ($F_{1,24}$ = 57.56, p < 0.001). Post hoc comparisons revealed that the increase of TNF-α induced by $A\beta_{1-42}$ was

significantly attenuated by 1-MT pretreatment ($p < 0.001$; **Fig. 7**C). As with the prefrontal cortex, statistical analysis of TNF-α in the hippocampus yielded a significant $A\beta_{1\text{-}42}$ × 1-MT interaction ($F_{1,24} = 83.26$, $p < 0.001$). Post hoc comparisons showed that the increased TNF-α levels observed in the hippocampus of Aβ-treated mice was mitigated by 1-MT pretreatment ($p < 0.001$; **Fig. 7**D).

Two-way ANOVA of IL-1β levels in the prefrontal cortex demonstrated a significant $A\beta_{1\text{-}42}$ × 1-MT interaction ($F_{1,24} = 41.51$, $p < 0.001$). Post hoc comparisons revealed that 1-MT pretreatment attenuated the increase of IL-1β levels caused by $A\beta_{1\text{-}42}$ (**Fig. 7**E). Similarly, two-way ANOVA of IL-1β levels in the hippocampus yielded a significant $A\beta_{1\text{-}42}$ × 1-MT interaction ($F_{1,24} = 45.58$, $p < 0.001$). Post hoc comparisons showed that the increased IL-1β levels observed in the hippocampus of Aβ-treated mice was mitigated by 1-MT pretreatment ($p < 0.001$; **Fig. 7**F).

Statistical analysis of IL-10 in the prefrontal cortex demonstrated a significant main effect of $A\beta_{1\text{-}42}$ ($F_{1,24} = 46.97$, $p < 0.001$) and 1-MT pretreatment ($F_{1,24} = 7.47$, $p < 0.05$). Post hoc comparisons revealed that $A\beta_{1\text{-}42}$ significantly reduced IL-10 levels in the prefrontal cortex of mice compared to sham group ($p < 0.001$). 1-MT pretreatment partially protected against the decrease of IL-10 induced by $A\beta_{1\text{-}42}$ ($p < 0.05$; **Fig. 7**G). In the hippocampus, statistical analysis of IL-10 yielded a significant $A\beta_{1\text{-}42}$ × 1-MT interaction ($F_{1,24} = 9.00$, $p < 0.05$). Post hoc comparisons showed that the decrease in IL-10 levels of Aβ-treated mice was mitigated by 1-MT pretreatment ($p < 0.05$; **Fig. 7**H).

3.7 The decrease of neurotrophic factors induced by $A\beta_{1\text{-}42}$ is markedly prevented by 1-MT

Two-way ANOVA of BDNF levels in the prefrontal cortex demonstrated a significant $A\beta_{1\text{-}42}$ × 1-MT interaction ($F_{1,24} = 15.26$, $p < 0.01$). Post hoc comparisons revealed that the Aβ-induced decrease of BDNF levels is normalized by 1-MT pretreatment ($p < 0.001$; **Table 2**). In the hippocampus, two-way ANOVA of BDNF levels yielded a significant $A\beta_{1\text{-}42}$ × 1-MT interaction ($F_{1,24} = 21.42$, $p < 0.001$). Post hoc comparisons showed that 1-MT

pretreatment significantly protected against Aβ-induced BDNF deficit ($p < 0.001$; **Table 2**).

Statistical analysis of NGF levels in prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × 1-MT interaction ($F_{1,24} = 6.43$, $p < 0.05$). Post hoc comparisons revealed that the decrease of NGF levels induced by $A\beta_{1-42}$ was significantly prevented by 1-MT pretreatment ($p < 0.01$; **Table 2**). As with the prefrontal cortex, statistical analysis of NGF in the hippocampus yielded a significant $A\beta_{1-42}$ × 1-MT interaction $F_{1,24} = 7.36$, $p < 0.05$. Post hoc comparisons showed that the decreased NGF levels observed in the hippocampus of Aβ-treated mice was also prevented by 1-MT pretreatment ($p < 0.01$; **Table 2**).

Two-way ANOVA of GDNF levels in the prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × 1-MT interaction ($F_{1,24} = 8.03$, $p < 0.05$). Post hoc comparisons revealed that 1-MT pretreatment attenuated the decrease of GDNF levels caused by $A\beta_{1-42}$ ($p < 0.01$; **Table 2**). In the hippocampus, two-way ANOVA of GDNF levels showed a significant main effect of $A\beta_{1-42}$ ($F_{1,24} = 17.69$, $p < 0.01$) and a main effect of 1-MT ($F_{1,24} = 88.62$, $p < 0.001$). Post hoc comparisons revealed that the injection of $A\beta_{1-42}$ significantly reduced the GDNF levels ($p < 0.05$). 1-MT pretreatment markedly abrogated the GDNF deficit induced by $A\beta_{1-42}$ ($p < 0.05$; **Table 2**).

3.8 1-MT blocks $A\beta_{1-42}$-induced brain IDO activation

Two-way ANOVA of IDO activity in the prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × 1-MT interaction ($F_{1,24} = 42.55$, $p < 0.001$). Post hoc comparisons revealed that $A\beta_{1-42}$ injection significantly increased IDO activity when compared to sham group ($p < 0.001$). 1-MT pretreatment notably blocked the induction of IDO induced by $A\beta_{1-42}$ ($p < 0.001$; **Fig. 8**A). In a similar manner, two-way ANOVA of IDO activity in the hippocampus demonstrated a significant $A\beta_{1-42}$ × 1-MT interaction ($F_{1,24} = 20.03$, $p < 0.001$). Post hoc comparisons showed that the injection of $A\beta_{1-42}$ significantly increased IDO activity in the hippocampus ($p < 0.001$). Once again 1-MT significantly blocked the induction of IDO induced by $A\beta_{1-42}$ ($p < 0.001$; **Fig. 8**B).

3.9 The increase of TRP levels and KYN production elicited by Aβ$_{1-42}$ is abrogated by 1-MT pretreatment

Two-way ANOVA of TRP levels in the prefrontal cortex demonstrated a significant Aβ$_{1-42}$ × 1-MT interaction ($F_{1,24}$ = 14.99, p < 0.01). Post hoc comparisons revealed that the Aβ-induced increase of TRP levels is normalized by 1-MT pretreatment (p < 0.001; **Fig. 9**A). Similarly, two-way ANOVA of TRP levels in the hippocampus revealed a significant Aβ$_{1-42}$ × 1-MT interaction ($F_{1,24}$ = 22.06, p < 0.001). Post hoc comparisons demonstrated that 1-MT pretreatment significantly abrogated the increase of TRP levels in the hippocampus induced by Aβ$_{1-42}$ (p < 0.001; **Fig. 9**B).

Statistical analysis of KYN levels in prefrontal cortex yielded a significant Aβ$_{1-42}$ × 1-MT interaction ($F_{1,24}$ = 106.00, p < 0.001). Post hoc comparisons revealed that the increase of KYN levels induced by Aβ$_{1-42}$ was significantly mitigated by 1-MT pretreatment (p < 0.001; **Fig. 9**C). As with the prefrontal cortex, statistical analysis of KYN levels in the hippocampus demonstrated a significant Aβ$_{1-42}$ × 1-MT interaction ($F_{1,24}$ = 43.44, p < 0.001). Post hoc comparisons showed that the increased KYN levels observed in the hippocampus of Aβ-treated mice was also prevented by 1-MT pretreatment (p < 0.001; **Fig. 9**D).

Two-way ANOVA of KYN/TRP ratio in the prefrontal cortex demonstrated a significant Aβ$_{1-42}$ × 1-MT interaction ($F_{1,24}$ = 118.10, p < 0.001). Post hoc comparisons revealed that the Aβ-induced increase of KYN/TRP ratio is attenuated by 1-MT pretreatment (p < 0.01; **Fig. 9**E). In similar manner, two-way ANOVA of KYN/TRP ratio in the hippocampus yielded a significant Aβ$_{1-42}$ × 1-MT interaction ($F_{1,24}$ = 36.06, p < 0.001). Post hoc comparisons showed that 1-MT pretreatment significantly abrogated the increase of TRP levels in the hippocampus induced by Aβ$_{1-42}$ (p < 0.01; **Fig. 9**F).

4. Discussion

In the present study we sought to investigate the potential mechanisms that link neuroinflammation and cognitive/emotional alterations induced by an

i.c.v. injection of $A\beta_{1-42}$ in mice. The $A\beta_{1-42}$ peptide was chosen due to its increased level of neurotoxicity (Chambon et al., 2011) and to its ability to induce IDO activation in contrast to $A\beta_{1-40}$ (Guillemin et al., 2003). Although more studies are needed to confirm this, a reasonable explanation for the activation of IDO by $A\beta_{1-42}$ and not by $A\beta_{1-40}$, is due to its strong stimulation of IFN-γ, a potent cytokine inducer of IDO (Yamada et al., 2009).

4.1 The i.c.v. injection of $A\beta_{1-42}$ induced cognitive and emotional disturbances

The present study demonstrates that $A\beta_{1-42}$-treated mice, as expected, displayed a poor performance in the object recognition test (ORT), which is highly dependent upon the function of the hippocampal and prefrontal cortex regions (Warburton and Brown, 2015). Because the ORT is used to evaluate declarative memory and object recognition has shown to be impaired in patients with AD (Winter et al., 2008), this test has an important application in the study of AD (Antunes and Biala, 2012). The time course shows that the impairment of recognition memory in the ORT occurred at 3 days and 7 days after $A\beta_{1-42}$ administration, with a maximal effect at 7 days. Due to the short retention time (90 minutes) used in the present study, we suggest that the $A\beta_{1-42}$ caused a deterioration in short-term memory, which is a common and early symptom of AD (Antunes and Biala, 2012). From a mechanistic perspective, the Aβ neurotoxicity could have effects on the encoding and consolidation of object characteristics that were reflected by a decreased preference for the novel object during the test phase.

In addition to progressive memory loss, AD patients frequently exhibited NPSD, including depression and anxiety, which are strongly correlated with the degree of functional and cognitive impairment, worsen the disease progress (Cerejeira et al., 2012). In order to investigate whether such behaviors are affected in $A\beta_{1-42}$-treated mice, we resort to the tail suspension test (TST) and elevated plus-maze test (EMPT), two behavioral paradigms widely used for the screening of antidepressants and anxiolytic drugs, respectively (Castagné et al., 2006). Our results demonstrate that the cognitive impairments in mice occurred together with emotional responses measured by the TST and EPMT 7 days following $A\beta_{1-42}$ administration, resembling the emotional disturbances observed

in AD patients. Thus, our data corroborate clinical observations and provides additional evidence that the cognitive dysfunction accompanies depression and psychobehaviorals signs and symptoms in early stages of AD (Cerejeira et al., 2012).

4.2 *The i.c.v. injection of $A\beta_{1-42}$ induced a neuroinflammatory response and a neurotrophic deficiency*

There is a considerable amount of evidence suggesting that neuroinflammation is involved in the mechanisms of Aβ-induced neurotoxicity (Chen et al., 2015; Passos et al., 2010; Souza et al., 2013) and AD pathogenesis (Heneka et al., 2015; Heppner et al., 2015; Wang et al., 2015). In both lipopolysaccharide (LPS)-induced and paraquat models of Parkinson's disease, a strong link has been made between depression-anxiety symptoms and inflammation (Litteljohn et al., 2008; Zang et al., 2012). In the present study, increased levels of IL-1β, TNF-α and IFN-γ and decreased levels of IL-10 were detected in the prefrontal cortex and hippocampus of mice 1 day, 3 days and 7 days following $A\beta_{1-42}$ administration. The maximal levels were found at 7 days, a time at which behavioral disturbances were also most evident. An important finding of our study was the biphasic response of IFN-γ in the prefrontal cortex, which was composed of an initial exponential increase 1 day after the $A\beta_{1-42}$ injection, followed by a decrease at the times 3 and 7 days. In regard to IL-10, it is one of the main anti-inflammatory cytokines that plays an important role in cell survival and neuronal homeostasis, playing a brain-protective effect (Zhang and Jiang, 2015). Together with the literature, the current study supports and extends the notion that an inflammatory response, reflected by a cytokine dysregulation in brain regions implicated with memory and executive function, can be an important neurobiological mechanism of Aβ-induced cognitive impairment and emotional alterations in mice.

Neurotrophic factors are proteins that play a vital role in regulating all aspects of neural circuit development and function in the mammalian brain, including neuronal survival, synaptic plasticity, neurogenesis, memory consolidation and the pathophysiology of AD (Allen et al., 2011, 2013; Budni et al., 2015; Park and Poo, 2013 Sopova et al., 2014). In this study, the time-

course curve shows that the i.c.v. injection of $A\beta_{1-42}$ induced a neurotrophic deficiency that peaked at the 7 day time point, as measured by the reduction in BDNF, NGF and GDNF levels in the prefrontal cortex and hippocampus of mice. Therefore, we suggest that the neurotrophic deficiency in the prefrontal cortex and hippocampus that occurs in response to $A\beta_{1-42}$ injection is related to the observed impairments in recognition memory, depressive-like and anxiety-like behaviors.

4.3 The i.c.v. injection of $A\beta_{1-42}$ increased IDO activity and KYN production without depleting TRP levels

It has been proposed that IDO plays a pivotal role in the pathogenesis of AD (Bonda et al., 2010; Kincses et al., 2010; Maddison and Giorgini, 2015; Tan et al., 2012). It is known that the increase in proinflammatory cytokines triggers IDO activation under conditions of brain inflammation (Guillemin and Brew, 2002). In the present study, we demonstrated that $A\beta_{1-42}$ induced an increase in IDO activity in the prefrontal cortex and hippocampus of mice 3 days and 7 days post-injection. This activation of IDO coincides temporally with the appearance of the memory impairment and emotional alterations, indicating a key role for this enzyme in early behavioral disturbances of AD. The increase of IDO activity in response to $A\beta_{1-42}$ occurred 48 h after the increase of pro-inflammatory cytokines, supporting the notion for a major role of these substances in the induction of IDO. It is worth highlighting that the IFN-γ demonstrated to be the main activating factor of IDO, corroborating a previous *in vitro* study that showed that the priming effect of $A\beta_{1-42}$ for the IDO induction is mediated by IFN-γ (Yamada et al., 2009). In accord with our study, Wu et al. (2013) have reported that higher immunoreactivity of IDO and tryptophan-2,3-dioxigenase (TDO), another initial enzyme of the KP, was observed in the hippocampus of AD patients. Therefore, we demonstrate for the first time that a single i.c.v. injection of $A\beta_{1-42}$ causes IDO activation in the prefrontal cortex and hippocampus of mice. Further investigation is also necessary to clarify the involvement of TDO in amyloid-related inflammation.

In our study, the increase in IDO activity induced by $A\beta_{1-42}$ was followed by increased levels of TRP in the prefrontal cortex and hippocampus. These

findings are somewhat counterintuitive, and certainly argue against the hypothesis that IDO activation depletes TRP bioavailability for 5-HT synthesis. In contrast with our results, earlier clinical studies have reported that TRP degradation leads to a serotonergic system deficiency which may exert its impact on cognitive symptoms in AD (Porter et al., 2000, 2003). Thereby, our study suggests that the neurobehavioral complications induced by $A\beta_{1-42}$ do not involve an acute TRP degradation. The increase in brain TRP caused by an inflammatory stimulus, such as $A\beta_{1-42}$ peptide infusion, could be explained as a compensatory mechanism of the brain in response to acute inflammation.

Most notably, we demonstrated that $A\beta_{1-42}$ injection precipitated behavioral disturbances coupled with increased kynurenine (KYN) levels and a KYN:TRP ratio with a compatible time course, supporting the involvement of brain KYN metabolism in driving AD symptoms. Corroborating our result, it has shown that a single peripheral injection of KYN was sufficient to induce a deficit in recognition memory in mice (Heisler and O'Connor, 2015). The observation that the $A\beta_{1-42}$ injection induced an increase in KYN levels independently of TRP depletion suggests an alternative hypothesis for the involvement of IDO in $A\beta_{1-42}$-induced behavioral disturbances, e.g., the generation of neuroactive TRP metabolites. For instance, Myint and Kim (2003) have previously proposed that the activation of IDO during inflammatory conditions switches the metabolism of KYN toward the production of neurotoxic metabolites by microglia cells and that these metabolites are important in the pathogenesis of AD (Guillemin and Brew, 2002). Of these metabolites, the N-metil-D-Aspartato (NMDA) receptor agonist quinolinic acid (QUIN) may be one of the critical factors in the pathomechanisms of neuronal damage and death in AD (Guillemin and Brew, 2002), mainly due its roles in reduction of the neurogenesis and the amplification of the inflammatory response within the brain (Heyes et al., 1993).

Thus, in the present study, we indicate that the activation of the KYN pathway, revealed by the increase of IDO activity and KYN levels in the brain, independently of TRP depletion, is involved in $A\beta_{1-42}$-induced cognitive and emotional alterations in mice. Although the QUIN levels were not measured in the current study, we speculate that a shift towards the neurotoxic arm of KP

could be driven by $A\beta_{1-42}$ injection. Further investigations should be aimed at addressing this possibility.

4.4 The inhibition of IDO activation with 1-MT blocked behavioral disturbances and abrogated neuroinflammatory response and neurotrophic deficiency induced by $A\beta_{1-42}$ injection

To determine the role of the enzyme IDO in $A\beta_{1-42}$-induced behavioral alterations, we administered the IDO inhibitor 1-MT twice daily for 1 week before i.c.v. $A\beta_{1-42}$ injection (experiment 2). The behavioral and neurochemical measurements were conducted at 7 days post $A\beta_{1-42}$ administration (i.e., the "peak effect" detected in the experiment 1). We demonstrated that the pharmacological blockade of IDO activation with 1-MT prevented the recognition memory impairment and depressive and anxiety-like responses induced by $A\beta_{1-42}$ infusion, similar to other studies (Lawson et al., 2013; O'Connor et al., 2009; Salazar et al., 2012). Therefore, we suggest that behavioral disturbances elicited by $A\beta_{1-42}$ are driven by IDO activity in the prefrontal cortex and hippocampus of mice.

In this study, we also demonstrated that 1-MT pretreatment attenuated the neuroinflammatory response and the neurotrophic deficiency in the prefrontal cortex and hippocampus of $A\beta_{1-42}$-treated mice. This result is relevant because it shows that most of the neuroinflammation caused by $A\beta_{1-42}$ is mediated by IDO. This also suggests that IDO is involved in the neurotrophic deficit found in AD. In the present study, 1-MT pretreatment normalized TRP levels and abrogated the increase in KYN production and the KYN:TRP ratio in the prefrontal cortex and hippocampus of $A\beta_{1-42}$-injected mice. Therefore, we suggest that increased KYN levels and possibly the neurotoxic end-products of the KP are the potential pathogenic precipitants of cognitive and neuropsychiatric-like behaviors induced by the $A\beta_{1-42}$ peptide. Here, we can further speculate that the broad spectrum of action of 1-MT was due to the blockage of QUIN production and thus the amplification of the inflammatory response, although possible off-target actions of 1-MT may be involved. Taken together, our study supports the idea that a single i.c.v. injection of $A\beta_{1-42}$ peptide induces cognitive impairment and emotional disturbances in mice

through IDO activation in the prefrontal cortex and hippocampus and that the neuroinflammatory response is the initial step. A summary of the mechanistic explanations can be seen in **Fig. 10**. The data in the present study are the first to suggest a mechanistic role for the KP in $A\beta_{1-42}$-induced neuroinflammation and behavioral abnormalities in mice.

5. Conclusions

Our data strongly suggest a critical role for IDO in mediating the cognitive impairment and emotional perturbations induced by $A\beta_{1-42}$-related neuroinflammation. The present study is an important step toward understanding the onset of cognitive and neuropsychiatric alterations associated with AD, specifically the link between early memory loss and depression and anxiety, as well as the role of brain cytokines and IDO activation in this mechanism. Thus, we suggest that the KP and its metabolites are important targets for novel therapeutic interventions in AD, providing an alternative or potential adjunct to anti-inflammatory and anti-amyloid therapies in alleviating AD symptoms.

Conflict of Interest

The authors have no conflicts of interest to report.

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Figure legends

Fig. 1. Overview of study design. $A\beta_{1-42}$, amyloid-beta peptide 1-42; i.c.v., intracerebroventricular; IDO, indoleamine-2,3-dyoxygenase; 1-MT, 1-methyltryptophan; PBS, phosphate buffered saline; s.c., subcutaneously.

Fig. 2. Effect of $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on the recognition index in the ORT (A), immobility time in the TST (B), time in the open arms of the EPMT (C) and total distance in the OFT (D) in groups tested 6 h, 1 day, 3 days and 7 days after $A\beta_{1-42}$ injection. Values are means ± S.E.M. (n=8). *P<0.05, **P<0.01, ***P<0.001 compared with the sham group (Two-way ANOVA, Bonferroni post hoc test).

Fig. 3. Effect of $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on the levels of IFN-γ (A and B), TNF-α (C and D), IL-1β (E and F) and IL-10 (G and H) in the prefrontal cortex (A, C, E and G) and hippocampus (B, D, F and H) of mice in groups tested 6 h, 1 day, 3 days and 7 days after $A\beta_{1-42}$ injection. Values are means ± S.E.M. (n=8). *P<0.05, **P<0.01, ***P<0.001 compared with the sham group (One-way ANOVA, Tukey post hoc test).

Fig. 4. Effect of $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on the levels of BDNF (A and B), NGF (C and D) and GDNF (E and F) in the prefrontal cortex (A, C and E) and hippocampus (B, D and F) of mice in groups tested 6 h, 1 day, 3 days and 7 days after $A\beta_{1-42}$ injection. Values are means ± S.E.M. (n=8). *P<0.05,

**P<0.01, ***P<0.001 compared with the sham group (One-way ANOVA, Tukey post hoc test).

Fig. 5. Effect of $A\beta_{1-42}$ (400 pmol; 3µl/site; i.c.v.) on IDO activity (A and B), TRP levels (C and D), KYN levels (E and F) and KYN/TRP ratio (G and H) in the prefrontal cortex (A, C, E and G) and hippocampus (B, D, F and H) of mice in groups tested 6h, 1 day, 3 days and 7 days after $A\beta_{1-42}$ injection. Values are means ± S.E.M. (n=8). *P<0.05, **P<0.01, ***P<0.001 compared with the sham group (One-way ANOVA, Tukey post hoc test).

Fig. 6. Effects of 1-MT (50 mg/kg; s.c.) on the recognition index (A) and total exploration time (B) in the ORT, immobility time in the TST (C) and time in open arms in the EPMT (D) 7 days after an i.c.v. injection of $A\beta_{1-42}$. Values are means ± S.E.M. (n=8). @P<0.05 when compared $A\beta_{1-42}$+vehicle with PBS+vehicle. #P<0.05 when compared $A\beta_{1-42}$/1-MT with $A\beta_{1-42}$+vehicle (Two-way ANOVA, Bonferroni post hoc test).

Fig. 7. Effects of 1-MT (50 mg/kg; s.c.) on the levels of IFN-γ (A and B), TNF-α (C and D), IL-1β (E and F) and IL-10 (G and H) in the prefrontal cortex (A, C, E and G) and hippocampus (B, D, F and H) of mice 7 days after an i.c.v. injection of $A\beta_{1-42}$. Values are means ± S.E.M. (n=8). @P<0.05 when compared $A\beta_{1-42}$+vehicle with PBS+vehicle. #P<0.05 when compared $A\beta_{1-42}$/1-MT with $A\beta_{1-42}$+vehicle (Two-way ANOVA, Bonferroni post hoc test).

Fig. 8. Effects of 1-MT (50 mg/kg; s.c.) on the IDO activity in the prefrontal cortex (A) and hippocampus (B) of mice 7 days after an i.c.v. injection of $A\beta_{1-42}$. Values are means ± S.E.M. (n=8). @P<0.05 when compared $A\beta_{1-42}$+vehicle with PBS+vehicle. #P<0.05 when compared $A\beta_{1-42}$/1-MT with $A\beta_{1-42}$+vehicle (Two-way ANOVA, Bonferroni post hoc test).

Fig. 9. Effects of 1-MT (50 mg/kg; s.c.) on TRP levels (A and B), KYN levels (C and D) and KYN/TRP ratio (E and F) in the prefrontal cortex (A, C and E) and hippocampus (B, D and F) of mice 7 days after an i.c.v. injection of $A\beta_{1-42}$.

Values are means ± S.E.M. (n=8). @P<0.05 when compared $A\beta_{1-42}$+vehicle with PBS+vehicle. #P<0.05 when compared $A\beta_{1-42}$/1-MT with $A\beta_{1-42}$+vehicle (Two-way ANOVA, Bonferroni post hoc test).

Fig. 10. Diagram showing the mechanistic links between neuroinflammation and neurobehavioral disturbances induced by $A\beta_{1-42}$ i.c.v. injection in mice. (+) increase; (-) decrease; underlined: mechanisms demonstrated in this study. First, the proinflammatory activation, as measured by the increase in the levels of proinflammatory cytokines following $A\beta_{1-42}$ injection caused IDO activation and subsequent increased levels of KYN, which may mediate the observed behavioral disturbances. Hence, KYN accumulation within the brain may activate neurodegenerative pathways via KYN neurotoxic metabolites that ultimately provoke a decrease in neurotrophic factor levels. This neurotrophic deficit could contribute to the observed neurobehavioral complications. Finally, the neuroinflammatory signature induced directly by $A\beta_{1-42}$ peptide may be caused neurotoxicity partially through IDO-independent mechanisms, such as the disruption of the neuroprotection via down-regulation of neurotrophic factors and anti-inflammatory cytokine IL-10.

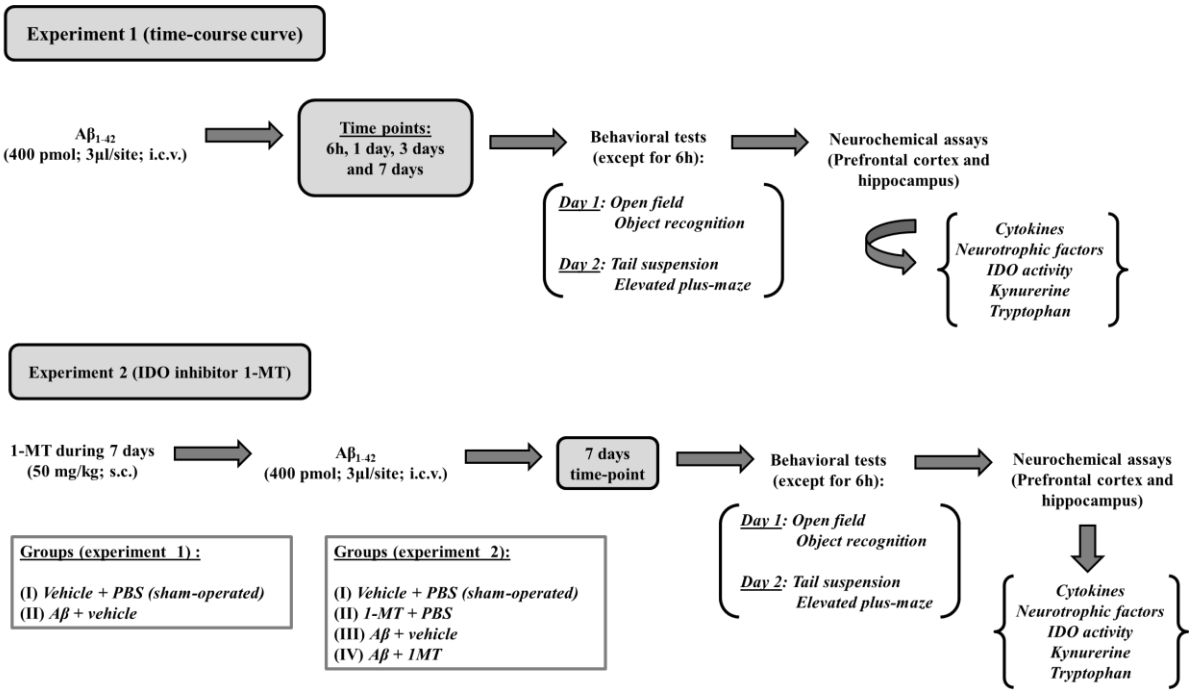
Experiment 1 (time-course curve)
Aβ1-42
(400 pmol; 3µl/site; i.c.v.)
Time points:
6h, 1 day, 3 days
and 7 days
Behavioral tests
(except for 6h):
Neurochemical assays
(Prefrontal cortex and
hippocampus)
Day 1: Open field
Object recognition
Day 2: Tail suspension
Elevated plus-maze
Cytokines
Neurotrophic factors
IDO activity
Kynurerine
Tryptophan
Experiment 2 (IDO inhibitor 1-MT)
1-MT during 7 days
(50 mg/kg; s.c.)
Aβ1-42
(400 pmol; 3µl/site; i.c.v.)
7 days
time-point
Behavioral tests
(except for 6h):
Neurochemical assays
(Prefrontal cortex and
hippocampus)
Day 1: Open field
Object recognition
Day 2: Tail suspension
Elevated plus-maze
Cytokines
Neurotrophic factors
IDO activity
Kynurerine
Tryptophan
Groups (experiment 1) :
(I) Vehicle + PBS (sham-operated)
(II) Aβ + vehicle
Groups (experiment 2):
(I) Vehicle + PBS (sham-operated)
(II) 1-MT + PBS
(III) Aβ + vehicle
(IV) Aβ + 1MT

Fig. 1

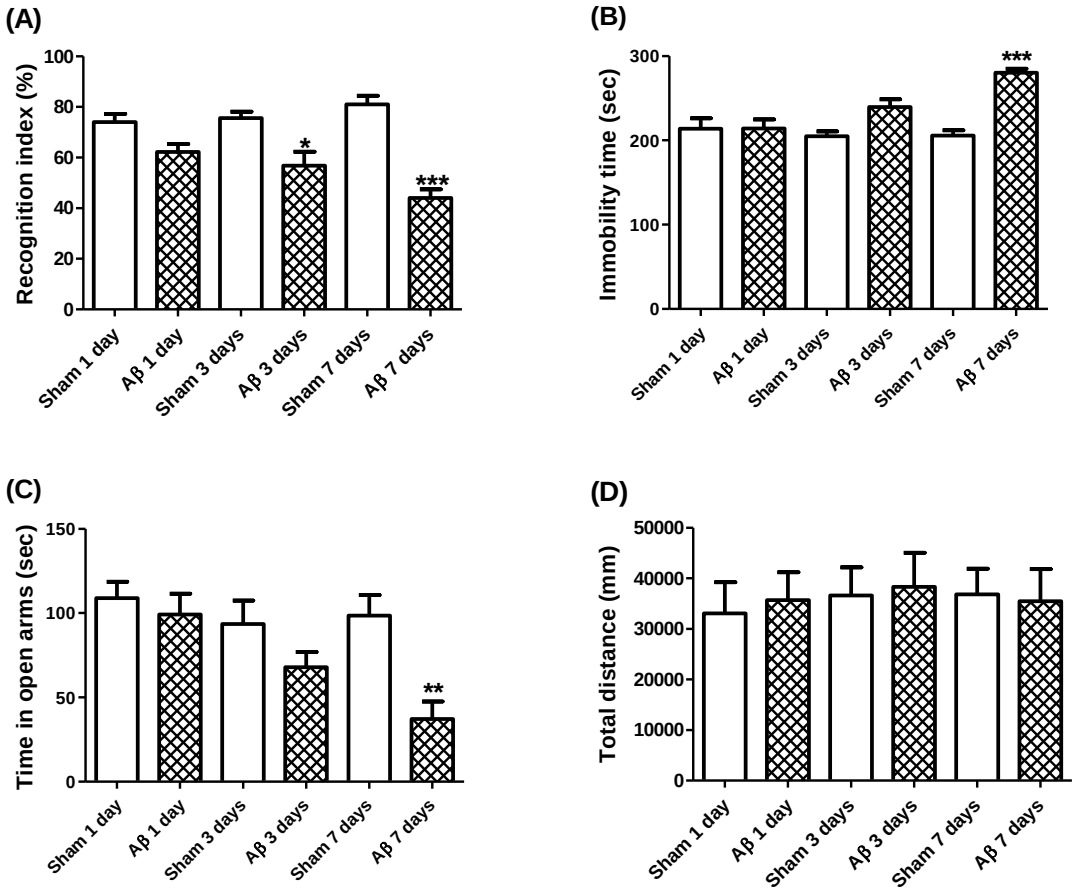
(A)
Recognition index (%)
100
80
60
40
20
0
*
***
Sham 1 day
Aβ 1 day
Sham 3 days
Aβ 3 days
Sham 7 days
Aβ 7 days
(B)
Immobility time (sec)
300
200
100
0
***
Sham 1 day
Aβ 1 day
Sham 3 days
Aβ 3 days
Sham 7 days
Aβ 7 days
(C)
Time in open arms (sec)
150
100
50
0
**
Sham 1 day
Aβ 1 day
Sham 3 days
Aβ 3 days
Sham 7 days
Aβ 7 days
(D)
Total distance (mm)
50000
40000
30000
20000
10000
0
Sham 1 day
Aβ 1 day
Sham 3 days
Aβ 3 days
Sham 7 days
Aβ 7 days

Fig. 2

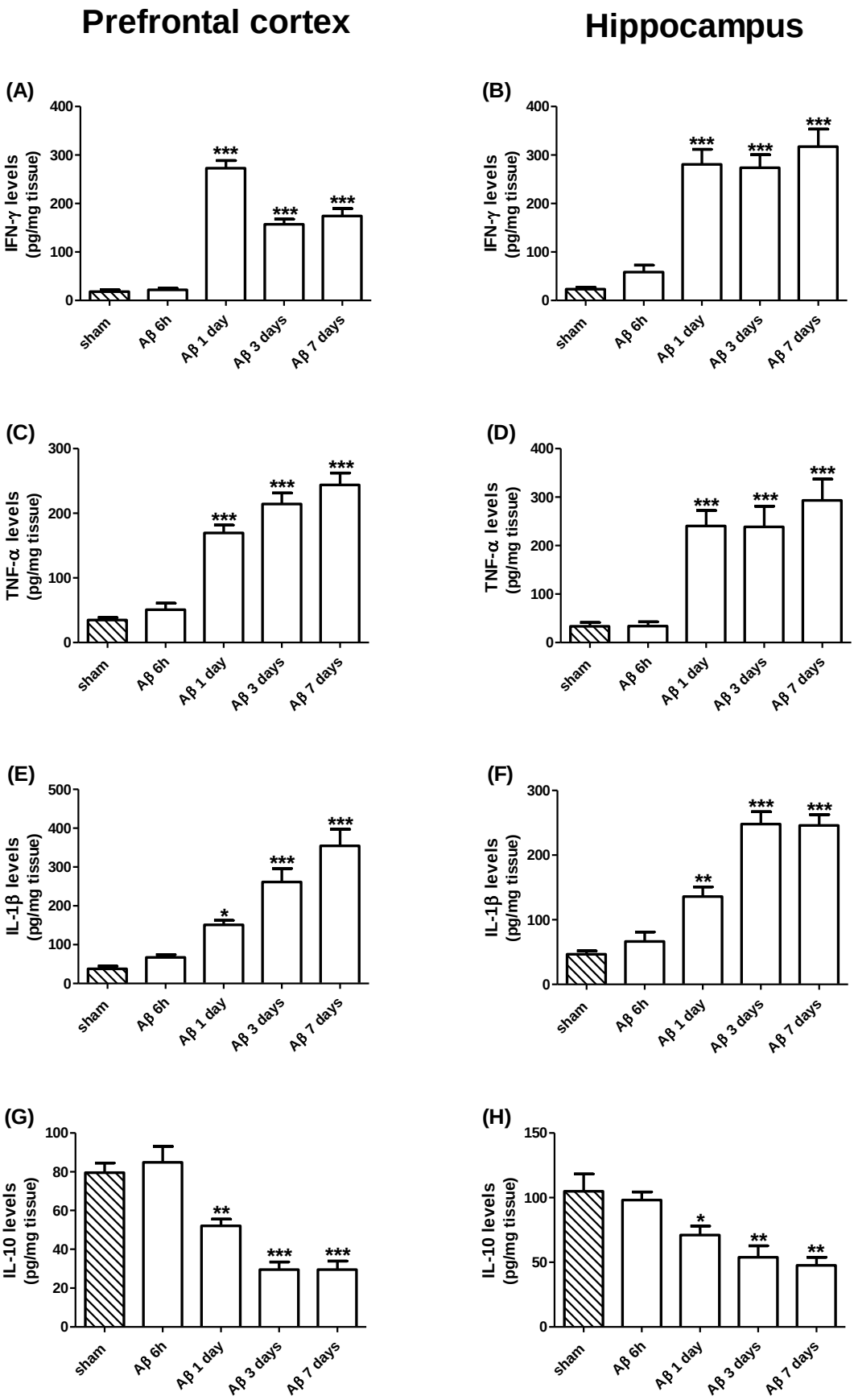
Prefrontal cortex
Hippocampus
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
IFN-γ levels (pg/mg tissue)
TNF-α levels (pg/mg tissue)
IL-1β levels (pg/mg tissue)
IL-10 levels (pg/mg tissue)
sham
Aβ 6h
Aβ 1 day
Aβ 3 days
Aβ 7 days

Fig. 3

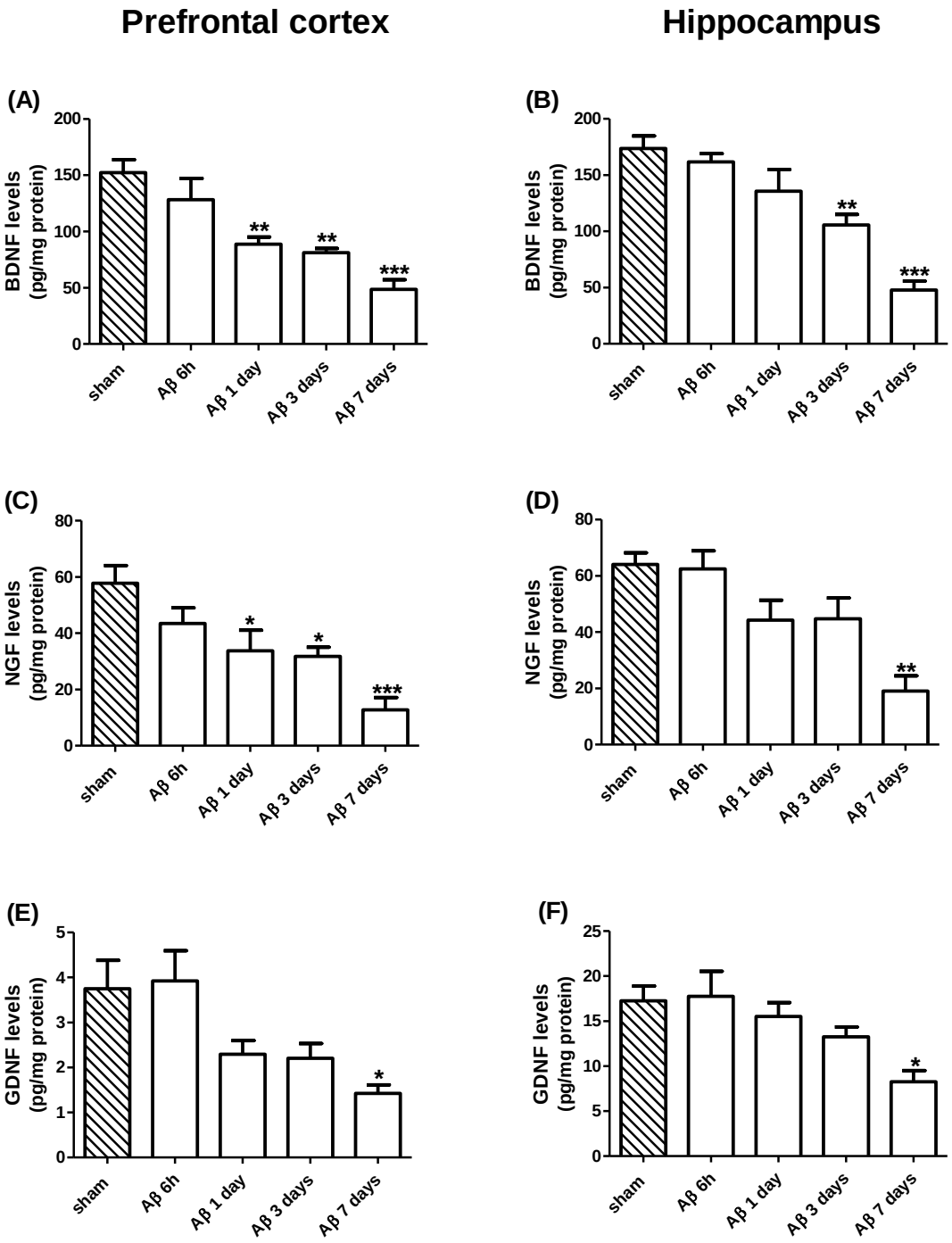
Prefrontal cortex
Hippocampus
(A)
(B)
(C)
(D)
(E)
(F)
BDNF levels (pg/mg protein)
NGF levels (pg/mg protein)
GDNF levels (pg/mg protein)
sham
Aβ 6h
Aβ 1 day
Aβ 3 days
Aβ 7 days

Fig. 4

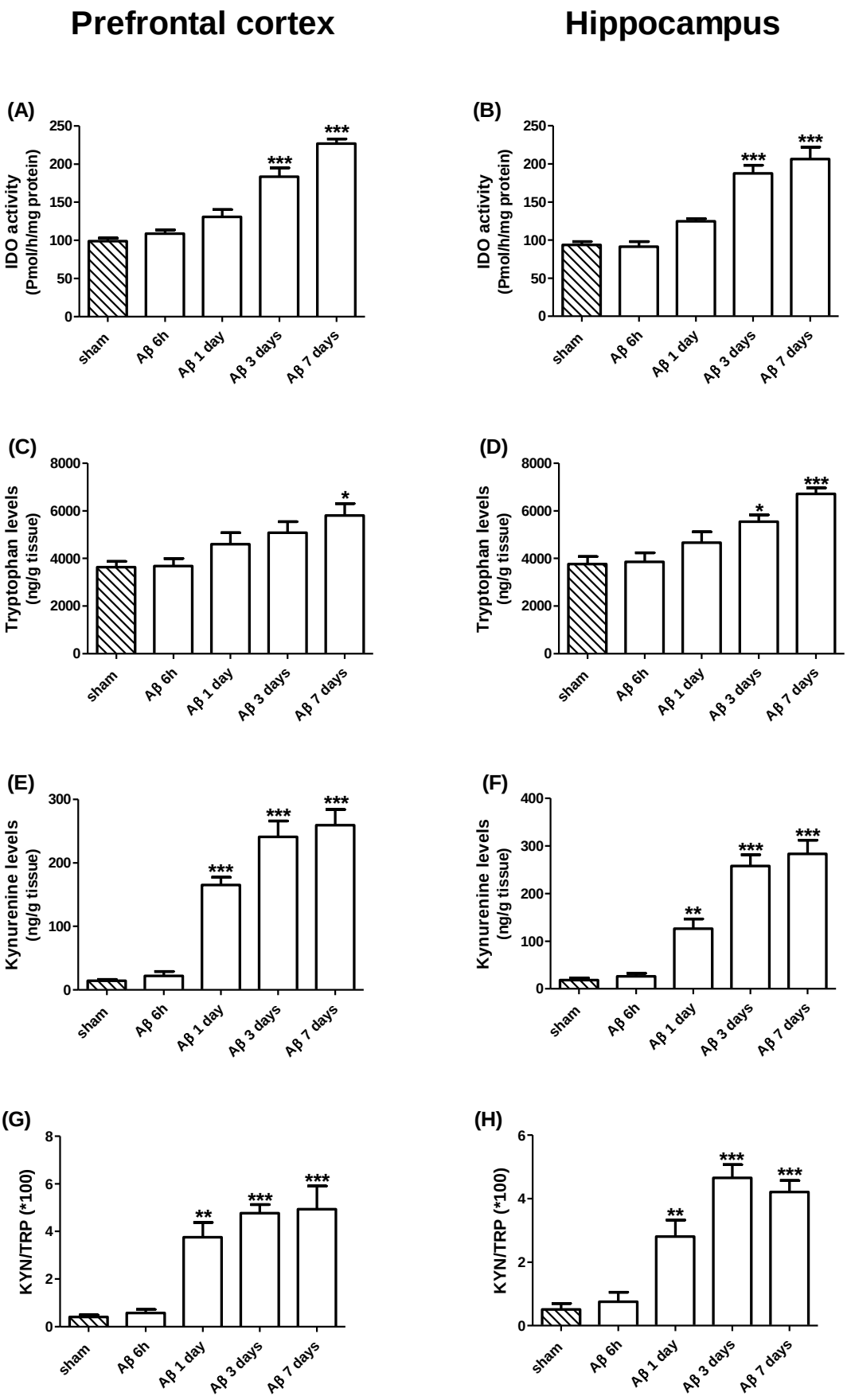
Prefrontal cortex
Hippocampus
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
IDO activity (Pmol/h/mg protein)
Tryptophan levels (ng/g tissue)
Kynurenine levels (ng/g tissue)
KYN/TRP (*100)
sham
Aβ 6h
Aβ 1 day
Aβ 3 days
Aβ 7 days

Fig. 5

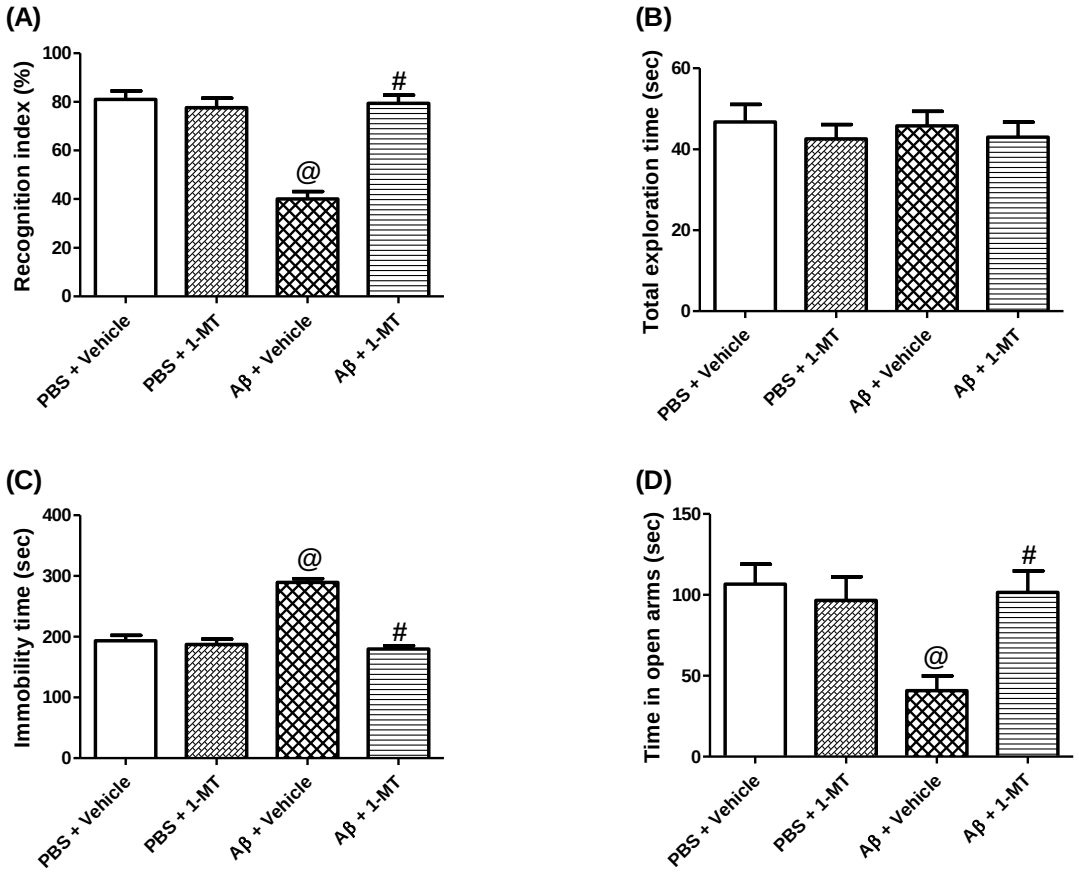
(A)
Recognition index (%)
100
80
60
40
20
0
@
#
PBS + Vehicle
PBS + 1-MT
Aβ + Vehicle
Aβ + 1-MT
(B)
Total exploration time (sec)
60
40
20
0
PBS + Vehicle
PBS + 1-MT
Aβ + Vehicle
Aβ + 1-MT
(C)
Immobility time (sec)
400
300
200
100
0
@
#
PBS + Vehicle
PBS + 1-MT
Aβ + Vehicle
Aβ + 1-MT
(D)
Time in open arms (sec)
150
100
50
0
@
#
PBS + Vehicle
PBS + 1-MT
Aβ + Vehicle
Aβ + 1-MT

Fig. 6

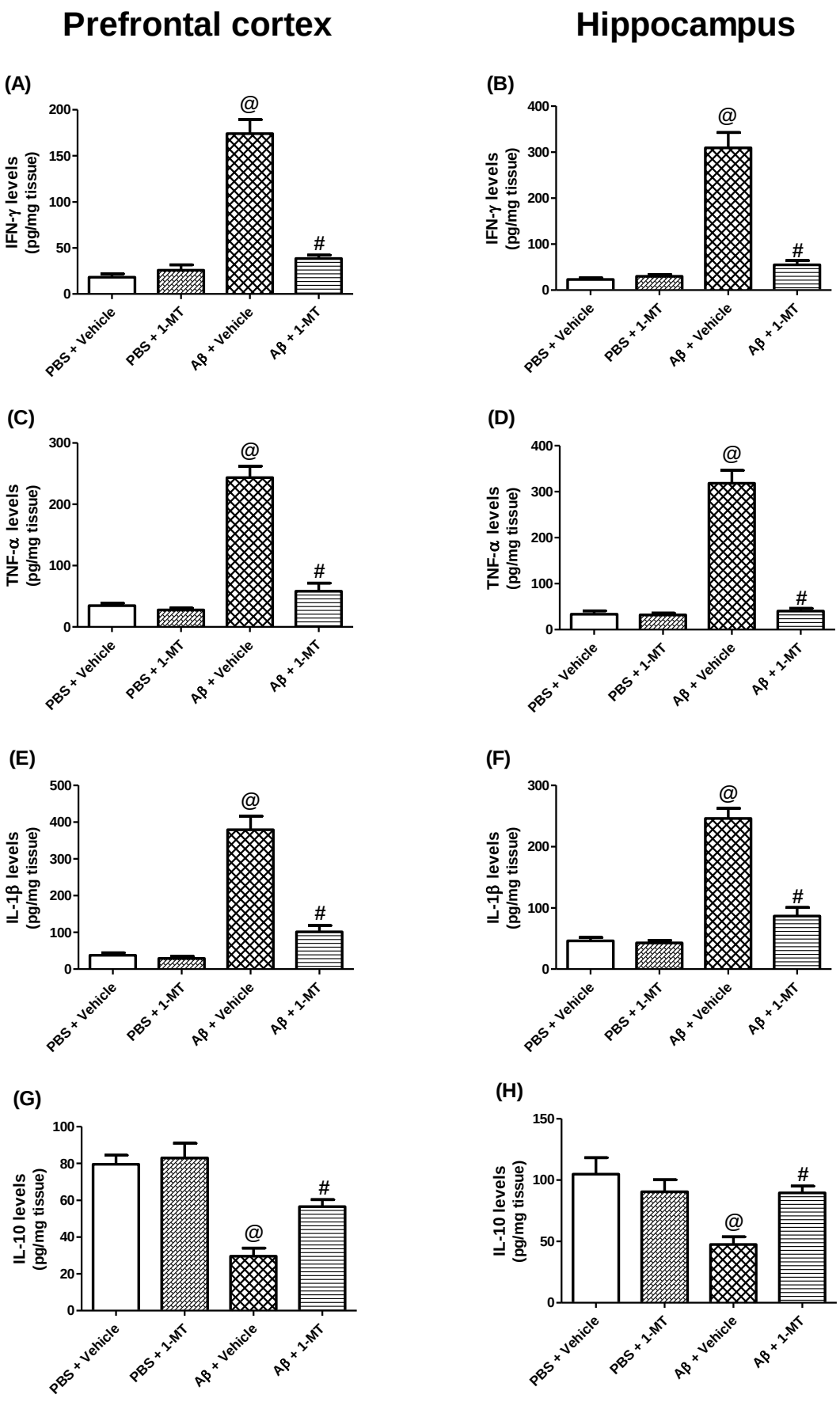
Prefrontal cortex
Hippocampus
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
IFN-γ levels (pg/mg tissue)
TNF-α levels (pg/mg tissue)
IL-1β levels (pg/mg tissue)
IL-10 levels (pg/mg tissue)
PBS + Vehicle
PBS + 1-MT
Aβ + Vehicle
Aβ + 1-MT
@
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Fig. 7

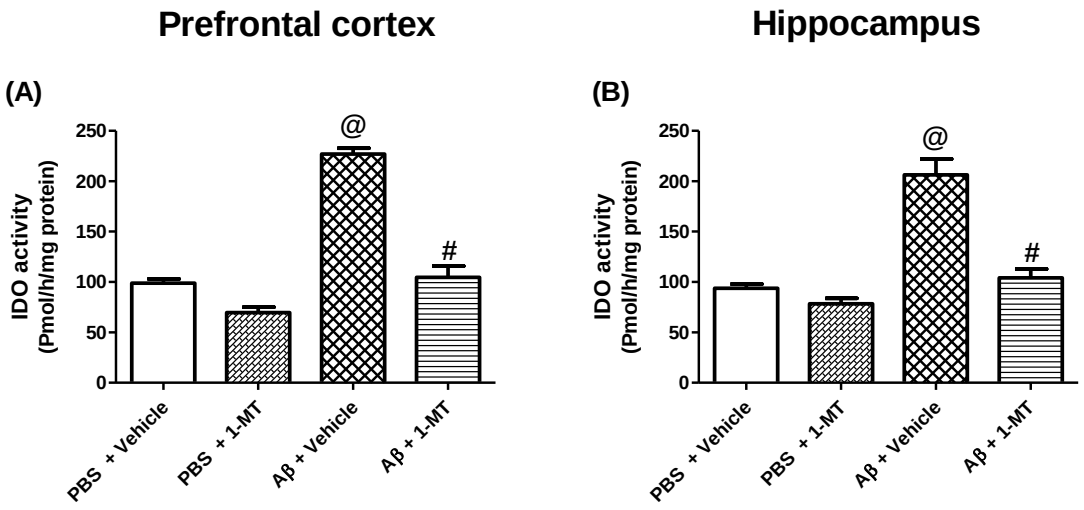
Prefrontal cortex
Hippocampus
(A)
(B)
IDO activity
(Pmol/h/mg protein)
250
200
150
100
50
0
@
#
PBS + Vehicle
PBS + 1-MT
Aβ + Vehicle
Aβ + 1-MT

Fig. 8

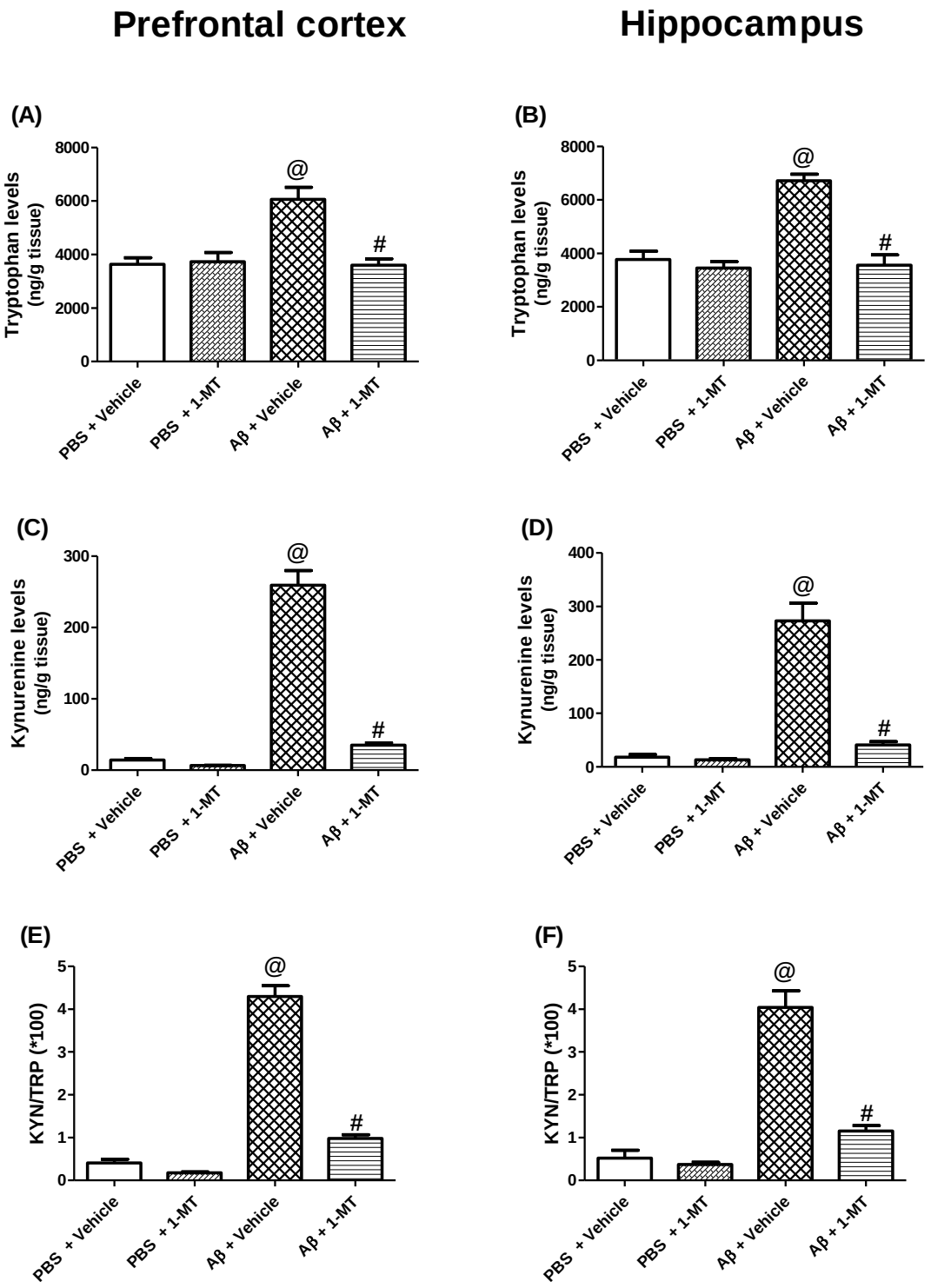
Prefrontal cortex
Hippocampus
(A)
(B)
(C)
(D)
(E)
(F)
Tryptophan levels (ng/g tissue)
Kynurenine levels (ng/g tissue)
KYN/TRP (*100)
PBS + Vehicle
PBS + 1-MT
Aβ + Vehicle
Aβ + 1-MT
@
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Fig. 9

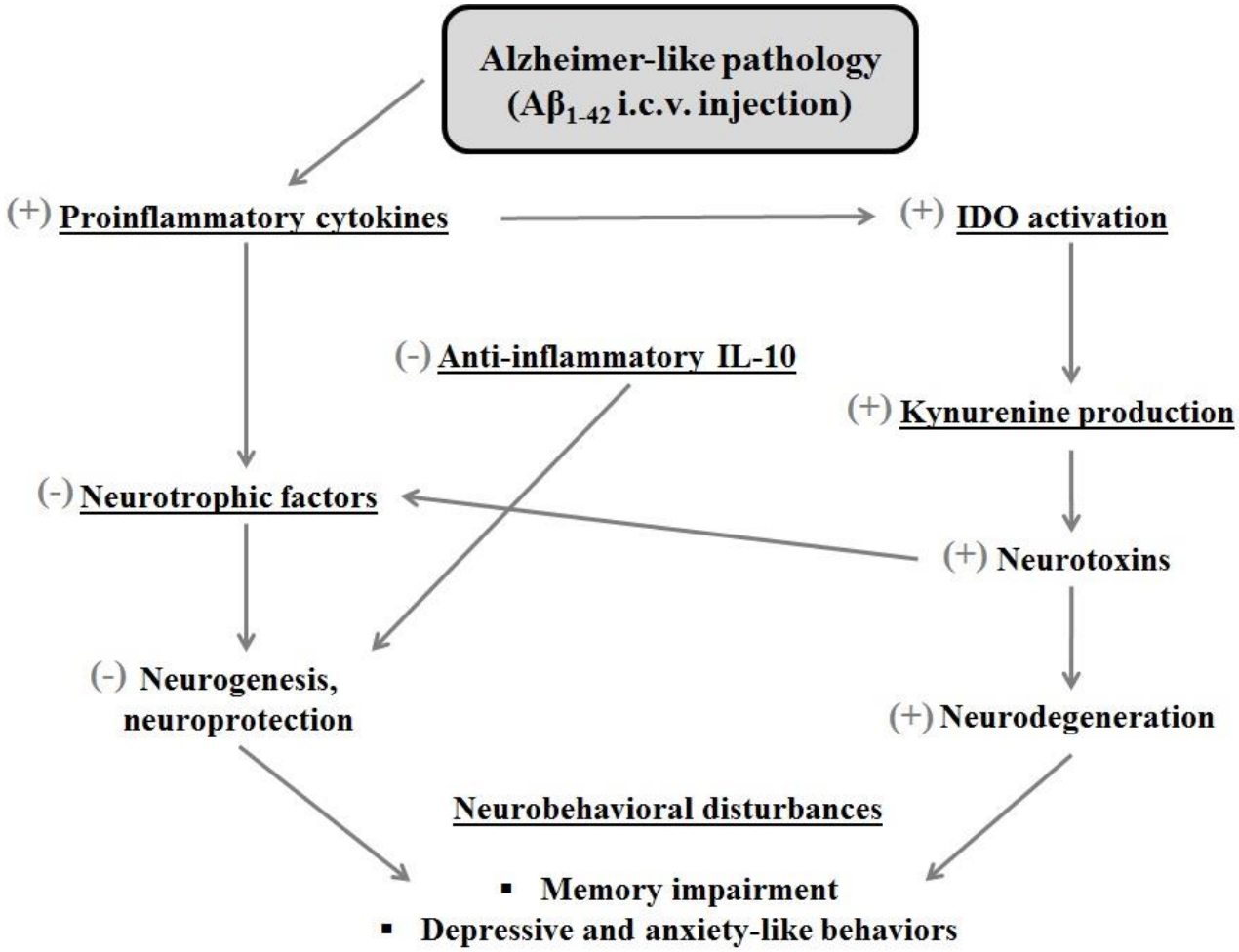
Alzheimer-like pathology
(Aβ1-42 i.c.v. injection)
(+) Proinflammatory cytokines
(+) IDO activation
(-) Anti-inflammatory IL-10
(+) Kynurenine production
(-) Neurotrophic factors
(+) Neurotoxins
(-) Neurogenesis, neuroprotection
(+) Neurodegeneration
Neurobehavioral disturbances
▪ Memory impairment
▪ Depressive and anxiety-like behaviors

Fig. 10

Table 1. Effect of $A\beta_{1-42}$ and 1-MT on locomotor activity of mice evaluated in the elevated plus-maze task and open-field test.

Behavioural test	Parameter	PBS+vehicle	PBS+1-MT	$A\beta_{1-42}$+vehicle	$A\beta_{1-42}$+1-MT
Elevated plus-maze	Open-arm entries	7.0±1.958	8.2±2.016	4.5±1.041	7.5±1.443
	Enclosed-arm entries	9.5±1.323	10.0±1.291	11.5±1.443	11.0±2.121
Open-field	Distance (mm)	34.869±4.159	32.766±3.779	35.645±3.859	33.888±4.560

Data are expressed as the mean ± S.E.M. (n = 8). Two-way ANOVA revealed no significant differences among experimental groups in any of the parameters evaluated.

Table 2. Effect of $A\beta_{1-42}$ and 1-MT on neurotrophin levels in the brain of mice.

(pg/mg protein)	PBS		$A\beta_{1-42}$	
	Vehicle	1-MT	Vehicle	1-MT
Prefrontal cortex				
BDNF	152.3±11.54	151.8±16.53	48.5±8.74[b]	149.8±10.96[d]
NGF	57.75±6.34	67.25±5.75	19.0±6.09[b]	56.50±3.37[d]
GDNF	4.37±0.50	4.07±0.27	1.42±0.19[b]	3.10±0.35[d]
Hippocampus				
BDNF	173.8±11.08	150.5±17.48	47.75±8.01[b]	137.0±9.92
NGF	64.0±4.26	69.75±6.39	21.50±4.33[b]	53.50±3.96[d]
GDNF	17.25±1.65	33.75±2.75[c]	8.25±1.25[b]	27.0±1.47[d]

The values were analyzed by two-way ANOVA and Bonferroni multiple comparison test.
Each value is expressed as the mean ± S.E.M. (n =6-8).
[b] $P < 0,05$ when compared PBS+vehicle with $A\beta_{1-42}$+vehicle.
[d] $P < 0.05$ when compared $A\beta_{1-42}$+1-MT with $A\beta_{1-42}$+vehicle.
[c] $P < 0.05$ when compared PBS+1-MT with PBS+Vehicle.

MANUSCRITO I

Aging exacerbates cognitive and anxiety alterations induced by an intracerebroventricular injection of amyloid-β_{1-42} peptide in mice: the role of neuroinflammation and indoleamine-2,3-dyoxigenase activation

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Aging exacerbates cognitive and anxiety alterations induced by an intracerebroventricular injection of amyloid-β_{1-42} peptide in mice: the roles of neuroinflammation and brain indoleamine-2,3-dyoxigenase activation

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Abstract

An increasing body of evidence indicates that the activation of indoleamine-2,3-dyoxigenase (IDO), a first and rate-limiting enzyme in the kynurenine (KYN) pathway, is involved in amyloid-beta $(A\beta)_{1-42}$-neurotoxicity and Alzheimer's disease (AD) pathogenesis. We have reported for the first time a mechanistic link between $A\beta_{1-42}$-induced neuroinflammation and behavioural disturbances via IDO activation in the brain of young adult mice. Because aging is characterized by a brain dyshomeostasis and it remains the most dominant risk factor for AD, the purpose of this study was to determine whether aging is associated with a higher sensitivity to behavioural and neurochemical alterations elicited by an intracerebroventricular (i.c.v.) injection of $A\beta_{1-42}$ (400 pmol/mice), and whether KYN pathway is involved in these effects. We confirmed that aged mice, when compared to young mice, displayed higher cognitive deficit in the object recognition test and higher anxiety-like behaviour in the elevated plus-maze test after the $A\beta_{1-42}$ administration. Aged mice also responded to $A\beta_{1-42}$ with a higher deficiency of brain-derived neurotrophic factor, glutathione levels and total radical-trapping antioxidant capacity, a higher IDO activity and a higher KYN and KYN/tryptophan ratio in the prefrontal cortex and hippocampus. These effects of $A\beta_{1-42}$ were associated with a higher proinflammatory status, as reflected by increased IL-6 and reduced IL-10 levels in these brain structures. These results represent primary evidence suggesting that age-associated inflammatory signature and down-regulation of neuroprotectants in the brain render aged mice more vulnerable to $A\beta_{1-42}$-induced memory loss, anxiety symptoms and KYN pathway dysregulation.

Keywords: Alzheimer's disease, aging, neuroinflammation, indoleamine-2,3-dyoxigenase, kynurenine, memory, depression, anxiety.

1. Introduction

Alzheimer´s disease (AD) is the most common neurodegenerative form of dementia. Aging is the most important risk factor for AD with the estimated annual incidence and prevalence rising dramatically with age. For example, the prevalence of AD is approximately 15% in people aged 65-74 and increases to about 44% over the age of 80 (Alzheimer´s Association, 2016). The primary presentation of AD is progressive cognitive decline, with memory loss being a relatively early sign of the disease (Wuwongse et al., 2010). Nevertheless, AD patients also frequently exhibit behavioral and psychological symptoms of dementia (BPSD), also known as neuropsychiatric symptoms, including depression, anxiety and psychosis (Cerejeira et al., 2012; Rosenberg et al., 2015). BPSD are clinical relevant as cognitive symptoms as they strongly correlate with the degree of functional and cognitive impairment, worsen the disability and caregiver burden (Cerejeira et al., 2012; Romero & Garrido, 2016). The classical amyloid-beta (Aβ) cascade hypothesis in AD pathogenesis postulates that the deposition of Aβ peptides and the activation of glial cells surround senile plaques in the brain triggers a marked synaptic dysfunction and neuronal death; finally leading to cognitive dysfunction (Ballard et al., 2011).

Despite the exact mechanisms of Aβ-induced neurotoxicity are not completely understood, there is a growing body of evidence suggesting that neuroinflammation plays a pivotal role (Calsolaro & Edison, 2016; Cameron and Landreth, 2010; Heneka et al., 2015; Hepner et al., 2015; Udeochu et al., 2016). In a recent series of studies, it has described that a mouse model of acute inflammation induced by an intracerebroventricular (i.c.v.) injection of $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides induced brain neuroinflammatory signature that precipitates changes in cognitive function and behaviour, mimicking the behavioural alterations observed in the early phase of AD (dos Santos et al., 2013; Souza et al., 2013; Souza et al., 2016; Piermartini et al., 2010). An important factor in this process is the imbalance between pro and anti-inflammatory cytokines in the brain (Heppner et al., 2015) that is related to contribute to cognitive and neuropsychiatric symptoms in AD (Holmgren et al., 2014; Souza et al., 2013; Wuwongse et al., 2010). In this context, proinflammatory cytokines can directly impact neuron function, resulting in production of oxidative species, but they can also act indirectly by the activation of the tryptophan (TRP) degrading

enzyme indoleamine-2,3-dyoxigenase (IDO), the first and rate-limiting enzyme in the kynurenine (KYN) pathway (Lawson et al., 2013). During inflammation, IDO initiates a metabolic cascade leading to increased levels of kynurenine (KYN) in the tissues (Maes et al., 2011). Moreover, increased IDO enzyme activity and elevated levels of KYN have been associated with inflammation-related depression and anxiety (Gibney et al., 2013; O'Connor et al., 2009; Salazar et al., 2012).

In fact, several strands of evidence reported that the overstimulation of the KYN pathway, the major route of tryptophan metabolism in mammalians brain, is strongly implicated in AD (Bonda et al., 2010; Maddison and Giorgini, 2015; Tan et al., 2012). For instance, previous preclinical research demonstrated that amyloid-beta ($A\beta_{1-42}$) peptide induce cytokine production and IDO induction which are involved in inflammatory process within senile plaques (Gulaj et al., 2010). Moreover, an increased KYN/TRP ratio was found in the blood and cerebrospinal fluid of AD patients compared to healthy subjects and this altered ratio coincides with increased levels of IDO in the brain (Bonda et al., 2010; Gulaj et al., 2010). In a recent study of our laboratory (Souza et al., 2016), we demonstrated that brain IDO activation plays a key role in mediating the memory and emotional disturbances in an experimental model based on $A\beta_{1-42}$-induced neuroinflammation. This KYN pathway activation was associated with down-regulation of neurotrophic factors in the prefrontal cortex and hippocampus, two key brain structures of the brain's memory system critically involved in cognitive and emotional regulation (Jin and Maren, 2015; Preston and Eichenbaum, 2013), contributing to behavioural deficits. However, these effects were observed in young adult animals and, as far as we know, there is no previous study addressing the role of $A\beta_{1-42}$ peptide on the activation of KYN pathway in aged animals.

Aging is characterized by a progressive decline in the efficiency of physiological function and by the increased susceptibility to disease and death (Gemma et al., 2007). It is well recognized that aging alters the functional integrity of the adult brain, characterized by impaired homeostasis in the brain, driving cognitive impairments and susceptibility to neurodegenerative disorders in healthy individuals (Udeochu et al., 2016). In fact, old age is a situation characterized by a chronic low-grade inflammatory state, with an

overexpression of circulating and brain inflammatory factors (Martilla et al., 2011; Mosher and Wyss-Coray, 2014), a phenomenon termed “inflammaging” (Franceschi et al., 2007). This dramatic changes in the brain microenvironment reflected by proinflammatory phenotypic changes could facilitate and exacerbate impairment in synaptic plasticity and cognitive function observed in the aged and AD brain (Udeochu et al., 2016). Accordingly, it is of paramount importance to the field of the AD research the study of the interaction between age factor and the neurotoxic mechanisms of $A\beta_{1-42}$ peptide.

Therefore, in the current study we evaluated the hypothesis that the effects of a single intracerebroventricular (i.c.v.) injection of $A\beta_{1-42}$ (400 pmol/mouse) on KYN pathway activation and behavioural complications are exacerbating by aging. For this aim, we investigated the effect of an i.c.v. injection of $A\beta_{1-42}$ on behavioural performance of young adult and aged Swiss albino mice in tests of memory (object recognition test), emotionality (tail suspension test and elevated plus-maze) and locomotion (open field). Moreover, neurochemical parameters related to inflammatory status (IL-6 and IL-10), neurotrophic factor (brain-derived neurotrophic factor, BDNF), non-enzymatic antioxidant defenses (glutathione, GSH and total radical-trapping antioxidant potential, TRAP) and KYN pathway activation (IDO activity, TRP levels and KYN levels) were evaluated in the prefrontal cortex and hippocampus in an attempt to elucidate potential events mediating the neuroinflammation and behavioral deficits, since there is considerable evidence suggesting that these parameters are involved in the mechanisms of Aβ neurotoxicity (Piermartini et al., 2010; Souza et al., 2013; Souza et al., 2016) and AD pathogenesis (Heppner et al., 2015; Leszek et al.,2016; Tong et al., 2009).

2. Methods

2.1 Animals

Male adult (3 months old, weighing 30–40 g) and aged (20 months old, weighing 40–50 g) Swiss Albino mice were kept in groups during aging and these animals were not exposed to any environmental enrichment. Animals

were maintained at 22-25ºC with free access to water and food, under a 12:12h light/dark cycle, with lights on at 7:00 a.m. All manipulations were carried out during light phase on the day. All efforts were made to minimize animal suffering and to reduce the number of animals used. The procedures of this study were conducted according to the guidelines of the Committee on Care and Use of Experimental Animals Resources and with the approval of Ethical Committee for Animal Use (CEUA protocol # 012/2012) of Federal University of Pampa, Brazil.

2.2 Experimental design

Mice were divided into four groups (n=8 animals per group): sham adult; Aβ adult; sham aged; and Aβ aged. Seven days after the i.c.v. injection of $A\beta_{1-42}$, mice were subjected to behavioural tests. Afterwards, they were euthanized and the prefrontal cortex and hippocampus were removed to neurochemical determinations (**Fig. 1**). The 7 days time-point was chosen based on our previous study (Souza et al., 2016), wherein the data of the time-course showed that the Aβ-induced neurochemical and behavioural alterations are more evident 7 days post injection.

2.3 I.c.v. injection of $A\beta_{1-42}$

$A\beta_{1-42}$ (Sigma-Aldrich) was prepared as stock solution at a concentration of 1 mg/ml in sterile 0.1M phosphate-buffered saline (PBS) (pH 7.4), and aliquots were stored at -20º C. $A\beta_{1-42}$ was aggregated by incubation at 37ºC for 4 days before use, as described previously (Souza et al., 2013; Souza et al., 2016). The aggregated form of $A\beta_{1-42}$ (400 pmol/mouse) was administered by i.c.v. route, using a microsyringe with a 28-gauge stainless-steel needle 3.0 mm long (Hamilton), as described beforehand (Cioanca et al., 2014; Piermartini et al., 2010). Briefly, mice were anesthetized with intraperitoneal (i.p.) injection of sodium pentobarbital (0.067 mg/g) and placed in a stereotaxic apparatus (Insight, Brazil). Under light anesthesia (i.e. just that necessary for loss of the postural reflex), the needle was inserted unilaterally 1mm to the right of the midline point equidistant from each eye and 1mm posterior to a line drawn through the anterior base of the eyes (used as external reference). $A\beta_{1–42}$ was administered right-unilaterally into the lateral ventricle. The injection volume of 3

μl of $A\beta_{1–42}$ or PBS was delivered gradually (1μl/min) using the following coordinates from bregma: anteroposterior (AP) = −0.1 mm, mediolateral (ML) = 1 mm, and dorsoventral (DV) = −3 mm. The sham-operated mice were injected with PBS (3 μl/site; i.c.v.). The advantage of this i.c.v. route of administration is the quick distribution of the peptide throughout the brain (Chambon et al., 2011). In order to confirm the accurate placement of the injection site (needle track) at the moment of dissection of the animals, two mice in each group were submitted to dye injection (Evans blue dye, 0,5μl) into the ventricles (Davisson et al., 1998).

2.4 Behavioural assessment

During a period of 2 days, starting 7 days after i.c.v. injection of $A\beta_{1-42}$, the animals were submitted to a battery of behavioural paradigms that include open-field test and object recognition test (day 1), and tail suspension test and elevated plus maze task (day 2). In order to minimize negative interferences of stress on cognitive performance of the animals, we separated the emotional tests from the object recognition test. Moreover, it is noteworthy that on day 1, the open-field test was also used as the habituation phase for object recognition test. All tests were carried out between 9:00 and 16:00h and they were scored by the same trained raters (who were blind to the experimental treatments) in an observation room where the mice had been habituated for at least 1 h before the beginning of the tests.

2.4.1 Open-field test (OFT)

The OFT was carried out to evaluate if the drugs produced effects on locomotor activity. The animals were submitted individually for a period of 5 min to an OFT apparatus (Insight Ltd., Ribeirao Preto, SP, Brazil). The total distance (unit: mm) was computed (Goes et al., 2014; Prut and Belzung, 2003).

2.4.2 Object recognition test (ORT)

After the OFT, the memory function of mice was assessed with the ORT. The task is based on the spontaneous tendency of rodents, previously exposed to two identical objects, to later explore one of the objects (preference of a novel object) for a longer time than they explore the familiar object. This test requires

no external motivation, reward, or punishment and a little training or habituation is required (Winters et al., 2008). The ORT was performed as described by Ennaceur and Delacour (1988) with some modifications. Mice were placed in an open box (similar to OFT) and allowed to explore two identical objects (sample phase) for 5 min and then returned to their home cage. To evaluate the short-term memory, mice were returned to the open box, after a delay of 90 min (intertrial interval), where they were exposed to two different objects (test phase), one identical to the one previously encountered in the sample phase, therefore now familiar, and the other is novel. The animals were allowed to explore both objects for more 5 min. After each trial, box and objects were cleaned with 70 % ethanol. The positions of the objects in the test and the objects used as novel or familiar were counterbalanced between the animals. Exploratory behavior was defined as sniffing or touching the object with the nose and/or forepaws. Any other behavior, such as sitting on or turning around the object was not considered as exploration. The amount of time each animal spent actively investigating the objects was manually scored and recognition index was calculated as the time exploring novel or familiar object divided by the total time spent exploring both objects.

2.4.3 Tail suspension test (TST)

The second day of behavioral assessments started with the TST. Mice were suspended 50 cm above the floor by adhesive tape placed approximately 1cm from de tip of the tail, and the immobility time was recorded for 6 min. The immobility behavioral was determined according to the method described by Steru et al. (1985).

2.4.4 Elevated plus-maze test (EPMT)

Three hours after the TST, the EPMT was used to evaluate anxiety-like behavior in mice, in accord with the method of Pellow et al. (1985). The experimental apparatus (Insight Ltd., Ribeirao Preto, SP, Brazil) was shaped like a ‘plus' sign and consisted of two open arms (30 × 5 cm) and two equal-sized closed arms (30 × 5 × 15 cm) extending from a common central platform (5 × 5 cm). The maze was made of opaque grey PVC and was kept elevated at a height of 50 cm above the floor. The experiments were conducted in a sound-

attenuated room under low intensity light (12 lx). The animals were individually placed in the central area of the maze facing an enclosed arm and were observed for 5 min. The apparatus was cleaned with ethanol solution (10% v/v) and dried with paper towels after each trial in order to avoid odor impregnation. During a 5 min test period, it was recorded the number of entries either the open or enclosed arms, plus the time spent in the open arms. An entry was defined as placing all four paws within the boundaries of the arm. The following measures were obtained from the test: (a) time spent in the open arms relative to the total time spent in the plus-maze (300 s); (b) number of entries into the open arms; (c) number of entries into the closed arms. The anxiolytic effectiveness of a drug is illustrated by a significant statistical augmentation of parameters in open arms (time and/or entries; Clénet et al., 2006).

2.5 Tissue Preparation for neurochemical determinations

After behavioral tests, mice were euthanized with barbiturate overdose (pentobarbital sodium 150 mg/kg; i.p. route) and transcardiacally perfused with 10 ml ice-cold saline via the aorta. The brain dissection was performed according to the method of Spijker (2011), a method to dissect multiple brain regions from a single brain based on existing atlases (Williams, 1999). Prefrontal cortex and hippocampus were bilaterally removed and rapidly homogenized in 50 mM Tris–Cl, pH 7.4. The homogenate was centrifuged at 2,400×*g* for 15 min at 4ºC and a low-speed supernatant fraction (S_1) was used for assays.

2.6 Neurochemical assays

2.6.1 Cytokine levels

Levels of interleukin-6 (IL6) and interleukin-10 (IL-10) in the prefrontal cortex and hippocampus were measured using sample aliquots of 100 µL and mouse cytokine ELISA DuoSet Kits from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's instructions (protein range of 31.25–2,000 pg). The level of cytokine was estimated by interpolation from a standard curve by colorimetric measurements at 450 nm (correction wavelength 540 nm) on an ELISA plate reader (Berthold Technologies-Apollo 8-LB 912, KG, Germany). Results are shown as pg/mg of protein.

2.6.2 Brain-derived neurotrophic factor (BDNF) levels

Protein levels of BDNF were measured using a commercially available sandwich enzyme-linked immune sorbent assay (ELISA) kit (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions. BDNF levels were evaluated in S_1 of prefrontal cortex and hippocampus. The BDNF levels were expressed as pg/mg wet weight of tissue.

2.6.3 Glutathione (GSH) levels

Levels of GSH were determined fluorometrically following Hissin and Hilf (1976) using o-phthalaldehyde (OPA) as fluorophore. Briefly, the samples were homogenized in 0.1 M $HClO_4$. Homogenates were centrifuged at 3100 *g* for 10 min, and the supernatants were separated for the measurement of GSH. S_1 (100 µL) was incubated with 100 µL of OPA (0.1% in methanol) and 1.8 mL of 0.1 M phosphate buffer (pH 8.0) for 15 min at room temperature in dark. Fluorescence was measured using a fluorescence spectrophotometer at excitation wavelength of 350 nm and at emission wavelength of 420 nm. GSH levels were expressed as nmol/g of tissue.

2.6.4. Total radical-trapping antioxidant potential (TRAP) assay

TRAP represents the total non-enzymatic antioxidant capacity of the tissue and was determined by measuring the luminol chemiluminescence intensity induced by 2,2_-azo-bis (2-amidinopropane) (ABAP) (Lissi et al., 1992) at room temperature. Prefrontal cortex and hippocampus were homogenized 1:10 (w/v) in 0.1M glycine buffer, pH 8.6, which was also used to prepare the other solutions. Four milliliter of 10 mM ABAP were added to the vial and the background chemiluminescence was measured. Ten microliter of 4 mM luminol were then added and the chemiluminescence was measured. This was considered to be the initial value. Ten microliter of 80 µM trolox or homogenates were added and chemiluminescence was measured until it reached the initial levels. The addition of trolox or tissue homogenate to the incubation medium reduces the chemiluminescence. The time necessary to return to the levels present before the addition was considered to be the induction time. The induction time is directly proportional to the antioxidant capacity of the tissue

and was compared to the induction time of trolox. The results are reported as nmol of trolox per mg protein.

2.6.5 Tryptophan (TRP) and kynurenine (KYN) levels

The levels of TRP and its metabolite KYN in the prefrontal cortex and hippocampus were performed in a Shimadzu LC-10A liquid chromatograph, according to Silva et al. (2002). The chromatographic separation was achieved using a 250- by 4.6-mm (inner diameter) C_{18} reverse-phase column (particle size, 4 µm; Aquapore RP-300 C-18). For TRP measurement, the column was eluted isocratically at flow rate of 1.0 ml/min with 0.015 M sodium acetate (pH 4.5) containing 15% methanol. For KYN determination, the column was eluted with acetonitrile at a 1:47 dilution in 0.1 M acetic acid–0.1 M ammonium acetate (pH 4.65). The absorbance of the column effluent was monitored at 280 and 365 nm for TRP and KYN respectively. The peaks of TRP or KYN were identified by comparison with the retention times of standard compounds (Sigma), and quantification was based on the ratios of the peak areas of compound to the internal standard. The tissue levels were expressed in pg/mg tissue.

2.6.6 Indoleamine-2,3-dyoxigenase (IDO) activity

IDO activity in the prefrontal cortex and hippocampus was determined as previously described (Lestage et al., 2002). The supernatants (0.2 ml) were added to 0.8 ml of the reaction mixture containing 400 µM L-tryptophan, 20mM ascorbate, 10 µM methylene blue, and 100 µg catalase in 50mM potassium phosphate buffer pH 6.5. The reaction was carried out at 37ºC under agitation for 60 min. Then, it was blocked by adding 0.2 ml of 30% trichloroacetic acid and further incubated at 50ºC for 30 min to convert the N-formylkynurenine to L-kynurenine. Samples were centrifuged at 13,000*g* for 10 min at 4ºC. The supernatants were filtered through microspin ultrafiltrates with a cut-off of 10,000M_r before being taken for measurement of IDO.

The amount of L-kynurenine formed from TRP was determined by reversed phase high pressure liquid chromatography (HPLC). One hundred µl of the reaction product was injected onto a Merck LiChrospher column (150mm long, 4.6mm diameter, packed with 5 lm silica beads holding 18C long carbon

chains). A cartridge guard column containing the same material as the analytical column was used. The mobile phase consisted of 0.1M ammonium acetate buffer (pH 4.65) with 5% acetonitrile. Flow rate was 1 ml/min. KYN was detected using a spectrometer measuring absorbency at a wavelength of 365nm and was quantified using known amounts of L-kynurenine. The retention time of KYN was around 5.35 min. All determinations were performed in duplicate. One unit of the activity was defined as 1 nmol KYN/h/mg protein at 37ºC.

2.7 Protein determination

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as the standard.

2.8 Statistical analysis

The data distribution was verified by applying the Kolmogorov-Smirnov test. Results are presented as means ± standard error medium (SEM). Comparisons between the experimental and the control groups were performed by two-way analysis of variance (ANOVA), followed by Bonferroni post hoc test, when appropriate. A value of $P < 0.05$ was considered to be statistically significant. All tests were carried out using the GraphPad software 5.0 (San Diego, California, USA).

3. Results

3.1 $A\beta_{1-42}$-induced cognitive and emotional symptoms without influencing locomotor activity in adult and aged mice

Two-way ANOVA of cognitive performance in the ORT demonstrated a significant main effect of $A\beta_{1-42}$ ($F_{1, 24} = 67.43$, $p < 0.001$) and age ($F_{1, 24} = 39.95$, $p < 0.001$). Post hoc comparisons revealed that i.c.v. injection of $A\beta_{1-42}$ significantly decreased the recognition index of adult and aged mice ($p > 0.001$) when compared to sham groups, thereby inducing a memory impairment. However, the Aβ-induced memory deficit was more pronounced in aged than in adult mice, supporting that aging aggravates the memory impairment ($p < 0.05$).

In addition, sham aged mice exhibited a decrease in recognition index as compared to the sham adult ($p < 0.001$), indicating that aging itself caused a cognitive decline (**Fig. 2**A). It is important to emphasize that the deficit in the ORT observed in Aβ-treated mice cannot be explained by a general reduced interest for novelty, as there were no differences observed among animal groups in the total investigation time (i.e., the sum of the time that animals spent investigating the two objects) during both the training and the test phases (data not show).

Statistical analysis of immobility time in TST yielded a significant effect of $A\beta_{1-42}$ ($F_{1, 24} = 51.49$, $p < 0.001$) age ($F_{1, 24} = 13.47$, $p < 0.01$) and $A\beta_{1-42}$ × age interaction ($F_{1, 24} = 15.23$, $p < 0.01$). Post hoc comparisons showed that i.c.v. injection of $A\beta_{1-42}$ significantly increased the duration of immobility of adult and aged mice when compared to sham groups ($p < 0.001$), which is interpreted as a depressive-like effect. Similar to ORT, age itself promotes depressive-like behavior ($p > 0.01$). Moreover, young and aged mice given $A\beta_{1-42}$ spent a similar amount of time immobile. (**Fig. 2**B).

When exposed to the EMPT, Aβ-treated mice showed a significant reduction in the time spent on open arms, indicating anxiety-like behavior ($A\beta_{1-42}$, $F_{1, 24} = 40.07$, $p < 0.001$; age, $F_{1, 24} = 14{,}69$, $p < 0.01$). Post hoc comparisons demonstrated that Aβ-treated and sham aged mice markedly spent less time in open arms in comparison with sham adult group ($p > 0.001$ and $p > 0.05$, respectively). However, the Aβ-induced anxiety-like behavior was more accentuated in aged than in young adult mice ($p > 0.05$; **Fig. 2**C). No significant differences among groups were observed in the number of open-arms and enclosed-arms entries (data not shown).

Two-way ANOVA of distance traveled in the OFT was not changed significantly by $A\beta_{1-42}$ ($F_{1, 24} = 0.21$, $p = 0.649$), age ($F_{1, 24} = 0.44$, $p = 0.516$) or their interaction ($F_{1, 24} = 0.01$, $p = 0.943$), ensuring that locomotor activity was not significantly affected in all groups tested (**Fig. 2**D).

3.2 Elevated IL-6 and diminished IL-10 levels in aged mouse brain after i.c.v. injection of $A\beta_{1-42}$

In order to determine the $A\beta_{1-42}$-induced neuroinflammation we sought to determine the degree to which brain IL-6 induction was related with a

corresponding reduction in IL-10 levels. To begin to address this question the prefrontal cortex and hippocampus was used as a representation of brain neuroinflammatory response.

Two-way ANOVA of IL-6 levels in the prefrontal cortex demonstrated a significant main effect $A\beta_{1\text{-}42}$ ($F_{1,\,24} = 56.37$, $p < 0.001$) and age ($F_{1,\,24} = 9.04$, $p < 0.05$). Post hoc comparisons revealed that i.c.v. injection of $A\beta_{1\text{-}42}$ significantly elevated IL-6 levels in the prefrontal cortex of adult and aged mice ($p < 0.001$). In addition, aged mice exhibited elevated IL-6 levels in comparison with sham adult ($p < 0.05$). A tendency for aging to further increase IL-6 levels in response to $A\beta_{1\text{-}42}$ was observed (**Fig. 3**A).

Statistical analysis of IL-6 levels in the hippocampus yielded a significant effect of $A\beta_{1\text{-}42}$ ($F_{1,\,24} = 46.25$, $p < 0.001$) and age ($F_{1,\,24} = 6.73$, $p < 0.05$). Post hoc comparisons revealed that i.c.v. injection of $A\beta_{1\text{-}42}$ significantly elevated IL-6 levels in the hippocampus of adult and aged mice ($p < 0.001$). In addition, aged mice exhibited elevated IL-6 levels in comparison with sham adult ($p < 0.05$). As with the prefrontal cortex, a tendency for aging to further increase IL-6 levels in response to $A\beta_{1\text{-}42}$ was also observed (**Fig. 3**B).

Two-way ANOVA of IL-10 levels in the prefrontal cortex demonstrated a significant main effect $A\beta_{1\text{-}42}$ ($F_{1,\,24} = 67.51$, $p < 0.001$) and age ($F_{1,\,24} = 32.96$, $p < 0.001$). Post hoc comparisons showed that i.c.v. injection of $A\beta_{1\text{-}42}$ significantly decreased IL-10 levels in the prefrontal cortex of adult and aged mice ($p > 0.001$). However, the decrease in IL-10 levels caused by $A\beta_{1\text{-}42}$ were more marked in aged than in adult mice ($p < 0.05$). In addition, aged mice exhibited diminished IL-10 levels in comparison with sham adult ($p < 0.01$; **Fig.** 3C).

Statistical analysis of IL-10 levels in the hippocampus showed a significant main effect $A\beta_{1\text{-}42}$ ($F_{1,\,24} = 25.99$, $p < 0.001$) and age ($F_{1,\,24} = 17.40$, $p < 0.01$). Post hoc comparisons revealed that i.c.v. injection of $A\beta_{1\text{-}42}$ significantly decreased IL-10 levels in the hippocampus of adult and aged mice ($p < 0.001$). However, the decrease in IL-10 levels caused by $A\beta_{1\text{-}42}$ was more marked in aged than in adult mice ($p < 0.05$). In addition, aged mice exhibited diminished IL-10 levels in comparison with sham adult ($p < 0.05$); **Fig. 3**D).

3.3 The i.c.v. injection of Aβ$_{1-42}$ caused a deficiency in non-enzymatic antioxidant defense in the prefrontal cortex and hippocampus of aged mice

We investigated the effect of Aβ$_{1-42}$ on some parameters of non-enzymatic antioxidant defense, namely GSH and TRAP (an index of total antioxidant capacity of the tissue). The ubiquitous tripeptide GSH is a major antioxidant defense mechanism against reactive free radicals and has also served as a marker of changes in oxidative stress (Tong et al., 2016).

Two-way ANOVA of GSH levels in the prefrontal cortex demonstrated a significant main effect Aβ$_{1-42}$ ($F_{1, 24}$ = 49.99, p < 0.001) and age ($F_{1, 24}$= 45.62, p < 0.001). Post hoc comparisons revealed that i.c.v. injection of Aβ$_{1-42}$ significantly decreased GSH levels in the prefrontal cortex of adult and aged mice (p < 0.001). However, the decrease in GSH levels caused by Aβ$_{1-42}$ was more pronounced in aged than in adult mice (p < 0.01). In addition, aged mice exhibited diminished GSH levels in comparison with sham adult (p < 0.001; **Fig. 4**A).

Statistical analysis of GSH levels in the hippocampus showed a significant main effect Aβ$_{1-42}$ ($F_{1, 24}$ = 54.99, p < 0.001) and age ($F_{1, 24}$= 17.95, p < 0.001). Post hoc comparisons showed that i.c.v. injection of Aβ$_{1-42}$ significantly decreased GSH levels in the hippocampus of adult and aged mice (p < 0.001). Aging process significantly decreased GSH levels in control conditions as compared to the adult (p < 0.05). GSH levels were further decreased in Aβ-treated aged mice when compared to Aβ-treated adult (p < 0.05; **Fig. 4**B).

Two-way ANOVA of TRAP in the prefrontal cortex yielded a significant main effect Aβ$_{1-42}$ ($F_{1, 24}$ = 49.91, p < 0.001) and age ($F_{1, 24}$= 33.73, p < 0.001). Post hoc comparisons revealed that i.c.v. injection of Aβ$_{1-42}$ significantly decreased TRAP in the prefrontal cortex of adult and aged mice (p < 0.01). However, the decrease in TRAP was more expressive after Aβ$_{1-42}$ injection in aged than in adult mice (p < 0.01). As expected, aged mice exhibited diminished TRAP in comparison with sham adult (p < 0.01; **Fig. 4**C).

Statistical analysis of TRAP in the hippocampus demonstrated a significant main effect Aβ$_{1-42}$ ($F_{1, 24}$ = 166.50, p < 0.001) and age ($F_{1, 24}$= 57.48, p < 0.001). Post hoc comparisons showed that i.c.v. injection of Aβ$_{1-42}$ significantly decreased TRAP in the hippocampus of adult and aged mice (p <

0.01); however, TRAP was further decreased in Aβ-treated aged mice ($p < 0.01$). Aging process significantly decreased TRAP in control conditions as compared to the adult ($p < 0.01$; **Fig. 4D**).

3.4 The i.c.v. injection of $A\beta_{1-42}$ provokes a down-regulation of BDNF levels in the prefrontal cortex and hippocampus of aged mice

Two-way ANOVA of BDF levels in the prefrontal cortex demonstrated a significant effect of $A\beta_{1-42}$ ($F_{1, 24} = 55.81$, $p < 0.001$), age ($F_{1,24} = 64.28$, $p < 0.001$) and $A\beta_{1-42}$ × age interaction ($F_{1, 24} = 19.53$, $p < 0.001$). Post hoc comparisons revealed that i.c.v. injection of $A\beta_{1-42}$ dramatically decreased BDNF levels in the prefrontal cortex of adult and aged mice ($p < 0.001$). However, the decrease in BDNF levels caused by $A\beta_{1-42}$ was more pronounced in aged than in adult mice ($p < 0.01$). As expected, aged mice exhibited an expressive reduction of BDNF levels in comparison with sham adult ($p < 0.001$; **Fig. 5**A).

Statistical analysis of BDNF levels in the hippocampus yielded a significant effect of $A\beta_{1-42}$ ($F_{1, 24} = 86.15$, $p < 0.001$), age ($F_{1,24} = 68.21$, $p < 0.001$) and $A\beta_{1-42}$ × age interaction ($F_{1, 24} = 9.24$, $p < 0.05$). Post hoc comparisons showed that i.c.v. injection of $A\beta_{1-42}$ expressively decreased BDNF levels in the hippocampus of adult and aged mice ($p <0.001$). Aging process significantly decreased BDNF levels in control conditions as compared to the adult ($p < 0.001$). BDNF levels were further decreased in Aβ-treated aged mice than Aβ-treated adult ($p < 0.001$; **Fig. 5**B).

3.5 Increased IDO activity in brain of aged mice in response to i.c.v. injection of $A\beta_{1-42}$

Two-way ANOVA of IDO activity in the prefrontal cortex demonstrated a significant main effect of $A\beta_{1-42}$ ($F_{1, 24} = 22.42$, $p < 0.001$) and age ($F_{1, 24} = 13.60$, $p < 0.01$). Post hoc comparisons showed that IDO activity in the prefrontal cortex was markedly increased in Aβ-treated mice, irrespective of age ($p < 0.01$). However, IDO activity was higher in aged mice receiving $A\beta_{1-42}$ than in adults receiving $A\beta_{1-42}$ ($p < 0.01$). Aging process significantly increased IDO activity in control conditions as compared to the adult ($p < 0.05$; **Fig. 6**A).

Statistical analysis of IDO activity in the hippocampus yielded a significant main effect of $A\beta_{1\text{-}42}$ ($F_{1,\ 24} = 32.81$, $p < 0.001$) and age ($F_{1,\ 24} = 9.16$ $p < 0.05$). As with the prefrontal cortex, post hoc comparisons revealed that IDO activity was higher in the hippocampus of aged mice compared to adults ($p < 0.05$), and $A\beta_{1\text{-}42}$ increased IDO activity irrespective of age ($p < 0.01$). However, Aβ-induced IDO activation was more marked in aged mice than in adults ($p < 0.05$; **Fig. 6**B).

3.6 Increased KYN pathway metabolism in brain of aged mice in response to i.c.v. injection of $A\beta_{1\text{-}42}$

Two-way ANOVA of TRP levels in prefrontal cortex demonstrated a significant main effect of $A\beta_{1\text{-}42}$ ($F_{1,\ 24} = 9.19$, $p < 0.05$). Post hoc comparisons revealed that i.c.v. injection of $A\beta_{1\text{-}42}$ significantly increased TRP levels in the prefrontal cortex of adult mice when compared to sham adult ($p < 0.05$). On the other hand, there was a tendency for an age-related decrease in TRP levels. In response to $A\beta_{1\text{-}42}$, aged mice did not show an increase in TRP levels, indicating that the effect of $A\beta_{1\text{-}42}$ on TRP levels is aging-independent (**Fig. 7**A). Similarly, Two-way ANOVA of TRP levels in the hippocampus showed a significant main effect $A\beta_{1\text{-}42}$ ($F_{1,\ 24} = 7.86$, $p < 0.05$). Post hoc comparisons revealed that i.c.v. injection of $A\beta_{1\text{-}42}$ significantly increased TRP levels in the hippocampus of adult ($p < 0.05$) but not aged mice. Again, there was a tendency for an age-related decrease in TRP levels (**Fig. 7**B).

Statistical analysis of KYN levels in the prefrontal cortex yielded a significant main effect of $A\beta_{1\text{-}42}$ ($F_{1,\ 24} = 74.49$, $p < 0.001$) and age ($F_{1,\ 24} = 5.37$, $p < 0.05$). Post hoc comparisons revealed that i.c.v. injection of $A\beta_{1\text{-}42}$ dramatically increased KYN levels in the prefrontal cortex of adult and aged mice, in an age-dependent manner ($p < 0.001$; **Fig. 7**C). Similar to the results for prefrontal cortex, $A\beta_{1\text{-}42}$ injection markedly increased KYN levels in the hippocampus of adult and aged mice ($p < 0.001$), in an age-dependent manner ($A\beta_{1\text{-}42,}$ $F_{1,\ 24} = 68.09$, $p < 0.001$); age, $F_{1,\ 24} = 8.68$, $p < 0.05$; **Fig. 7**D).

Two-way ANOVA of KYN/TRP ratio in the prefrontal cortex demonstrated a significant effect of $A\beta_{1\text{-}42}$ ($F_{1,\ 24} = 96.36$, $p < 0.001$), age ($F_{1,24} = 26.65$, $p < 0.001$) and $A\beta_{1\text{-}42}$ × age interaction ($F_{1,\ 24} = 5.19$, $p < 0.05$). Post hoc comparisons revealed that i.c.v. injection of $A\beta_{1\text{-}42}$ significantly increased

KYN/TRP ratio in the prefrontal cortex of adult and aged mice ($p < 0.001$). However, KYN/TRP ratio was higher in Aβ-treated aged mice than Aβ-treated adults ($p < 0.001$; **Fig. 7**E). Similarly, Two-way ANOVA of KYN/TRP ratio in the hippocampus yielded a significant effect of $A\beta_{1-42}$ ($F_{1, 24} = 41.61$, $p < 0.001$), age ($F_{1,24} = 15.58$, $p < 0.01$) and $A\beta_{1-42}$ × age interaction ($F_{1, 24} = 8.64$, $p < 0.05$). Post hoc comparisons showed that i.c.v. injection of $A\beta_{1-42}$ significantly increased KYN/TRP ratio in the hippocampus of adult and aged mice ($p < 0.05$ and $p < 0.001$, respectively). However, KYN/TRP ratio was higher in Aβ-treated aged mice than Aβ-treated adults ($p < 0.001$), indicating that Aβ-induced KYN pathway activation is exacerbated by aging (**Fig. 7**F).

4. Discussion

In a recent study, we have reported that a single i.c.v. injection of $A\beta_{1-42}$ in young adult mice induced neuroinflammation and neurotrophic deficiency in the prefrontal cortex and hippocampus, that were paralleled by cognitive impairment and depressive and anxiety-like behaviour, and these events were mediated by IDO activation (Souza et al., 2013). Because aging remains the most dominant risk factor for AD, in the current study we investigated whether aging was associated with a higher sensitivity to behavioural and neurochemical alterations elicited by i.c.v. injection of $A\beta_{1-42}$ and whether KYN pathway is involved in these effects.

Our study demonstrated that $A\beta_{1-42}$-treated mice and sham aged mice displayed a poor performance in the object recognition test, as revealed by the reduction of recognition index. As expected, aged mice were more sensitive to the memory impairment induced by $A\beta_{1-42}$. This is an important finding, supporting the view that in response to $A\beta_{1-42}$, this early symptom of AD (Antunes and Biala, 2012, Ballard et al., 2011) is exacerbated by aging. Here, the cognitive impairment in $A\beta_{1-42}$-treated mice occurred together with increased emotional responses in the tail suspension test (TST) and elevated plus-maze test (EPMT), resembling the emotional disturbances observed in AD patients (Wuwongse et al., 2010) and transgenic mouse model of AD (Filali et al., 2012; Lalonde et al., 2012). Notably, aged mice injected with $A\beta_{1-42}$ displayed higher levels of anxiety, indicating that aged mice are more prone to develop anxiety-

like behaviour in response to $A\beta_{1-42}$. It is important to emphasize that the anxiety-like behaviour in the EPMT was higher in sham aged mice than sham adult mice, suggesting a susceptibility to anxious disorders in elderly animals. In the TST, both aged and adult mice exhibited depressive-like behaviour after an i.c.v. injection of $A\beta_{1-42}$. Contrary to our hypothesis, the duration of immobility time in response to $A\beta_{1-42}$ did not differ between adult and aged mice. However, when compared aged mice with adult in control conditions, we observed that aging process precipitates depressive state, corroborating previous reports that aging is associated with an increased risk of depression (Djernes, 2006; Godbout and Johnson, 2006). Thus, the current data are tally with our previous work (Souza et al., 2016) and with the studies of other laboratories (dos Santos et al., 2010; Piermartini et al.,2010), indicating that $A\beta_{1-42}$ induces psychobehaviorals deficits in mice. In addition, our results extend this notion, showing that Aβ-induced memory impairment and anxiety-like behaviour are aggravated by aging. It is noteworthy to mention that the i.c.v. injection of $A\beta_{1-42}$ does not cause gross motor alterations (as evaluated in the open field test) that would preclude the assessment of emotional functions.

Emerging evidence from preclinical and clinical studies have established that neuroinflammation contribute to and drive AD pathogenesis (Calsolaro and Edison, 2016; Heneka et al., 2015; Hepner et al., 2015; Udeochu et al., 2016). In fact, it has been widely recognized that chronic neuroinflammation, induced by a number of pro-inflammatory cytokines, such as interleukin-6 (IL-6) released from activated microglia and astrocytes, is an early and continuous feature of AD brain (Zang and Jiang et al., 2015). This increase in inflammatory cytokines is also accompanied by a decrease in the levels of IL-10, that is one of the main anti-inflammatory cytokines, indicating the concept that in the AD brain there is a markedly cytokine imbalance (Wang et al., 2015).

Supporting these reports, the present study demonstrated that i.c.v. injection of $A\beta_{1-42}$ induced an expressive increase in IL-6 levels and decrease in IL-10 in the prefrontal cortex and hippocampus of adult and aged mice. Importantly, we observed an amplification in the IL-10 deficiency and a tendency to further increase the IL-6 levels in the Aβ-treated aged mice when compared to Aβ aged mice. These results are in line with the literature, since old age is a situation characterized by a chronic low-grade inflammatory state,

with an overexpression of inflammatory factors, such as IL-6 to the detriment of anti-inflammatory factors, such as interleukin-10 (Fagiolo et al., 1993; Franceschi et al., 2000; Xia et al., 2016). Elevated IL-6 has found to be correlated with age-related cognitive decline in humans (Weaver et al., 2002,), playing a complex role in regulate neuroinflammation both in aging as in AD (Wang et al., 2015). In this context, in several rodent models of aging, the excessive neuroinflammatory cytokine response is coupled with a behavior complications, including cognitive impairment and depressive-like behaviour (Chen et al., 2008; Godbout et al., 2008). Together with literature, the present study indicates that the excessive neuroinflammation in the brain of aged mice may be permissive to the worsening of $A\beta_{1-42}$-induced behavioural disturbances.

In our study, we observed that $A\beta_{1-42}$ administration caused a down-regulation in BDNF levels in the prefrontal cortex and hippocampus of adult and aged mice. The reduction of the neurotrophin BDNF can lead reduced neuronal protection and neurogenesis, ultimately resulting in the development of cognitive and emotional symptoms (Gibney et al., 2013; Martinowich et al., 2007; Sopova et al., 2014). It has been proposed that changes in BDNF expression and function in limbic structures of the brain, such as prefrontal cortex and hippocampus, results in impaired neurogenesis, dendrite length and spine density, supporting a “neurotrophic hypothesis of depression” and antidepressant response (Duman and Monteggia, 2006). Moreover, impaired neurogenesis and neuroplasticity play major roles in the pathophysiology of AD (Sopova et al., 2014). Most notably, in the present study the BDNF deficit is more pronounced in aged $A\beta_{1-42}$-treated than adult $A\beta_{1-42}$-treated mice. This result is in agreement with previous studies, which have reported an age-related down-regulation of BDNF in the brain of humans and rodents (Souza et al., 2015; Tapia-Arancibia et al., 2008). Thus, in the present study we demonstrated an exaggerated BDNF deficit in the brain of $A\beta_{1-42}$-treated aged mice, reinforcing that aging is a permissive process that could be act in a synergistic way in the neurotrophin deficiency mediated by $A\beta_{1-42}$, increasing the risk for AD symptoms.

In recent years, alterations in the KYN pathway has been identified in several neurological and more specifically neurodegenerative diseases, such as Huntington chorea, multiple sclerosis, Parkinson’s disease and AD (Lovelace et

al., 2017; Maddison and Giorgini, 2015; Tan et al., 2012). Accumulating data suggest that IDO, the rate-limiting step on the KYN pathway, plays a central role in the AD pathogenesis (Bonda et al., 2010; Gulaj et al. 2010; Kincses et al., 2010). It has been previously reported that the increase of proinflammatory cytokines, such as interleukin-1 beta, tumor necrosis factor-alpha, interferon-gamma and IL-6 triggers IDO activation in brain inflammatory conditions (Myint and Kim, 2003; Guillemin and Brew, 2002). We also highlighted recently that hippocampal IDO activation is mediated by proinflammatory cytokines in i.c.v. streptozotocin-induced depressive-like behaviour (Souza et al., 2017). In line with our previous works (Souza et al., 2016, 2017), in the current study we observed that the i.c.v. injection of $A\beta_{1-42}$ induced an increase in IDO activity in the prefrontal cortex and hippocampus in an age-dependent manner. In regard to aging, it was described that an IDO elevation in brains of older rats and humans could be initiated by pro-inflammatory cytokines and these changes are consistent with a low-grade inflammation in the elderly, mainly due to the elevation of IL-6 levels (Badawi et al., 2017; Capuron et al., 2009). A chronic low-grade inflammation in aging is also associated with alterations in enzymatic IDO activity (Martilla et al., 2011), suggesting that these alterations might precipitate the neuropsychiatric symptoms in elderly persons (Capuron et al., 2011). Therefore, corroborating and extending previous data, our study demonstrates for the first time that the increased brain IDO activity in response to $A\beta_{1-42}$ is aggravated by aging process.

In our study, the increase of IDO activity induced by $A\beta_{1-42}$ was followed by increased levels of TRP in the prefrontal cortex and hippocampus of adult mice, but this effect did not occur in aged mice. Although the increase in TRP levels are somewhat counterintuitive, and certainly argue against the hypothesis that IDO activation depletes TRP bioavailability for 5-HT synthesis, previous studies had already shown that this response could be argued as a compensatory mechanism of the brain is response to inflammatory stimuli (Gibney et al., 2013; Souza et al., 2016). The absence of this compensatory mechanism in aged animals could be explained by the decrease in tryptophan levels due to the aging process (Badawy et al., 2017; Capuron et al., 2011). Thereby, our study reinforces the view that the behavioural disturbances induced by $A\beta_{1-42}$ are not involved with reduced TRP availability for serotonin

synthesis in the brain of adult mice; however, we cannot rule out that this effect can occur in the aged animals. Thus, further studies are need to investigate this possibility.

Of great interest, we demonstrated that a single i.c.v. injection of $A\beta_{1-42}$ precipitated behavioural disturbances coupled with increased KYN levels and KYN:TRP ratio in adult and aged mice, supporting that the causative role of brain KYN metabolism in driving AD symptoms. With regard to aging, the foregoing data indicate that older age together with inflammation was correlated negatively with tryptophan levels and positively with KYN levels and with KYN/TRP ratio (Capuron et al., 2011). In contradistinction with these reports, we verified in our study that control aged mice did not showed significant higher levels of KYN either KYN/TRP ratio in the prefrontal cortex and hippocampus when compared to control adults; although a trend towards these alterations have been observed. Therefore, the current study provides additional evidence that the increase in KYN levels, independently of TRP depletion, is an important molecular event linked to $A\beta_{1-42}$-induced behavioural disturbances. The fact that the aged mice receiving $A\beta_{1-42}$ have shown a greater increase in KYN levels supports the view that aging is a predisposing factor to Aβ-induced KYN pathway dysregulation.

From a mechanistic perspective, Myint and Kim (2003) have earlier proposed that inflammation-induced IDO activation switches the metabolism of KYN towards the production of neurotoxic metabolites by microglia cells. Of these neurotoxic metabolites, the N-methyl-D-aspartate agonist and free-radical inductor quinolinic acid (QUIN) may be one of the critical factors in the pathogenesis of neuronal damage in AD (Guillemin and Brew, 2002; Kincses et al., 2010). It has been reported that QUIN not only induced oxidative stress through the production of reactive species, but also appears to provoke an impairment in antioxidant mechanisms, such as the reduction of glutathione (GSH) levels (Rodríguez-Martínez et al., 2000). In agreement with these findings, the present study showed that an i.c.v. injection of $A\beta_{1-42}$ caused a non-enzymatic antioxidant deficiency in the brain mice, reflected by the depletion of GSH levels and the decrease in total radical-trapping antioxidant potential (TRAP) in the prefrontal cortex and hippocampus of adult and aged mice. Similar to the other biomarkers, we found that this impairment on non-

enzymatic antioxidant capacity occurred in an age-dependent fashion, wherein higher antioxidant deficit was found in Aβ-treated mice and control aged mice. It is an important finding, since the reduction in antioxidant potential and a loss of glutathione in brain senescence, compromise the ability of the aging brain to meet the demands of oxidative stress (Tong et al., 2016). Furthermore, these findings are in line with the "free radical theory of aging" which postulates that aging and its related diseases are the consequences of changes in endogenous antioxidant defenses which cause an inability to counterbalance the free radical-induced damage to cellular macromolecules, resulting in oxidative stress (Cui et al., 2012; Gemma et al., 2007; Tong et al., 2016). According to these findings, it is reasonable to assume that the aging process render the aged mice more susceptible to the deleterious effect of Aβ peptide by affecting the cellular machinery implicated in neuroprotection. Despite a direct impairment in antioxidant defenses caused by Aβ peptide has been extensively demonstrated (dos Santos et al., 2013; Piermartini et al., 2010; Souza et al., 2013), here we conjecture that this deficit could be indirectly caused by increased KYN and its neurotoxic metabolites in the brain. However, further investigations are needed to confirm this hypothesis.

In summary, our study supports the notion that a single i.c.v. injection of $A\beta_{1-42}$ peptide induces cognitive impairment and emotional disturbances in mice through IDO activation in the prefrontal cortex and hippocampus. The most important finding, however, was that aged mice displayed higher cognitive deficit and higher anxiety-like behaviour after the $A\beta_{1-42}$ administration. Aged mice also responded to $A\beta_{1-42}$ with a higher deficiency of BDNF, GSH and TRAP, a higher IDO activity and a higher KYN and KYN:TRP ratio in the prefrontal cortex and hippocampus. These effects of $A\beta_{1-42}$ were associated with a higher proinflammatory status, as measured by prefrontal cortex and hippocampal levels of IL-6 and IL-10. In addition, our study represents primary evidence suggesting that age-associated inflammatory signature and down-regulation of endogenous neuroprotectants in the brain render aged mice more vulnerable to $A\beta_{1-42}$-induced memory loss, anxiety symptoms and KYN pathway dysregulation, in an overlapping mechanism way (see the synopsis in **Fig. 9**).

5. Conclusions

Taken together, our data demonstrates for the first time that the neurochemical alterations and behavioural deficits (markedly cognitive decline and anxiety-like behavior) induced by an i.c.v. injection of $A\beta_{1-42}$ is aggravated by aging. In addition, our data confirm that Aβ-induced behavioural disturbances occurs concurrently with elevations in brain KYN levels. Despite additional experiments are necessary to fully determine the role of KYN metabolites in mediating the behavioral effects of $A\beta_{1-42}$-associated neuroinflammation, our study strongly confirm IDO as a key mediator of $A\beta_{1-42}$-induced neurotoxicity. It is reasonable to assume that prophylactic approaches aimed at decreasing neuroinflammation associated with aging and amyloid pathology could be important for preventing KYN pathway dysregulation and cognitive and neuropsychiatric symptoms in the elderly and AD patients.

Conflict of Interest

The authors have no conflicts of interest to report.

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Figure legends

Figure 1. Overview of study design. $A\beta_{1-42}$, amyloid-beta peptide 1-42; i.c.v., intracerebroventricular; IDO, indoleamine-2,3-dyoxygenase; IL-6, interleukin-6; IL-10, interleukin-10; BDNF, brain-derived neurotrophic factor; GSH, glutathione; TRAP, total radical-trapping antioxidant potential.

Figure 2. Effect of $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on the recognition index in the ORT (A), immobility time in the TST (B), time in the open arms of the EPMT (C) and total distance in the OFT (D) in adult and aged mice. Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ adult with Sham adult; #P<0.05 when compared sham aged with sham adult; @P<0.05 when compared Aβ aged with Aβ adult (Two-way ANOVA, Bonferroni post hoc test).

Figure 3. Effect of $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on the levels of IL-6 (A and B) and IL-10 (C and D), in the prefrontal cortex (A, C) and hippocampus (B, D) of adult and aged mice. Values are means ± S.E.M. (n=8). Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ adult with sham adult; #P<0.05 when compared sham aged with sham adult; @P<0.05 when compared Aβ aged with Aβ adult; ***P<0.05 when compared Aβ aged with sham aged (Two-way ANOVA, Bonferroni post hoc test).

Figure 4. Effect of $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on the levels of GSH (A and B) and TRAP (C and D) in the prefrontal cortex (A and C) and hippocampus (B and D) of adult and aged mice. Values are means ± S.E.M. (n=8). Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ adult with sham adult; #P<0.05 when compared sham aged with sham adult; @P<0.05 when compared Aβ aged with Aβ adult (Two-way ANOVA, Bonferroni post hoc test).

Figure 5. Effect of $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on the levels of BDNF in the prefrontal cortex (A) and hippocampus (B) of adult and aged mice. Values are means ± S.E.M. (n=8). Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ adult with sham adult; #P<0.05 when compared sham aged with sham adult; @P<0.05 when compared Aβ aged with Aβ adult (Two-way ANOVA, Bonferroni post hoc test).

Figure 6. Effect of $A\beta_{1-42}$ (400 pmol; 3µl/site; i.c.v.) on IDO activity in the prefrontal cortex (A) and hippocampus (B) of adult and aged mice. Values are means ± S.E.M. (n=8). Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ adult with sham adult; #P<0.05 when compared sham aged with sham adult; @P<0.05 when compared Aβ aged with Aβ adult (Two-way ANOVA, Bonferroni post hoc test).

Figure 7. Effect of $A\beta_{1-42}$ (400 pmol; 3µl/site; i.c.v.) on TRP levels (A and B), KYN levels (C and D) and KYN/TRP ratio (E and F) in the prefrontal cortex (A, C and E) and hippocampus (B, D and F) of adult and aged mice. Values are means ± S.E.M. (n=8). Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ adult with sham adult; #P<0.05 when compared sham aged with sham adult; @P<0.05 when compared Aβ aged with Aβ adult (Two-way ANOVA, Bonferroni post hoc test).

Figure 8. A figure illustrating the common neurobiological alterations shared between $A\beta_{1-42}$-induced neurotoxicity and aging process in mice, described in our study. $A\beta_{1-42}$ neurotoxicity is characterized by inflammation and oxidative stress in parallel with IDO activation and neurotrophic deficiency. Aging on the other hand is characterized by endogenous antioxidant deficit, low-grade inflammatory state and BDNF deficiency. These two factors appear to share common overlapping mechanisms implicated in cognitive impairment and neuropsychiatric-like symptoms.

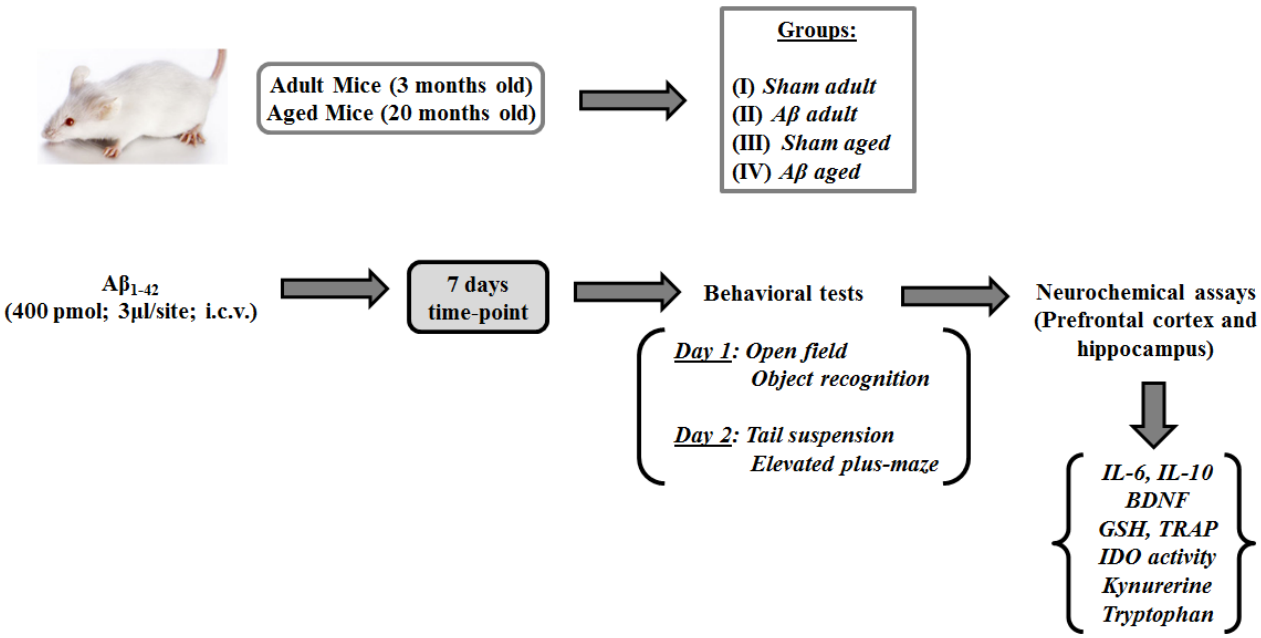
Adult Mice (3 months old)
Aged Mice (20 months old)
Groups:
(I) Sham adult
(II) Aβ adult
(III) Sham aged
(IV) Aβ aged
Aβ1-42
(400 pmol; 3µl/site; i.c.v.)
7 days
time-point
Behavioral tests
Day 1: Open field
Object recognition
Day 2: Tail suspension
Elevated plus-maze
Neurochemical assays
(Prefrontal cortex and
hippocampus)
IL-6, IL-10
BDNF
GSH, TRAP
IDO activity
Kynurerine
Tryptophan

Fig. 1

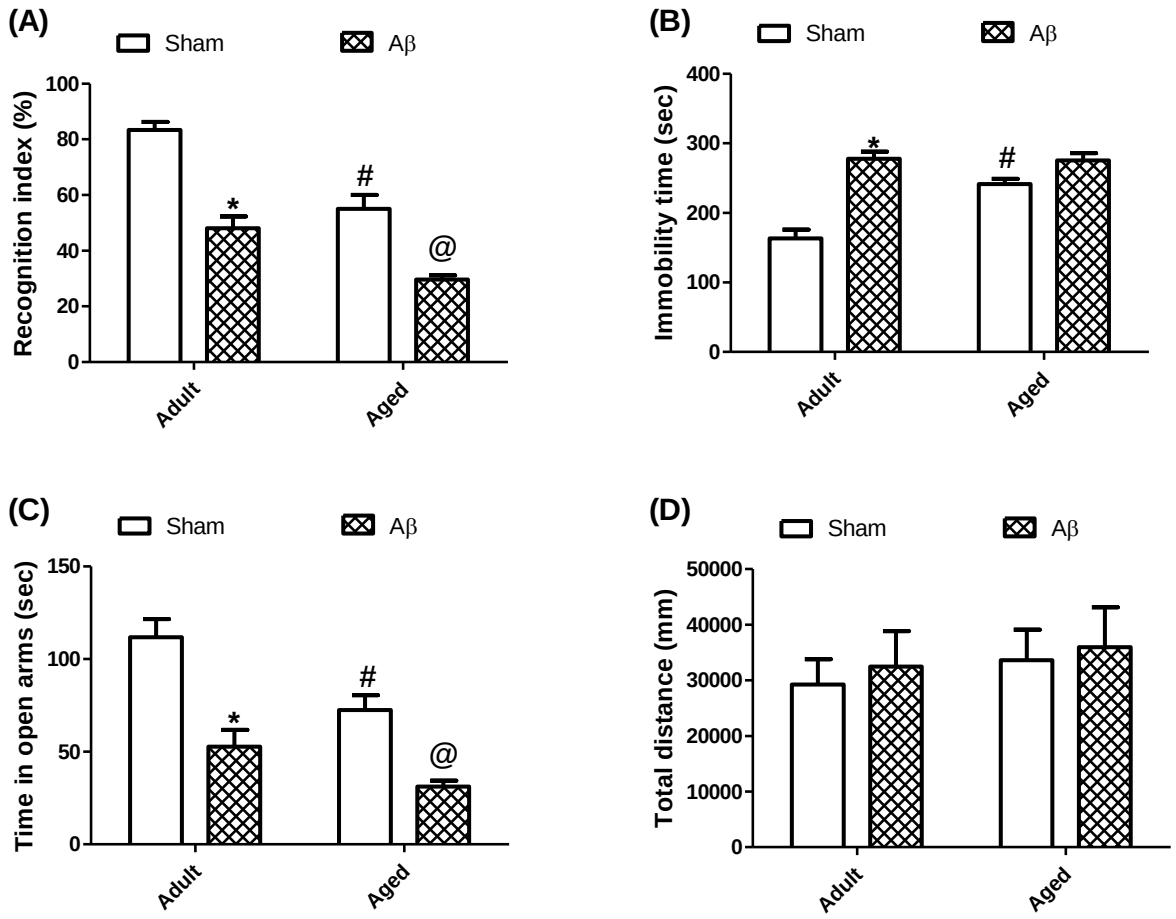
(A)
Sham
Aβ
Recognition index (%)
100
80
60
40
20
0
*
#
@
Adult
Aged
(B)
Sham
Aβ
Immobility time (sec)
400
300
200
100
0
*
#
Adult
Aged
(C)
Sham
Aβ
Time in open arms (sec)
150
100
50
0
*
#
@
Adult
Aged
(D)
Sham
Aβ
Total distance (mm)
50000
40000
30000
20000
10000
0
Adult
Aged

Fig. 2

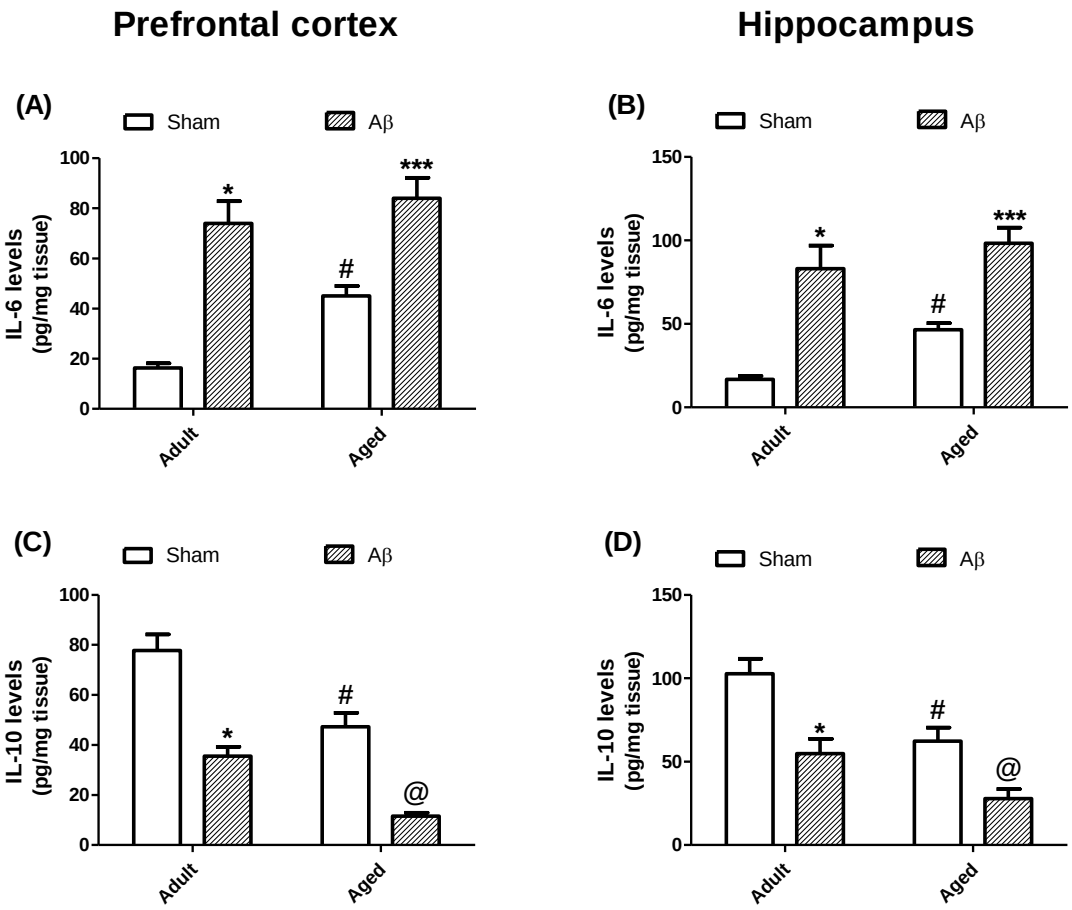
Prefrontal cortex
Hippocampus
(A)
(B)
(C)
(D)
Sham
Aβ
IL-6 levels (pg/mg tissue)
IL-10 levels (pg/mg tissue)
Adult
Aged
*
#
***
@

Fig.3

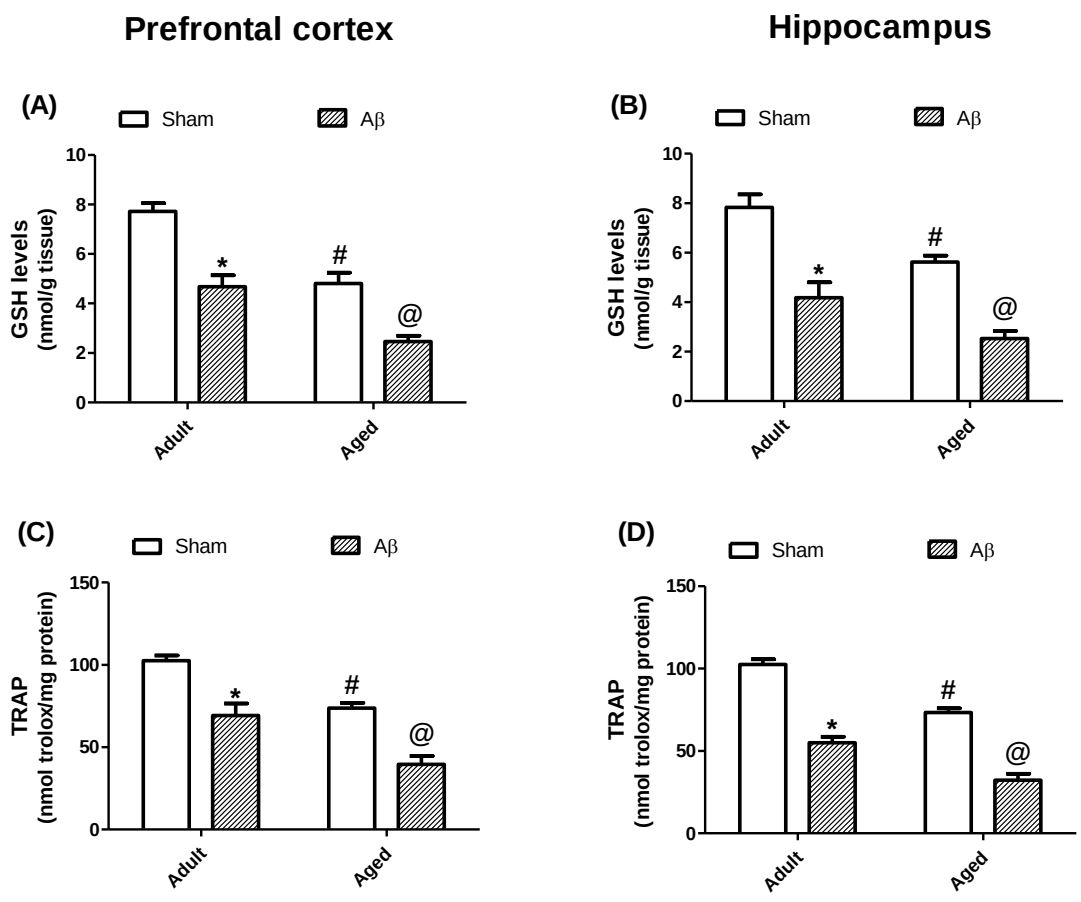
Prefrontal cortex
Hippocampus
(A)
(B)
(C)
(D)
Sham
Aβ
GSH levels (nmol/g tissue)
TRAP (nmol trolox/mg protein)
Adult
Aged
*
#
@

Fig. 4

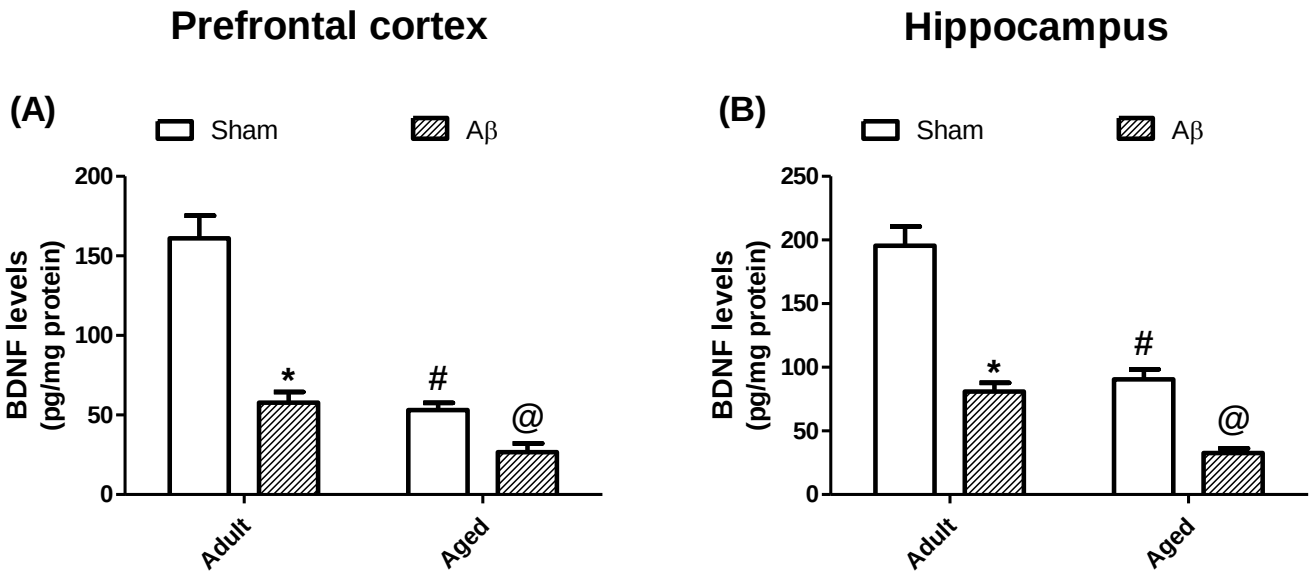
Prefrontal cortex
Hippocampus
(A)
(B)
Sham
Aβ
BDNF levels
(pg/mg protein)
200
150
100
50
0
250
*
#
@
Adult
Aged

Fig. 5

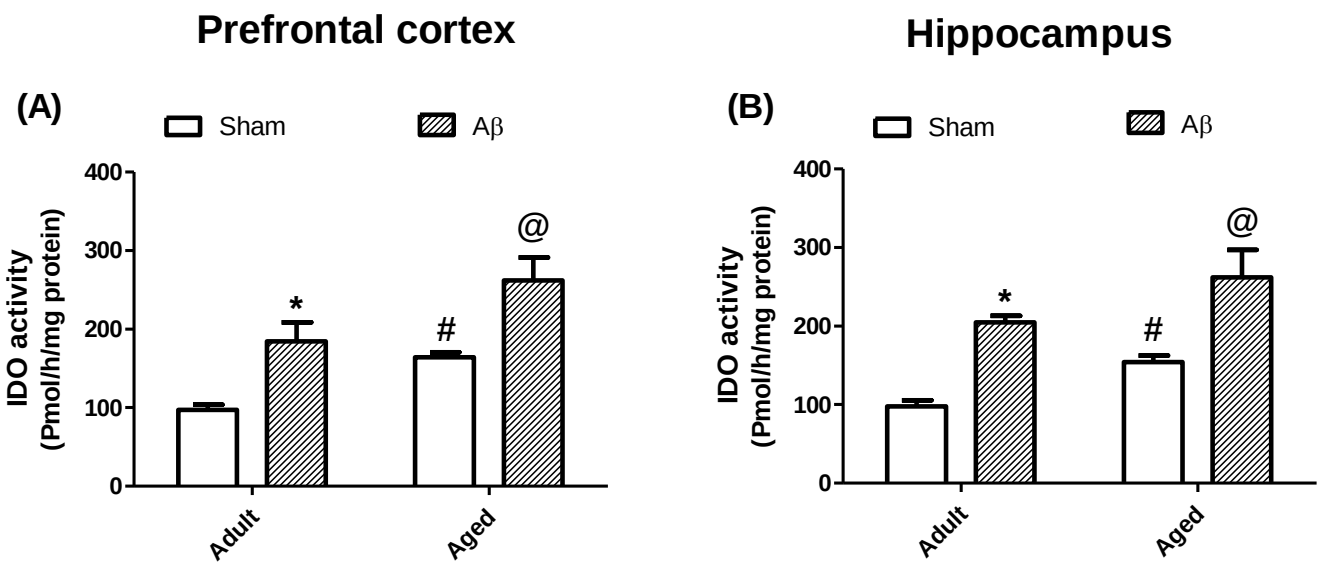
Prefrontal cortex
(A)
Sham
Aβ
IDO activity
(Pmol/h/mg protein)
400
300
200
100
0
*
#
@
Adult
Aged
Hippocampus
(B)
Sham
Aβ
IDO activity
(Pmol/h/mg protein)
400
300
200
100
0
*
#
@
Adult
Aged

Fig. 6

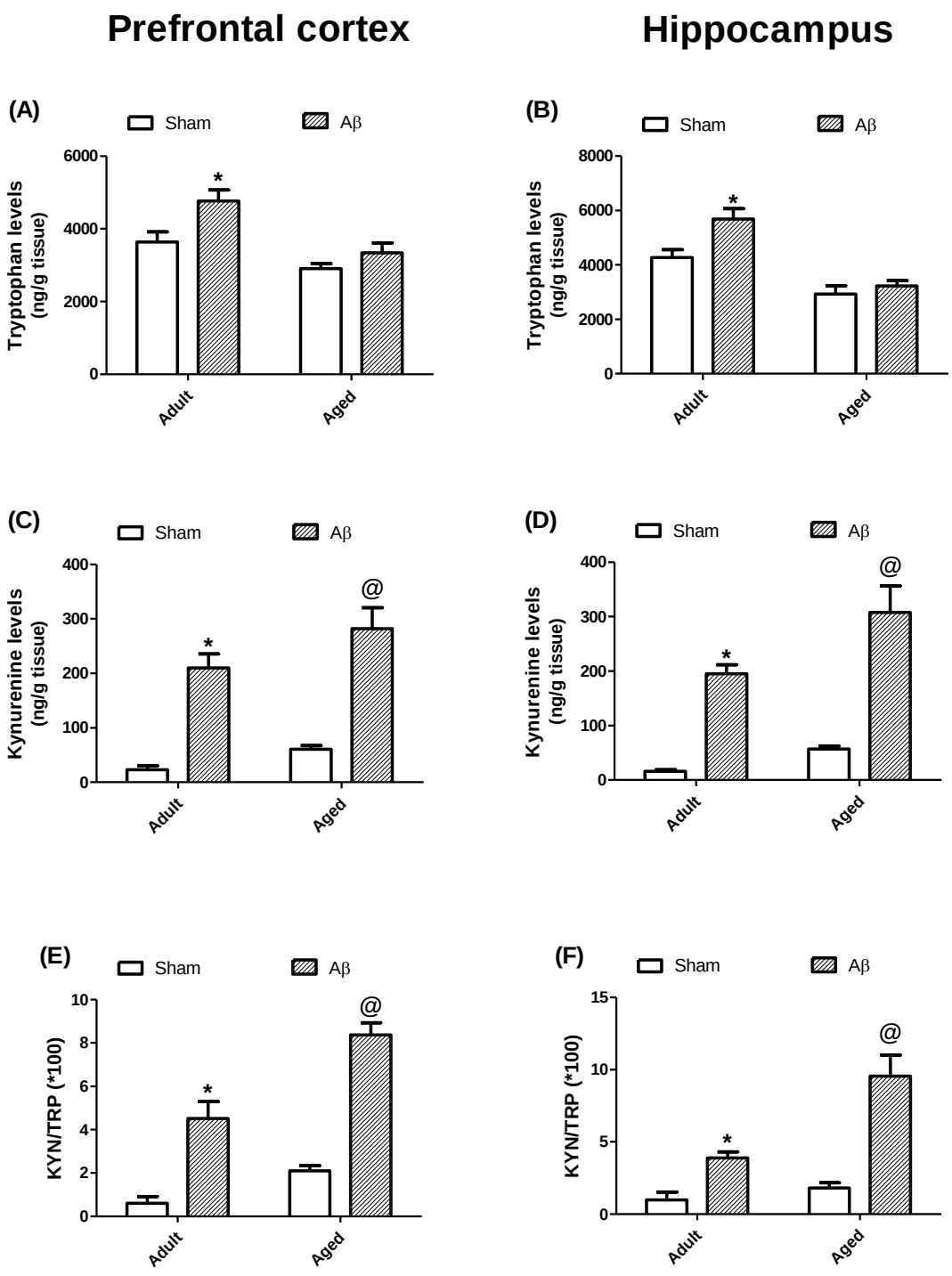
Prefrontal cortex
Hippocampus
(A)
(B)
(C)
(D)
(E)
(F)
Sham
Aβ
Tryptophan levels (ng/g tissue)
Kynurenine levels (ng/g tissue)
KYN/TRP (*100)
Adult
Aged
*
@

Fig. 7

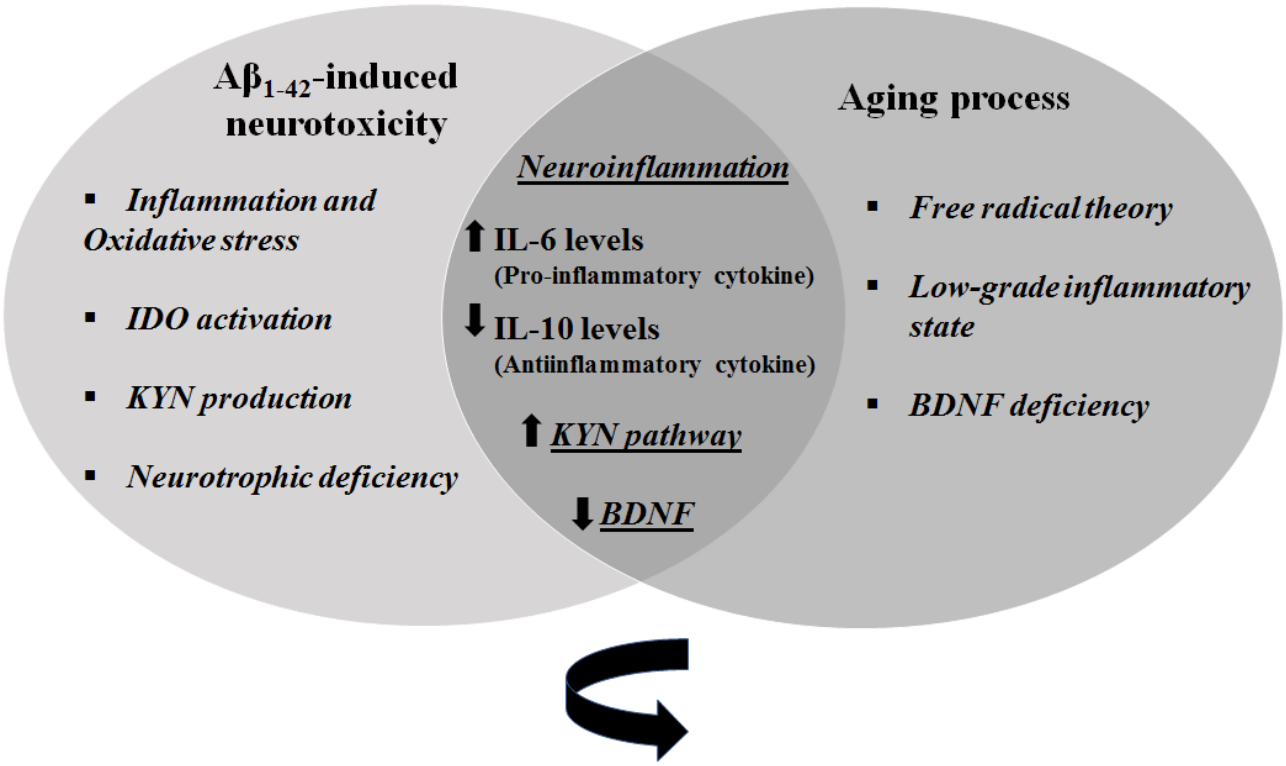
Aβ1-42-induced neurotoxicity
Inflammation and Oxidative stress
IDO activation
KYN production
Neurotrophic deficiency
Neuroinflammation
IL-6 levels
(Pro-inflammatory cytokine)
IL-10 levels
(Antiinflammatory cytokine)
KYN pathway
BDNF
Aging process
Free radical theory
Low-grade inflammatory state
BDNF deficiency
Cognitive impairment and neuropsychiatric-like symptoms

Fig. 8

MANUSCRITO II

Swimming exercise prevents behavioural disturbances induced by an intracerebroventricular injection of amyloid-β_{1-42} peptide through modulation of cytokine/NF-kappaB pathway and indoleamine-2,3-dyoxigenase in mouse brain

Leandro Cattelan Souza, Cristiano R. Jesse, Lucian Del Fabbro, Marcelo Gomes de Gomes, André Tiago Rossito Goes, Silvana Peterini Boeira

Submetido à Behavioural Brain Research.

Swimming exercise prevents behavioural disturbances induced by an intracerebroventricular injection of amyloid-β_{1-42} peptide through modulation of cytokine/NF-kappaB pathway and indoleamine-2,3-dyoxigenase in mouse brain

Leandro Cattelan Souza, Cristiano R. Jesse*, Lucian Del Fabbro, Marcelo Gomes de Gomes, André Tiago Rossito Goes, Silvana Peterini Boeira

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Abstract

Emerging evidence indicates that the activation of indoleamine-2,3-dyoxigenase (IDO), a first and rate-limiting enzyme in the kynurenine (KYN) pathway, is involved in amyloid-beta ($A\beta_{1-42}$)-neurotoxicity and Alzheimer's disease (AD) pathogenesis. Physical exercise has been considered an effective intervention in AD, attenuating or limiting their progression. Nevertheless, the neurobiological mechanisms underlying the neuroprotective effects of exercise have not yet been fully elucidated. In present study, we investigated the protective effect of an 8-week swimming training (ST) exercise on cognitive and non-cognitive functions and its role in modulating biomarkers of KYN pathway, before an intracerebroventricular (i.c.v.) injection of $A\beta_{1-42}$ (400 pmol/animal; 3µl/site) peptide in mice. Our results demonstrated that ST was effective in preventing the following behavioural disturbances caused by $A\beta_{1-42}$ injection: memory impairment in the object recognition test and depressive/anxiety-like behaviour in the tail suspension test and elevated plus-maze test, respectively. ST abrogated the neuroinflammatory response and neurotrophic deficiency in the prefrontal cortex and hippocampus induced by $A\beta_{1-42}$. Also, $A\beta_{1-42}$ increased IDO activity, KYN and tryptophan (TRP) levels and KYN:TRP ratio in the prefrontal cortex and hippocampus – alterations that were blocked by ST. It can be concluded that ST prevented behavioural and neurobiological deficits induced by $A\beta_{1-42,}$ and suggest that these neuroprotective effects are likely to involve the inhibition of inflammation/IDO activation and up-regulation of neurotrophic factors in brain of mice. Thus, it is possible that physical exercise can be used as a non-pharmacological approach to alleviates both cognitive and non-cognitive symptoms of AD.

Keywords: Alzheimer's disease, swimming exercise, memory, depression, anxiety, indoleamine-2,3-dyoxigenase, neuroinflammation, neurotrophic factors

Abbreviations: $A\beta_{1-42}$, amyloid-beta peptide 1-42; i.c.v., intracerebroventricular; ST, swimming training; IDO, indoleamine-2,3-dyoxygenase; IL-6, interleukin-6; IL-4, interleukin-4; BDNF, brain-derived neurotrophic factor; GDNF, glial cell-derived neurotrophic factor; NGF, nerve growth factor; NT-3, neurotrophin-3;

NFκB, nuclear factor-kappa B; KYN, kynurenine; TRP, tryptophan; TRYCATs, neurotoxic tryptophan catabolites.

1. Introduction

Dementia is an emerging global public health problem that affects an estimated 47.5 million people worldwide and is projected to increase to 75.6 million in 2030 and almost triple by 2050, reaching to 135.5 million [1]. Alzheimer's disease (AD) is the leading cause of dementia, which is a fatal neurodegenerative disorder that is characterized by a progressive cognitive and functional decline [2]. AD affects not only cognition but also mood and behavior, with most AD patients developing behavioural and psychological symptoms of dementia (BPSD), also called neuropsychiatric symptoms in dementia, which consist of an array of behavioural, emotional and psychological symptoms, such as depression and anxiety [3]. BPSD constitute a major component of the disease burden for both patient and family, worsening the disability and accelerating the progression of disease [3,4].

The amyloid cascade hypothesis has been the major pathogenic concept in the field of AD research for the past few decades. It states that the pathological sequence of events leading to AD are the accumulation of the amyloid-β peptide (Aβ), followed by the deposition of neurofibrillary tangles, which triggers the onset of synaptic and neuronal dysfunction and loss, finally leading to cognitive impairments [2]. The current evidence points compelling towards a central role for neuroinflammation in the mechanisms of Aβ-induced neurotoxicity and AD pathogenesis [5-9]. It has been reported that this inflammation is mediated by pro-inflammatory cytokines released from activated microglia, which is thought to contribute and exacerbate AD pathology [7,10]. Aβ peptide has been shown to be capable of directly inducing the production of inflammatory cytokines by glial cells [11]. This inflammatory response leads to neuronal damages and an increase in Aβ synthesis via activation of amyloidogenic pathway, thereby contributing to a pathological and self-sustained vicious cycle [12]. The mechanism by which Aβ activates glial cells may include nuclear factor-kappa B (NF-κB)-dependent pathway. In other words, Aβ is able to stimulate NF-κB transcription, an inducible transcription

factor complex that regulates proinflammatory gene expression and lead to the production of inflammatory molecules [11,13]. Besides, elevated levels of inflammatory cytokines in the brain have been implicated with the development of behavioural disturbances in AD, such as cognitive impairment and BPSD [14,15].

The precise mechanisms of how neuroinflammation is involved in AD pathogenesis remains not fully understood, but the kynurenine (KYN) pathway of tryptophan (TRP) metabolism is identified [16-18]. Pro-inflammatory cytokines, including interleukin-6 (IL-6) has been shown to cause an activation of the TRP-metabolizing enzyme indoleamine-2,3-dyoxigenase (IDO) [19]. Recently, a number of studies have shown that overexpression of IDO is significantly associated with inflammation and behavioural alterations in mice, both in cognitive and non-cognitive domains [20-22]. In regard with AD, previous preclinical research demonstrated that amyloid-beta ($A\beta_{1-42}$) peptide induce cytokine production and IDO activation which are involved in inflammatory process within senile plaques [23]. An increased KYN/TRP ratio was found in the blood and CSF of AD patients compared to healthy subjects and this altered ratio coincides with increased levels of IDO in the brain [23,24]. In addition, it has indicated that under brain inflammatory conditions, IDO activation results in increased KYN production and its neurotoxic downstream metabolites, which have been closely related to neurodegenerative pathways of AD [18,25]. In a recent study of our laboratory [9], we demonstrated for the first time that brain IDO activation plays a key role in mediating the memory and emotional disturbances in an experimental model based on $A\beta_{1-42}$-induced neuroinflammation. This KYN pathway activation was associated with down-regulation of neurotrophic factors in the prefrontal cortex and hippocampus, two key brain structures of the brain's memory system critically involved in cognitive and emotional regulation [26,27], contributing to behavioural deficits. Thus, it has been proposed that dysregulation of KP, mainly associated with an increased IDO activation, has been implicated in the pathophysiology of AD.

A large of evidence suggests that physical exercise presents a promising non-pharmacological option to delay the onset of or slow down the progression of AD [28-31]. Clinical reports indicated that physical exercise improves cognitive function in elderly people [32] and ameliorates memory in older adults

with mild cognitive impairment [33], supporting that exercise as a clinically relevant option toward the prevention of AD [30]. Moreover, exercise interventions in mouse models of AD have also suggested that exercise training reduces amyloid pathology and prevented cognitive and non-cognitive dysfunctions [34-36]. Exercise also has been shown to be as effective as pharmacological treatments or psychotherapies for anxiety and depressive disorders [37,38], as well as showed a positive effect in alleviating BPSD in AD patients [39].

Whilst the neurobiological mechanisms by which physical exercise alleviates AD deficits are yet unclear, a number of recent studies have expanded the research question by investigating potential pro-neurogenic and anti-inflammatory effects of exercise [29-31]. In this regard, aerobic physical exercise has been suggested to increase the synthesis of neurotrophic factors, thereby enhancing neurogenesis and synaptogenesis, which in turn improve memory and cognitive functions [29]. Similarly, we have reported that swimming exercise training prevented the cognitive decline, oxidative stress and neuroinflammation in a mouse model of acute inflammation induced by i.c.v. injection of $A\beta_{1-40}$ peptide, that resembles the early phases of AD [40]. However, up to now, there is no previous studies addressing the impact of physical exercise on brain IDO activation and its role in the prevention of concomitant behavioural disturbances in a mouse model of AD. Furthermore, only a few studies have focused on the protective role of exercise in non-cognitive symptoms associated with AD in experimental models.

In view of this, in the present study we sought to approach this question by investigating whether swimming exercise prevents the cognitive/emotional disturbances induced by i.c.v. administration of $A\beta_{1-42}$ peptide through inflammation-induced IDO activation in the brain. In addition, neurochemical parameters related to inflammatory response, neurotrophic factors and KP metabolism were evaluated in the prefrontal cortex and hippocampus in an attempt to determine the underlying mechanisms of neuroprotective effects of physical exercise. Swimming was chosen as paradigm of exercise as there is still scarce information regarding the impact of this kind of exercise on mouse models of AD. Moreover, swim is a natural behaviour of rodents and imposes less mechanical stress due to water thrust and recruitment of different muscles

[41]. Swimming is considered an effective kind of exercise to enhance muscle oxidative capacity and promotes adaptation to physical training in rodents similar to those observed in human beings. Likewise, swim lacks the presence of electric stimulus as stress factor [42].

2. Methods

2.1 Animals

Experiments were performed using male Swiss Albino mice (3 months old, weighing 30–40 g). Animals were maintained at 22-25ºC with free access to water and food, under a 12:12h light/dark cycle, with lights on at 7:00 a.m. All manipulations were carried out during light phase on the day. All efforts were made to minimize animal suffering and to reduce the number of animals used. The procedures of this study were conducted according to the guidelines of the Committee on Care and Use of Experimental Animals Resources and with the approval of Ethical Committee for Animal Use (CEUA protocol # 012/2012) of Federal University of Pampa, Brazil.

2.2 Experimental design

Mice were randomly assigned into four groups (n=8 per group): (1) sham sedentary; (2) $A\beta_{1-42}$ sedentary; (3) sham exercise and (4) $A\beta_{1-42}$ exercise. In this experimental design, exercise groups were submitted to swimming training for 8 weeks with a progressive increase of both intensity and time. Sedentary groups were maintained in physical inactivity. After 24h of last bout of ST, mice received intracerebroventricular (i.c.v.) injection of $A\beta_{1-42}$ or vehicle. Seven days after i.c.v. injection, mice underwent a battery of behavioural tests for 2 days and, finally, submitted to euthanasia. The prefrontal cortex, hippocampus and quadriceps femoris muscle were removed for assays (**Fig. 1**). The 7 days time-point was chosen based on our previous study [9], wherein the data of the time-course showed that the Aβ-induced neurochemical and behavioural alterations are more evident 7 days post injection.

2.3 Exercise training protocol

Mice in the exercise groups were submitted to an 8-week swimming training (ST) program with a customized regimen as previously described by us [40]. In the first week, mice were acclimatized to the unfamiliar activity (swimming adaption period) in 250-L water-filled tank with the temperature kept at 31º ± 2ºC, in order to decrease the stress of swimming activity. In the adaptation period, animals could swim or stand in the tank with water depth of 5cm. In the beginning of the second week, water deep was increased to 20cm, so that the hind limbs of the animals could not reach the bottom of the tank. Progressively, larger weights were attached to the proximal portions of animal's tails in order to increase the exercise intensity; the weights were 0-3%, in correspondence to body weight (BW) (**Table 1**). This intensity is considered below to anaerobic threshold for swimming training, in which was demonstrated by literature that workloads of up to 6% BW for rats [43] and 4% BW for mice [44] can be considered 'sub-threshold' and is indicated to improvement of aerobic capacity [43]. The swimming bouts were performed five times per week, and animals swam individually in group of 8 animals, separated by opaque acrylic boards. After each daily swimming bouts, animals were towel dried and placed near a heater until the hair dried.

2.4 I.c.v. injection of $A\beta_{1-42}$

$A\beta_{1-42}$ (Sigma-Aldrich) was prepared as stock solution at a concentration of 1 mg/ml in sterile 0.1M phosphate-buffered saline (PBS) (pH 7.4), and aliquots were stored at -20º C. $A\beta_{1-42}$ was aggregated by incubation at 37ºC for 4 days before use [9,40]. The aggregated form of $A\beta_{1-42}$ (400pmol/mouse) was administered by i.c.v. route, using a microsyringe with a 28-gauge stainless-steel needle 3.0 mm long (Hamilton), as described previously [8,45]. Briefly, mice were anesthetized with intraperitoneal (i.p.) injection of sodium pentobarbital (0.067mg/g) and placed in a stereotaxic apparatus (Insight, Brazil). Under light anesthesia (i.e. just that necessary for loss of the postural reflex), the needle was inserted unilaterally 1mm to the right of the midline point equidistant from each eye and 1mm posterior to a line drawn through the anterior base of the eyes (used as external reference). $A\beta_{1–42}$ was administered right-unilaterally into the lateral ventricle. The injection volume of 3µl of $A\beta_{1–42}$ or

PBS was delivered gradually (1µl/min) using the following coordinates from bregma: anteroposterior (AP) = −0.1 mm, mediolateral (ML) = 1 mm, and dorsoventral (DV) = −3 mm. The sham-operated mice were injected with PBS (3µl/site; i.c.v.). The advantage of this i.c.v. route of administration is the quick distribution of the peptide throughout the brain [46]. In order to confirm the accurate placement of the injection site (needle track) at the moment of dissection of the animals, two mice in each group were submitted to dye injection (Evans blue dye, 0,5 µl) into the ventricles [47].

2.5 Behavioural assessment

During a period of 2 days, starting 7 days after i.c.v. injection of $A\beta_{1-42}$, the animals were submitted to a battery of behavioural paradigms that include open-field test and object recognition test (day 1), and tail suspension test and elevated plus maze task (day 2). In order to minimize negative interferences of stress on cognitive performance of the animals, we separated the emotional tests from the object recognition test. Moreover, it is noteworthy that on day 1, the open-field test was also used as the habituation phase for object recognition test. All tests were carried out between 9:00 and 16:00h and they were scored by the same trained raters (who were blind to the experimental treatments) in an observation room where the mice had been habituated for at least 1 h before the beginning of the tests.

2.5.1 Open-field test (OFT)

The OFT was carried out to evaluate if the drugs produced effects on locomotor activity. The animals were submitted individually for a period of 5 min to an OFT apparatus (Insight Ltd., Ribeirao Preto, SP, Brazil). The total distance (unit: mm) was computed [49,49].

2.5.2 Object recognition test (ORT)

After the OFT, the memory function of mice was assessed with the ORT. The task is based on the spontaneous tendency of rodents, previously exposed to two identical objects, to later explore one of the objects (preference of a novel object) for a longer time than they explore the familiar object. This test requires no external motivation, reward, or punishment and a little training or habituation

is required [50]. The ORT was performed as described by Ennaceur and Delacour [51] with some modifications. Mice were placed in an open box (similar to OFT) and allowed to explore two identical objects (sample phase) for 5 min and then returned to their home cage. To evaluate the short-term memory, mice were returned to the open box, after a delay of 90 min (intertrial interval), where they were exposed to two different objects (test phase), one identical to the one previously encountered in the sample phase, therefore now familiar, and the other is novel. The animals were allowed to explore both objects for more 5 min. After each trial, box and objects were cleaned with 70 % ethanol. The positions of the objects in the test and the objects used as novel or familiar were counterbalanced between the animals. Exploratory behavior was defined as sniffing or touching the object with the nose and/or forepaws. Any other behavior, such as sitting on or turning around the object was not considered as exploration. The amount of time each animal spent actively investigating the objects was manually scored and recognition index was calculated as the time exploring novel or familiar object divided by the total time spent exploring both objects.

2.5.3 Tail suspension test (TST)

The second day of behavioral assessments started with the TST. Mice were suspended 50 cm above the floor by adhesive tape placed approximately 1cm from de tip of the tail, and the immobility time was recorded for 6 min. The immobility behavioral was determined according to the method described by Steru et al. [52].

2.5.4 Elevated plus-maze test (EPMT)

Three hours after the TST, the EPMT was used to evaluate anxiety-like behavior in mice, in accord with the method of Pellow et al. [53]. The experimental apparatus (Insight Ltd., Ribeirao Preto, SP, Brazil) was shaped like a ‘plus' sign and consisted of two open arms (30 × 5 cm) and two equal-sized closed arms (30 × 5 × 15 cm) extending from a common central platform (5 × 5 cm). The maze was made of opaque grey PVC and was kept elevated at a height of 50 cm above the floor. The experiments were conducted in a sound-attenuated room under low intensity light (12 lx). The animals were individually

placed in the central area of the maze facing an enclosed arm and were observed for 5 min. The apparatus was cleaned with ethanol solution (10% v/v) and dried with paper towels after each trial in order to avoid odor impregnation. During a 5 min test period, it was recorded the number of entries either the open or enclosed arms, plus the time spent in the open arms. An entry was defined as placing all four paws within the boundaries of the arm. The following measures were obtained from the test: (a) time spent in the open arms relative to the total time spent in the plus-maze (300 s); (b) number of entries into the open arms; (c) number of entries into the closed arms. The anxiolytic effectiveness of a drug is illustrated by a significant statistical augmentation of parameters in open arms (time and/or entries) [54].

2.6 Tissue preparation for assays

After behavioral tests, mice were euthanized with barbiturate overdose (pentobarbital sodium 150 mg/kg; i.p. route) and transcardiacally perfused with 10 ml ice-cold saline via the aorta. The brain dissection was performed according to the method of Spijker [55], a method to dissect multiple brain regions from a single brain based on existing atlases [56]. Prefrontal cortex and hippocampus were bilaterally removed and rapidly homogenized in 50 mM Tris–Cl, pH 7.4. The homogenate was centrifuged at 2,400×*g* for 15 min at 4ºC and a low-speed supernatant fraction (S_1) was used for assays. Quadriceps femoris muscle was removed and stored at -80ºC for determination of citrate synthase (CS) activity, in order to determine the efficacy of exercise protocol.

2.7 Neurochemical assays

2.7.1 Cytokine levels

Levels of interleukin-6 (IL-6) and interleukin-4 (IL-4) in the prefrontal cortex and hippocampus were measured using sample aliquots of 100 µL and mouse cytokine ELISA DuoSet Kits from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's instructions (protein range of 31.25–2,000 pg). The level of cytokine was estimated by interpolation from a standard curve by colorimetric measurements at 450 nm (correction wavelength 540 nm) on an ELISA plate reader (Berthold Technologies-Apollo 8-LB 912, KG, Germany). Results are shown as pg/mg of protein.

2.7.2 Neurotrophic factors levels

Protein levels of brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and neurotrophin-3 (NT) were measured using a commercially available sandwich enzyme-linked immune sorbent assay (ELISA) kit (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions. BDNF and NGF levels were evaluated in S1 of prefrontal cortex and hippocampus. The BDNF, NGF and GDNF levels were expressed as pg/mg wet weight of tissue.

2.7.3 Nuclear factor-kappa B (NF-kB) levels

The NF-kB levels were determined using the NF-kB p65 (Total and Phosphorylated) InstantOne ELISA Assay kit (Colorimetric Detection, catalog number 85-86083, eBioscience Ready-SET-Go, USA) according to the manufacturer's instructions. Briefly, the hippocampi were homogenized with specific kit lysis buffer, lysates were centrifuged and the supernatant was removed for analysis. Sample, negative control and positive control were incubated with antibody cocktail followed Detection Reagent. The Stop Solution was added and the absorbance was measured on a microplate reader (450 nm). The Pierce BCA Protein Assay kit was used to determine the protein concentration of each sample. The NF-kB levels were expressed as relative optical density/mg protein.

2.7.4 Tryptophan (TRP) and kynurenine (KYN) levels

The levels of TRP and its metabolite KYN in the prefrontal cortex and hippocampus were performed in a Shimadzu LC-10A liquid chromatograph, according to Silva et al. [57]. The chromatographic separation was achieved using a 250- by 4.6-mm (inner diameter) C_{18} reverse-phase column (particle size, 4 µm; Aquapore RP-300 C-18). For TRP measurement, the column was eluted isocratically at flow rate of 1.0 ml/min with 0.015 M sodium acetate (pH 4.5) containing 15% methanol. For KYN determination, the column was eluted with acetonitrile at a 1:47 dilution in 0.1 M acetic acid–0.1 M ammonium acetate (pH 4.65). The absorbance of the column effluent was monitored at 280 and 365 nm for TRP and KYN respectively. The peaks of TRP or KYN were identified by comparison with the retention times of standard compounds

(Sigma), and quantification was based on the ratios of the peak areas of compound to the internal standard. The tissue levels were expressed in pg/mg tissue.

2.7.5 Indoleamine-2,3-dyoxigenase (IDO) activity

IDO activity in the prefrontal cortex and hippocampus was determined as previously described [58]. The supernatants (0.2 ml) were added to 0.8 ml of the reaction mixture containing 400 µM L-tryptophan, 20mM ascorbate, 10 µM methylene blue, and 100 µg catalase in 50mM potassium phosphate buffer pH 6.5. The reaction was carried out at 37ºC under agitation for 60 min. Then, it was blocked by adding 0.2 ml of 30% trichloroacetic acid and further incubated at 50ºC for 30 min to convert the N-formylkynurenine to L-kynurenine. Samples were centrifuged at 13,000*g* for 10 min at 4ºC. The supernatants were filtered through microspin ultrafiltrates with a cut-off of 10,000M_r before being taken for measurement of IDO.

The amount of L-kynurenine formed from TRP was determined by reversed phase high pressure liquid chromatography (HPLC). One hundred µl of the reaction product was injected onto a Merck LiChrospher column (150mm long, 4.6mm diameter, packed with 5 lm silica beads holding 18C long carbon chains). A cartridge guard column containing the same material as the analytical column was used. The mobile phase consisted of 0.1M ammonium acetate buffer (pH 4.65) with 5% acetonitrile. Flow rate was 1 ml/min. KYN was detected using a spectrometer measuring absorbency at a wavelength of 365nm and was quantified using known amounts of L-kynurenine. The retention time of KYN was around 5.35 min. All determinations were performed in duplicate. One unit of the activity was defined as 1 nmol KYN/h/mg protein at 37ºC.

2.8 Aerobic metabolism assay

2.8.1 Citrate synthase (CS) activity

CS activity was measured from quadriceps femoris using commercially citrate synthase activity assay kit (Sigma Aldrich). Briefly, muscle tissue from each animal was homogenized in an extraction buffer (50 mM Tris-Hcl and 1 mM EDTA, pH 7.4). After centrifugation at 13,000 rpm for 1min at 4ºC, aliquots

of supernatants were used for the measurement of the enzyme activity. The activity of CS was expressed as units/mg protein.

2.9 Protein determination

Protein concentration was measured by the method of Bradford [59], using bovine serum albumin as the standard.

2.10 Statistical analysis

The data distribution was verified by applying the Kolmogorov-Smirnov test. Results are presented as means ± standard error medium (SEM). Comparisons between the experimental and the control groups were performed by one-way (exercise = independent variable) or two-way ANOVA ($A\beta_{1-42}$ X exercise treatments = independent variables) followed by Bonferroni post hoc test, when appropriate. A value of $P < 0.05$ was considered to be statistically significant. All tests were carried out using the GraphPad software 5.0 (San Diego, California, USA).

3. Results

3.1 Effect of swimming training (ST) on the activity of citrate synthase (CS) in quadriceps muscle

Citrate synthase (CS) activity is a validated biomarker for mitochondrial density in skeletal muscle. CS activity is also used as a biochemical marker of the skeletal muscle oxidative adaptation to a training intervention in experiments with rodents [60] and humans [61]. In light of this, the activity of CS was analyzed in quadriceps femoris muscle to assay the efficacy of exercise training protocol.

One-way ANOVA revealed that exercise increased significantly CS activity in trained animals in relation to sedentary animals ($F_{4,35} = 11.05$, $p < 0.001$; **Fig. 2**), ensuring that exercise protocol was effective in enhancing the muscle oxidative metabolism.

3.2 Effect of ST and Aβ$_{1-42}$ i.c.v. injection on cognitive and non-cognitive functions

3.2.1 Memory in the object recognition test (ORT)

Two-way ANOVA of cognitive performance in the ORT demonstrated a significant effect of Aβ$_{1-42}$ ($F_{1, 24}$ = 45.06 p < 0.001) exercise ($F_{1, 24}$ = 31.50, p < 0.001) and Aβ$_{1-42}$ × exercise interaction ($F_{1, 24}$ = 16.13, p < 0.001). Post hoc comparisons revealed that i.c.v. injection of Aβ$_{1-42}$ significantly decreased the recognition of mice compared to sham group, thereby inducing a memory impairment (p < 0.001). Exercise protected against Aβ$_{1-42}$-induced memory deficits, normalizing the recognition index in ORT (p < 0.001; **Fig. 3**A). The total time exploration in ORT was not changed significantly by Aβ$_{1-42}$ ($F_{1, 24}$ = 0.13 p = 0.72), exercise ($F_{1, 24}$ = 0.02, p = 0.89) and Aβ$_{1-42}$ × exercise interaction ($F_{1, 24}$ = 0.01, p = 0.95), ensuring that the performance in the tests was not influenced by motivational interferences or locomotor deficits (**Fig. 3**B)

3.2.2 Non-cognitive symptoms in the tail suspension test (TST) and elevated plus-maze test (EPMT) and locomotor activity in the open-field test (OFT)

Statistical analysis of immobility time in TST yielded significant effect of Aβ$_{1-42}$ ($F_{1, 24}$ = 21.23 p < 0.001) exercise ($F_{1, 24}$ = 73.18, p < 0.001) and Aβ$_{1-42}$ × exercise interaction ($F_{1, 24}$ = 8.55, p < 0.001). Post hoc comparisons showed that i.c.v. injection of Aβ$_{1-42}$ significantly increased the duration of immobility of mice when compared to sham group (p < 0.001), which is interpreted as a depressive-like effect. Exercise protected against the increase of immobility time induced by Aβ$_{1-42}$ (p < 0.001). In addition, exercise itself reduced the time spent immobile, showing an antidepressant-like effect (p < 0.01; **Fig. 4**A).

Two-way ANOVA of time spent in open arms in the EPMT demonstrated a significant effect of Aβ$_{1-42}$ ($F_{1, 24}$ = 10.74 p < 0.01) exercise ($F_{1, 24}$ = 13.95, p < 0.01) and Aβ$_{1-42}$ × exercise interaction ($F_{1, 24}$ = 7.95, p < 0.05). Post hoc comparisons showed that Aβ$_{1-42}$-treated mice experienced increased anxiety-like behaviour as they spent significantly lower time in the open arms, when compared to sham group (p < 0.001). Exercise prevented the appearance of anxiety-like behaviour evoked by Aβ$_{1-42}$, maintaining the time spent in open

arms at the level of control group (p < 0.001; **Fig. 4**B). No significant differences among groups were observed in the number of open-arms and enclosed-arms entries (data not shown).

Two-way ANOVA of distance traveled in the OFT was not changed significantly by $A\beta_{1-42}$ ($F_{1, 24}$ = 0.21, p = 0.88), exercise ($F_{1, 24}$ = 0.39, p = 0.53) or their interaction ($F_{1, 24}$ = 0.03, p = 0.85), ensuring that locomotor activity was not significantly affected in all groups tested (**Fig. 4**C).

3.3 Effect of ST and $A\beta_{1-42}$ i.c.v. injection on cytokine levels

In order to determine the $A\beta_{1-42}$-induced neuroinflammation we sought to determine the levels of the pro-inflammatory cytokine interleukin-6 (IL-6) and the levels of the anti-inflammatory cytokine interleukin-4 (IL-4).

Two-way ANOVA of IL-6 in the prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × exercise interaction ($F_{1,24}$ = 6.36, p < 0.01). Post hoc comparisons revealed that exercise fully prevented the increase of IL-6 levels caused by $A\beta_{1-42}$ (p < 0.001; **Fig. 5**A). Similarly, two-way ANOVA of IL-6 in the hippocampus yielded a significant $A\beta_{1-42}$ × exercise interaction ($F_{1,24}$ = 6,71, p < 0.01). Post hoc comparisons showed that the increased IL-6 levels observed in the hippocampus of Aβ-treated mice was markedly abrogated by exercise (p < 0.001). In addition, trained animals exhibited lower levels of IL-6 than sedentary animals in control conditions, supporting the anti-inflammatory effect of exercise (p < 0.05; **Fig. 5**B).

Statistical analysis of IL-4 in the prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × exercise interaction ($F_{1,24}$ = 23.06, p < 0.001). Post hoc comparisons revealed that the increase of IL-4 levels induced by $A\beta_{1-42}$ was significantly attenuated by exercise (p < 0.001; **Fig. 5**C). As with the prefrontal cortex, statistical analysis of IL-4 in the hippocampus yielded a significant $A\beta_{1-42}$ × exercise interaction ($F_{1,24}$ = 78.45, p < 0.001). Post hoc comparisons showed that the increased IL-4 levels observed in the hippocampus of Aβ-treated mice was fully prevented by exercise (p < 0.001; **Fig. 5**D).

3.4 Effect of ST and Aβ$_{1-42}$ i.c.v. injection on nuclear factor-kappa B (NFκB) levels

Two-way ANOVA of *NF-κB* in the prefrontal cortex demonstrated a significant effect of Aβ$_{1-42}$ ($F_{1, 24}$ = 28.33, p < 0.001), exercise ($F_{1, 24}$ = 15.59, p < 0.001) and Aβ$_{1-42}$ × exercise interaction ($F_{1, 24}$ = 6.99, p < 0.01). Post hoc comparisons revealed that exercise fully prevented the increase of *NF-κB* levels caused by Aβ$_{1-42}$ (p < 0.001). In addition, *NF-κB* levels in sham exercised mice were higher when compared to that of sham sedentary mice, but not reach statistical significance (**Fig. 6**A). Similarly, two-way ANOVA of *NF-κB* in the hippocampus yielded a significant effect of Aβ$_{1-42}$ ($F_{1, 24}$ = 98.31, p < 0.001) exercise ($F_{1, 24}$ = 101.4, p < 0.001) and Aβ$_{1-42}$ × exercise interaction ($F_{1, 24}$ = 29.83, p < 0.001). Post hoc comparisons showed that the increased *NF-κB* observed in the hippocampus of Aβ-treated mice was markedly abrogated by exercise (p < 0.001). Once again, higher levels of *NF-κB* was found in sham exercised mice when compared to their counterparts sedentary, but not reach statistical significance (**Fig. 6**B).

3.5 Effect of ST and Aβ$_{1-42}$ i.c.v. injection on neurotrophic levels

3.5.1 Levels of Brain-derived neurotrophic factor (BDNF) and glial cell-line derived neurotrophic factor (GDNF)

Two-way ANOVA of BDNF in the prefrontal cortex demonstrated a significant Aβ$_{1-42}$ × exercise interaction ($F_{1,24}$ = 11.64, p < 0.01). Post hoc comparisons revealed that the Aβ-induced decrease of BDNF levels is normalized by exercise (p < 0.001). In addition, BDNF levels in sham exercised mice were significantly increased compared to that of sham sedentary mice (p < 0.01), supporting an exercise-induced upregulation of BDNF protein (**Fig. 7**A). In the hippocampus, two-way ANOVA of BDNF yielded a significant Aβ$_{1-42}$ × exercise interaction ($F_{1,24}$ = 14.51, p < 0.001). Post hoc comparisons showed that exercise significantly prevented the loss of BDNF levels induced by Aβ$_{1-42}$ (p < 0.001). Moreover, exercise itself increased the BDNF levels in hippocampus of mice (p < 0.01; **Fig. 7**B).

Statistical analysis of GDNF in the prefrontal cortex demonstrated a significant Aβ$_{1-42}$ × exercise interaction ($F_{1,24}$ = 13.47, p < 0.01). Post hoc

comparisons revealed that the decrease of GDNF levels induced by $A\beta_{1-42}$ was significantly abrogated by exercise ($p < 0.001$). In addition, exercise itself increased the GDNF levels in the prefrontal cortex of mice ($p < 0.01$; **Fig. 7**C). As with the prefrontal cortex, statistical analysis of GDNF in the hippocampus yielded a significant $A\beta_{1-42}$ × exercise interaction ($F_{1,24} = 8.66$, $p < 0.01$). Post hoc comparisons showed that the decreased GDNF levels observed in the hippocampus of Aβ-treated mice was also normalized by exercise ($p < 0.001$). Once again, exercised mice showed higher levels of GDNF in comparison with sedentary mice, indicating that exercise significantly modulated GDNF content in hippocampus ($p < 0.01$; **Fig. 7**D).

3.5.2 Levels of Nerve growth factor (NGF) and neurotrophin-3 (NT-3)

Two-way ANOVA of NGF in the prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × exercise interaction ($F_{1,24} = 7.59$, $p < 0.01$). Post hoc comparisons revealed that exercise totally protected against the decrease of NGF levels caused by $A\beta_{1-42}$ ($p < 0.001$; **Fig. 8**A). In the hippocampus, two-way ANOVA of NGF showed a significant $A\beta_{1-42}$ × exercise interaction ($F_{1,24} = 8.27$, $p < 0.01$). Post hoc comparisons revealed that exercise totally prevented the loss in NGF levels induced by $A\beta_{1-42}$ ($p < 0.001$; **Fig. 8**B).

Statistical analysis of NT-3 in prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × 1-MT interaction ($F_{1,24} = 10.58$, $p < 0.01$). Post hoc comparisons revealed that the decrease of NT-3 levels induced by $A\beta_{1-42}$ was attenuated by exercise ($p < 0.01$; **Fig. 8**C). As with the prefrontal cortex, statistical analysis of NT-3 in the hippocampus yielded a significant $A\beta_{1-42}$ × exercise interaction ($F_{1,24} = 8.21$, $p < 0.01$). Post hoc comparisons showed that the decreased NT-3 levels observed in the hippocampus of Aβ-treated mice was also mitigated by exercise ($p < 0.05$; **Fig. 8**D).

3.6 Effect of ST and $A\beta_{1-42}$ i.c.v. injection on biomarkers of Kynurenine (KYN) pathway

3.6.1 Indoleamine-2,3-dyoxigenase (IDO) activity

Two-way ANOVA of IDO activity in the prefrontal cortex demonstrated a significant effect of $A\beta_{1-42}$ ($F_{1, 24} = 97.01$, $p < 0.001$), exercise ($F_{1, 24} = 42.60$, $p <$

0.001) and $A\beta_{1-42}$ × exercise interaction ($F_{1, 24}$ = 25.87, p < 0.001). Post hoc comparisons revealed that $A\beta_{1-42}$ injection significantly increased IDO activity when compared to sham group (p < 0.001). Exercise notably mitigated the induction of IDO induced by $A\beta_{1-42}$ (p < 0.001; **Fig. 9**A). In a similar manner, two-way ANOVA of IDO activity in the hippocampus demonstrated a significant effect of $A\beta_{1-42}$ ($F_{1, 24}$ = 83.94, p < 0.001), exercise ($F_{1, 24}$ = 29.46, p < 0.001) and $A\beta_{1-42}$ × exercise interaction ($F_{1, 24}$ = 15.08, p < 0.01). Post hoc comparisons showed that the injection of $A\beta_{1-42}$ significantly increased IDO activity in the hippocampus (p < 0.001). Once again, exercise significantly attenuated the induction of IDO induced by $A\beta_{1-42}$ (p < 0.001; **Fig. 9**B).

3.6.2 Levels of KYN and tryptophan (TRP) and KYN:TRP ratio

Two-way ANOVA of TRP levels in the prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × exercise interaction ($F_{1,24}$ = 6.03, p < 0.05). Post hoc comparisons revealed that the Aβ-induced increase of TRP levels were normalized by exercise (p < 0.001; **Fig. 10**A). Similarly, two-way ANOVA of TRP levels in the hippocampus revealed a significant $A\beta_{1-42}$ × exercise interaction ($F_{1,24}$ = 51.75, p < 0.001). Post hoc comparisons demonstrated that exercise significantly abrogated the increase of TRP levels in the hippocampus induced by $A\beta_{1-42}$ (p < 0.001; **Fig. 10**B).

Statistical analysis of KYN levels in prefrontal cortex yielded a significant $A\beta_{1-42}$ × exercise interaction ($F_{1,24}$ = 43.75, p < 0.001). Post hoc comparisons revealed that the increase of KYN levels induced by $A\beta_{1-42}$ was notably mitigated by exercise (p < 0.001; **Fig. 10**C). As with the prefrontal cortex, statistical analysis of KYN levels in the hippocampus demonstrated a significant $A\beta_{1-42}$ × exercise interaction ($F_{1,24}$ = 11.40, p < 0.01). Post hoc comparisons showed that the increased KYN levels observed in the hippocampus of Aβ-treated mice was also attenuated by exercise (p < 0.001; **Fig. 10**D).

Two-way ANOVA of KYN/TRP ratio in the prefrontal cortex demonstrated a significant $A\beta_{1-42}$ × exercise interaction ($F_{1,24}$ = 14.56, p < 0.01). Post hoc comparisons revealed that the Aβ-induced increase of KYN/TRP ratio is attenuated by exercise (p < 0.001; **Fig. 10**E). In similar manner, two-way

ANOVA of KYN/TRP ratio in the hippocampus yielded a significant $A\beta_{1-42}$ × exercise ($F_{1,24}$ = 3.46, $p < 0.05$). Post hoc comparisons showed that exercise significantly mitigated the increase of TRP levels in the hippocampus induced by $A\beta_{1-42}$ ($p < 0.01$; **Fig. 10**F).

4. Discussion

Numerous studies have highlighted the role of brain IDO activation by proinflammatory cytokines in the mechanisms of Aβ-induced neurotoxicity and AD pathogenesis [9,16-18]. Ample evidence suggests that physical exercise constitutes an effective intervention in AD, attenuating or limiting their progression [28-31]. Nevertheless, the neurobiological mechanisms underlying the neuroprotective effects of exercise in preventing AD have not yet been fully elucidated. In addition, we observed in literature that the effect of exercise on the regulation of IDO and KYN pathway activation in animal models of AD is scarce or absent. In order to fill these gaps, the present study investigated the neuroprotective effect of an 8-week swimming training (ST) exercise on cognitive and non-cognitive functions and its role in modulating biomarkers of KYN pathway, using a mouse model of $A\beta_{1-42}$-induced neuroinflammation.

4.1 ST protects against cognitive and emotional disturbances induced by an i.c.v. injection of $A\beta_{1-42}$

The present study showed that an i.c.v. injection of $A\beta_{1-42}$ peptide (400 pmol/mice; 3µl/site) induced a deficit in object recognition memory and non-cognitive symptoms include depressive/anxiety-like behaviour as shown in the TST and EPMT. Consistent with our previous works [9,40], the current data showed that in the ORT, the cognitive performance of $A\beta_{1-42}$-treated mice was severely impaired, as reflected by a decreased recognition index. During non-cognitive behavioural assessment, we also observed that the $A\beta_{1-42}$-treated mice exhibited a longer immobility time in the TST and spent dramatically less time in the open arms in the EPMT. Thus, our findings successfully recapitulated the cognitive and non-cognitive disturbances observed in AD patients [3,4] and AD mouse models [62,63].

On the other hand, the ST protocol used in our study proved to be beneficial in counteracting the detrimental effects of $A\beta_{1-42}$ peptide by preventing the memory impairment and BPSD-like behaviour observed. Moreover, our results demonstrated that sham exercise group exhibited less time immobile in the TST than the sham sedentary group, showing that swimming training itself had effect on depressive-like behaviour. These experimental data correspond well with the pro-cognitive, antidepressant and anxiolytic effects of exercise in AD patients reported in the clinical literature [30,31,39,64]. In agreement with our data, it has been demonstrated that regular exercise ameliorates spatial and non-spatial memories [36,63,65,66] as well as alleviates emotional alterations [63,67-69] in multiple lines of transgenic mice modeling AD. García-Mesa et al. [63] have shown that voluntary running wheel ameliorated cognitive deterioration and BPSD-like behaviors such as anxiety and the startle response in 3xTg-AD mouse. Taken together, our results are complementary to experimental and clinical studies, extending the notion that ST is an effective paradigm of exercise to protect against cognitive and emotional disturbances elicited by $A\beta_{1-42}$ peptide or even improve these alterations in AD patients.

4.2 ST blocks the neuroinflammatory response induced by $A\beta_{1-42}$

Converging evidence indicates that brain inflammatory process has a fundamental role in the pathogenesis of AD [5-7]. It has been reported that Aβ is able to induce proinflammatory gene expression in microglial cells resulting in the elevation of proinflammatory cytokines, such as tumor necrosis factor-alpha, interleukin-1β and interleukin-6 (IL-6), which lead to tau hyperphosphorylation and neuronal loss [11]. IL-6 is a pleiotropic inflammatory cytokine that stimulates microglia and astrocytes to release a cascade of proinflammatory cytokines, contributing to neurodegeneration in AD [10]. Corroborating these reports, in the present study elevated IL-6 levels was found in the prefrontal cortex and hippocampus of mice following $A\beta_{1-42}$ administration. Another important finding that emerged from this study was that the i.c.v. injection of $A\beta_{1-42}$ caused an exponential increase in the levels of the anti-inflammatory cytokine IL-4 in the prefrontal cortex and hippocampus of mice. IL-4 has marked inhibitory effects on the expression and release of the proinflammatory

cytokines, exerting protective effects in the brain [70]. An *in vitro* study of Szczepanik et al. [71] have indicated that IL-4 in the central nervous system have immune modulating activity against $A\beta_{1-42}$ activation of microglia and the subsequent production of inflammatory cytokine. Thus, the elevated levels of IL-4 found in our study could be explained as a compensatory mechanism of the brain to counterbalance the Aβ-induced up-regulation of IL-6. On the other hand, we found that our ST regimen markedly blocked the Aβ-induced neuroinflammatory response, supporting the previous studies that have reported anti-inflammatory effects of exercise [31]. One of the putative mechanisms whereby exercise regulates inflammation is through the increased production and release of anti-inflammatory cytokine IL-10 [72], an evidence that has already been demonstrated by a previous study of our laboratory [40].

The involvement of nuclear factor-kappa B (NF-κB) pathway in proinflammatory cytokines transcription factors in the pathophysiology of AD has been described [11]. In the present study, the levels of NF-κB phosphorylated, which indicates an activation of NF-κB, was increased in the prefrontal cortex and hippocampus of $A\beta_{1-42}$-treated mice, supporting previous studies that indicated that the pro-inflammatory state induced by $A\beta_{1-42}$ is modulated by NF-κB activation [13,73]. It is an important finding, since it has been demonstrated that NF-κB activation accompanied by secretion of the proinflammatory cytokines is associated with neuronal degeneration in the brains of patients with AD [74]. In this study, we found that the NF-κB activation in the prefrontal cortex and hippocampus of $A\beta_{1-42}$-treated mice was prevented by ST. On the other hand, we observed a higher but not significant NF-κB levels in the prefrontal cortex and hippocampus of sham exercised mice in comparison with their sedentary counterparts. Although this result is apparently conflicting, it is noteworthy to mention that the activation of NF-κB may be an important molecular regulator of adaptation to exercise training. Exercise-induced increases in reactive species generation are likely to stimulate the redox sensitive NF-κB, resulting in the up-regulation of antioxidant gene expression [75]. This is in line with our previous study [40], in which we showed an antioxidant effect of this same swimming training protocol against $A\beta_{1-40}$-induced oxidative stress. Together, the results of current study represent additional evidence that physical exercise is an effective approach to inhibition

of NF-κB activation and neuroinflammatory response in the brain of $A\beta_{1-42}$-treated mice, interrupting this vicious cycle of $A\beta_{1-42}$ neurotoxicity. The exercise-induced modulation of inflammatory signaling pathway can be an important neurobiological mechanism against Aβ-induced cognitive impairment and emotional alterations in mice.

4.3 ST prevents $A\beta_{1-42}$-induced neurotrophic deficiency in the prefrontal and hippocampus

Neurotrophic factors are proteins which play a vital role in regulating all aspects of neural circuit development and function in mammalian brain, including neuronal survival, maintenance and regeneration, synaptic plasticity, neurogenesis and memory consolidation [76]. Converging evidence have shown that impaired neurogenesis and neuroplasticity play major roles in the pathophysiology of AD [77,78]. It has been reported that the levels of many neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), nerve-growth factor (NGF) and neurotrophin-3 (NT) are altered in CSF and brain tissues in both AD patients and animal models of AD [77,79].

Here, we demonstrated that i.c.v. injection of $A\beta_{1-42}$ induced a neurotrophic deficiency revealed by the reduction in BDNF, GDNF, NGF and NT-3 levels in the prefrontal cortex and hippocampus of mice. In contrast, we found that the levels of these neurotrophic factors in these brain regions were notably modulated by ST. The down-regulated neurotrophins induced by $A\beta_{1-42}$ was prevented by ST preserving the trophic factors of these structures at the levels of the sham sedentary group. Moreover, we also found significantly enhanced levels of BDNF and GDNF and a trend towards increased NGF and NT-3 levels of theses brain regions in sham exercised mice in comparison with their counterparts sedentary. Therefore, our results highlight that our ST protocol acts more forcefully on the regulation of BDNF and GDNF in the brain of mice, although further investigations are need to confirm this results. Our findings are in line with multiple lines of evidence, which have indicated that neurotrophic factors are essential effectors for the role of exercise on brain plasticity and prevention of cognitive and neuropsychiatric disorders

[30,34,35,66,80]. In this regard, BDNF is considered to be the most downstream factor mediating the upregulation of hippocampal neurogenesis of exercise [80]. The role of GDNF in AD pathogenesis has not been fully explored and how these neurotrophic molecule is regulated by physical exercise in AD mouse models is not well-established. It has been indicated that GDNF levels are significantly down-regulated in cerebrospinal fluid of patients with early stage of AD [81]. Revilla et al. [82] have showed that GDNF was down-regulated in cerebral cortex of 3xTgAD mice and were recovered by wheel running exercise. Together with literature, our results suggest that exercise-induced protection and up-regulation of neurotrophic factors, mainly by the increased BDNF and GDNF levels, are important processes that mediate the neuroprotective effect of exercise against $A\beta_{1-42}$-induced behavioural disturbances. In addition, our study demonstrates for the first time a modulatory effect of exercise on brain GDNF levels in an experimental model of amyloid-like pathology, providing new insights to understand the biological basis for exercise-induced neuroprotection in AD.

4.4 ST markedly mitigates IDO activation and abrogates increased TRP levels and KYN production elicited by $A\beta_{1-42}$

Emerging evidence has suggested that IDO plays a pivotal role in the AD pathogenesis [16-18]. IDO has been assumed to represent a link between proinflammatory cytokines and the neurotoxic effects of $A\beta_{1-42}$ peptide [23]. In a recent study of our research group [9], we demonstrated that the pharmacological inhibition of IDO with 1-methyltryptophan abrogates behavioural disturbances in a mouse model of $A\beta_{1-42}$-induced neuroinflammation. It has been proposed that IDO can be induced by acute or chronic inflammation [19]. Inflammation-induced IDO activation switches the metabolism of KYN toward the production of neurotoxic tryptophan catabolites (TRYCATs), rather than serotonin (5-HT), which decreases the availability of TRP for 5-HT synthesis [83]. An increasing body of evidence has demonstrated that the detrimental effects of TRYCATs, such as 3-hydroxyanthranilic acid (3-HAA) and quinolinic acid (QUIN), are involved in the pathophysiology of AD [18,25]. Of these TRYCATs, the N-methyl-D-asparte agonist and free-radical

generator quinolinic acid (QUIN) may be one of the critical factors in the pathomechanisms of neuronal damage and death in AD [84,85], mainly due its roles in reduction of neurogenesis and the amplification of inflammatory response in the brain [86].

In the present study, the i.c.v. injection of $A\beta_{1-42}$ caused an increase in IDO activity followed by increased levels of KYN, TRP and KYN/TRP ration in the prefrontal cortex and hippocampus, supporting our previous work that $A\beta_{1-42}$ causes KYN pathway activation in the brain of mice. Although the increase in TRP levels are somewhat counterintuitive, and certainly argue against the hypothesis that IDO activation depletes TRP bioavailability for 5-HT synthesis, previous studies had already shown that this response could be argued as a compensatory mechanism of the brain is response to inflammatory stimuli [9,20]. On the other hand, our protocol of swimming training consistently blunted the IDO activation and the alterations of KYN pathway markers elicited by $A\beta_{1-42}$. Our results are consistent with a previous study of Liu et al. [87], which has reported that swimming exercise, by reducing the levels of proinflammatory cytokines, subsequently reduced IDO levels in prefrontal cortex of rats, which have contributed to exercise ameliorating depression induced by chronic unpredictable mild stress. Therefore, we suggest that the anti-inflammatory action of ST could mitigated the IDO activation in the brain, preventing the appearance of behavioural disturbances induced by $A\beta_{1-42}$. Our study provides another important finding for the mechanistic studies of exercise-induced neuroprotection, indicating for the first time that physical exercise may confer protective effects against $A\beta_{1-42}$ peptide through modulation of KYN pathway in the brain of mice.

In summary, the current study shows two main findings. First, we confirmed that the activation of the KYN pathway, revealed by the increase of IDO activity and KYN levels in the brain, is involved in the $A\beta_{1-42}$ induced cognitive and emotional alterations in mice; and the neuroinflammatory response is the initial step. Hence, KYN accumulation within the brain may be activated neurodegenerative pathways via TRYCATs production that ultimately provoked a decrease in neurotrophic factors levels, which could contribute to the behavioural deficits observed. Considering that the transcription factor NF-κB is known to be a redox-sensitive factor, it is reasonable to speculate that a

free radical generation induced by neurotoxic metabolite QUIN could be lead to a harmful vicious cycle of inflammation between $A\beta_{1-42}$ peptide, activated microglia and stressed neurons. Second, we demonstrated that an 8-week ST performed at intensity above the anaerobic threshold is effective in preventing the behavioural disturbances induced by $A\beta_{1-42}$ peptide, mainly due to its anti-inflammatory and pro-neurogenic effects. Importantly, our ST protocol may have caused an interruption in the self-sustained cycle of $A\beta_{1-42}$-induced neuroinflammation by modulating cytokine/NF-κB signaling pathway and the activation of IDO in the brain. A summary of the mechanistic explanations can be seen in **Fig. 11.** A greater understanding of these mechanisms and their potential relationships with exercise will be invaluable in providing biomarkers for investigating the efficacy of differing exercise regimes to alleviates AD symptoms.

5. Conclusions

Despite the exact neurobiological mechanisms by which physical exercise exerts a protective effect on brain function are still under investigation, the findings from the present study provide valuable insights into molecular pathways that could be implicated in both AD and regular exercise. In short, it can be concluded that swimming exercise prevented behavioural and neurobiological deficits induced by $A\beta_{1-42}$ peptide, and suggest that these neuroprotective effects are likely to involve the inhibition of inflammation/IDO activation and up-regulation of BDNF and GDNF in brain of mice. Thus, it is possible that physical exercise can be used as a non-pharmacological approach to alleviates both cognitive and non-cognitive symptoms of AD.

Conflict of Interest

The authors have no conflicts of interest to report.

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Figure legends

Fig. 1. Overview of study design. $A\beta_{1-42}$, amyloid-beta peptide 1-42; i.c.v., intracerebroventricular; BW, body weight; IDO, indoleamine-2,3-dyoxygenase; IL-6, interleukin-6; IL-4, interleukin-4; BDNF, brain-derived neurotrophic factor; GDNF, glial cell-derived neurotrophic factor; NGF, nerve growth factor; NT-3, neurotrophin-3; NFκB, nuclear factor-kappa B; KYN, kynurenine.

Fig. 2. Effect of ST on CS activity in quadriceps femoris muscle (Values are means ± S.E.M. (n=8). #P<0.05 when compared sham exercise with sham sedentary; @P<0.05 when compared Aβ exercise with Aβ sedentary (One-way ANOVA, Bonferroni post hoc test).

Fig. 3 Effect of ST and $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on the recognition index (A) and total exploration time (B) in the ORT. (Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ sedentary with sham sedentary; @P<0.05 when compared Aβ exercise with Aβ sedentary (Two-way ANOVA, Bonferroni post hoc test).

Fig. 4 Effect of ST and $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on immobility time in the TST (A), time spent in open arms in the EPMT (B) and total distance in the OFT (C). Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ sedentary with sham sedentary; #P<0.05 when compared sham exercise with

sham sedentary; @P<0.05 when compared Aβ exercise with Aβ sedentary (Two-way ANOVA, Bonferroni post hoc test).

Fig. 5. Effect of ST and $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on the levels of IL-6 (A and B) and IL-4 (C and D), in the prefrontal cortex (A, C) and hippocampus (B, D) of mice. Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ sedentary with sham sedentary; #P<0.05 when compared sham exercise with sham sedentary; @P<0.05 when compared Aβ exercise with Aβ sedentary (Two-way ANOVA, Bonferroni post hoc test).

Fig. 6. Effect of ST and $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on NFκB in the prefrontal cortex (A) and hippocampus (B) of mice. Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ sedentary with sham sedentary; @P<0.05 when compared Aβ exercise with Aβ sedentary (Two-way ANOVA, Bonferroni post hoc test).

Fig. 7 Effect of ST and $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on the levels of BDNF (A and B) and GDNF (C and D) in the prefrontal cortex (A and C) and hippocampus (B and D) of mice. Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ sedentary with sham sedentary; #P<0.05 when compared sham exercise with sham sedentary; @P<0.05 when compared Aβ exercise with Aβ sedentary (Two-way ANOVA, Bonferroni post hoc test).

Fig. 8. Effect of ST and $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on the levels of NGF (A and B) and NT-3 (C and D) in the prefrontal cortex (A and C) and hippocampus (B and D) of mice. Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ sedentary with sham sedentary; @P<0.05 when compared Aβ exercise with Aβ sedentary (Two-way ANOVA, Bonferroni post hoc test).

Fig. 9 Effect of ST and $A\beta_{1-42}$ (400 pmol; 3 µl/site; i.c.v.) on IDO activity in the prefrontal cortex (A) and hippocampus (B) of mice. Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ sedentary with sham sedentary; @P<0.05 when compared Aβ exercise with Aβ sedentary (Two-way ANOVA, Bonferroni post hoc test).

Fig. 10 Effect of ST and $A\beta_{1-42}$ (400 pmol; 3µl/site; i.c.v.) on TRP levels (A and B), KYN levels (C and D) and KYN/TRP ratio (E and F) in the prefrontal cortex (A, C and E) and hippocampus (B, D and F) of mice. Values are means ± S.E.M. (n=8). *P<0.05 when compared Aβ sedentary with sham sedentary; @P<0.05 when compared Aβ exercise with Aβ sedentary (Two-way ANOVA, Bonferroni post hoc test).

Fig. 11. Diagram showing the mechanistic links between neuroinflammation and neurobehavioral disturbances induced by $A\beta_{1-42}$ i.c.v. injection and the neuroprotective effects of ST against these alterations. (+) increase/activation; (-) decrease/inhibition; underlined: mechanisms demonstrated in this study. On the one hand, the neuroinflammatory response, as measured by the increase in the levels of IL-6, IL-4 and NFκB following $A\beta_{1-42}$ injection caused IDO activation and subsequent increased levels of KYN, which may mediate the observed behavioral disturbances. Hence, KYN accumulation within the brain may activate neurodegenerative pathways via KYN neurotoxic metabolites that ultimately provoke a decrease in neurotrophic factor levels. This neurotrophic deficit could contribute to the observed neurobehavioral complications (clear arrows). On the other hand, an 8-week ST performed at intensity above the anaerobic threshold prevented the behavioural disturbances induced by $A\beta_{1-42}$ peptide, mainly due to its anti-inflammatory and pro-neurogenic effects. Moreover, ST protocol may have caused an interruption in the self-sustained cycle of $A\beta_{1-42}$-induced neuroinflammation by modulating cytokine/NF-κB pathway and the activation of IDO in the brain (dark arrows).

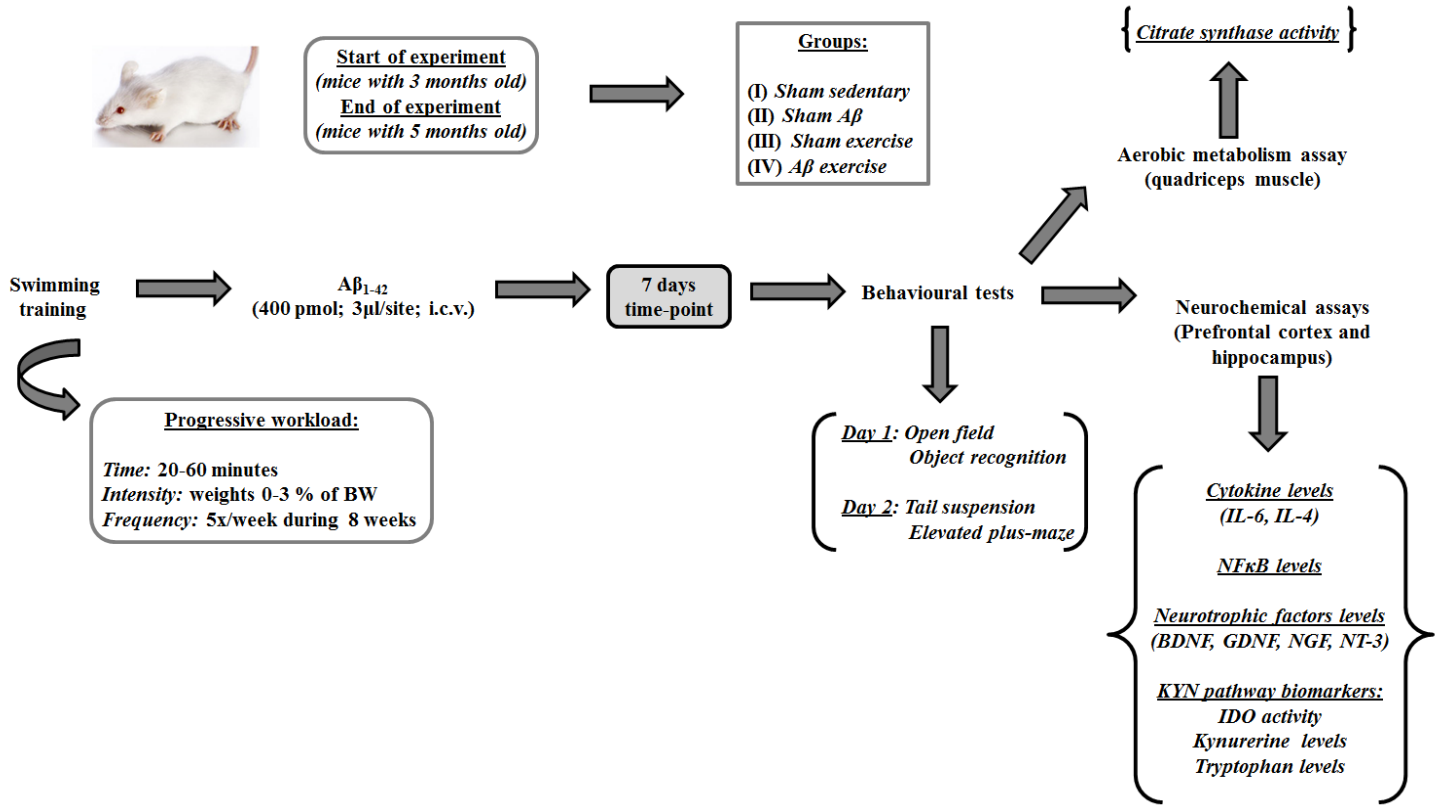
Start of experiment
(mice with 3 months old)
End of experiment
(mice with 5 months old)
Groups:
(I) Sham sedentary
(II) Sham Aβ
(III) Sham exercise
(IV) Aβ exercise
Citrate synthase activity
Aerobic metabolism assay
(quadriceps muscle)
Swimming training
Aβ1-42
(400 pmol; 3µl/site; i.c.v.)
7 days time-point
Behavioural tests
Neurochemical assays
(Prefrontal cortex and hippocampus)
Progressive workload:
Time: 20-60 minutes
Intensity: weights 0-3 % of BW
Frequency: 5x/week during 8 weeks
Day 1: Open field
Object recognition
Day 2: Tail suspension
Elevated plus-maze
Cytokine levels
(IL-6, IL-4)
NFκB levels
Neurotrophic factors levels
(BDNF, GDNF, NGF, NT-3)
KYN pathway biomarkers:
IDO activity
Kynurerine levels
Tryptophan levels

Fig. 1

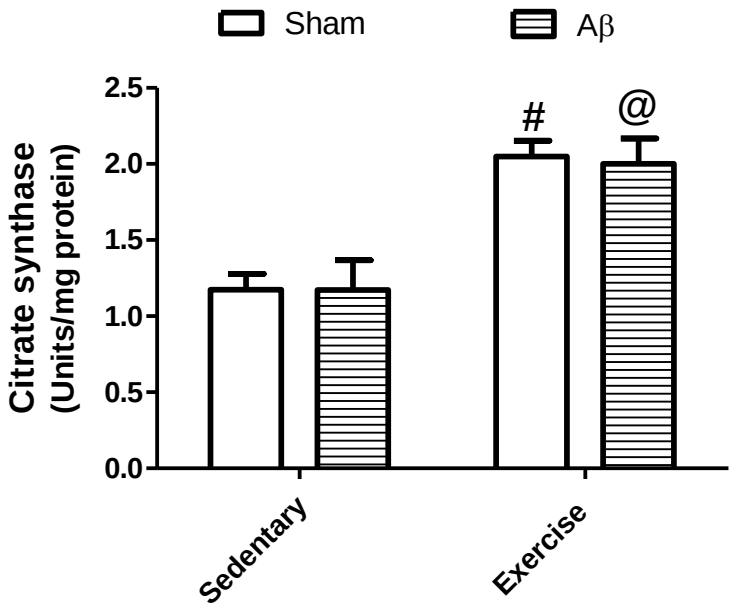
Sham
Aβ
Citrate synthase
(Units/mg protein)
2.5
2.0
1.5
1.0
0.5
0.0
#
@
Sedentary
Exercise

Fig. 2

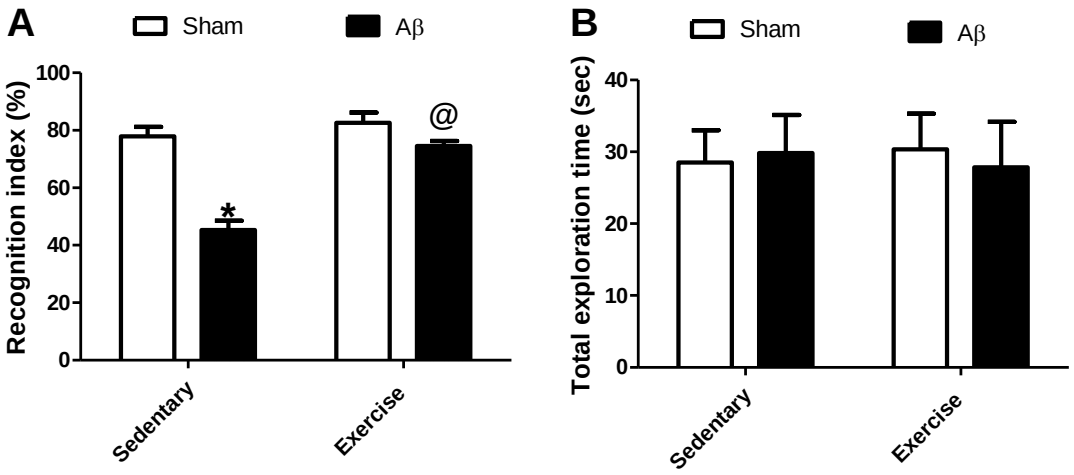
A
Sham
Aβ
Recognition index (%)
100
80
60
40
20
0
*
@
Sedentary
Exercise
B
Sham
Aβ
Total exploration time (sec)
40
30
20
10
0
Sedentary
Exercise

Fig. 3

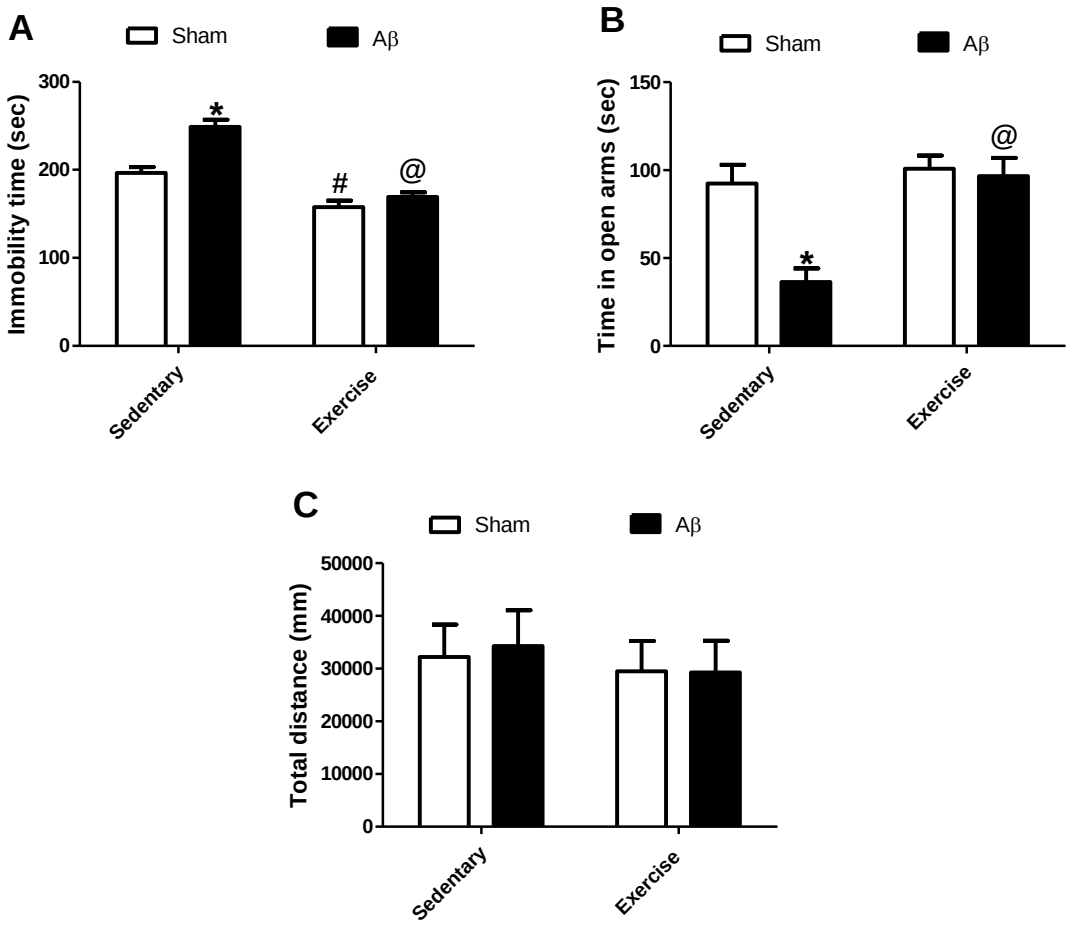
A
Sham
Aβ
Immobility time (sec)
300
200
100
0
*
#
@
Sedentary
Exercise
B
Sham
Aβ
Time in open arms (sec)
150
100
50
0
*
@
Sedentary
Exercise
C
Sham
Aβ
Total distance (mm)
50000
40000
30000
20000
10000
0
Sedentary
Exercise

Fig. 4

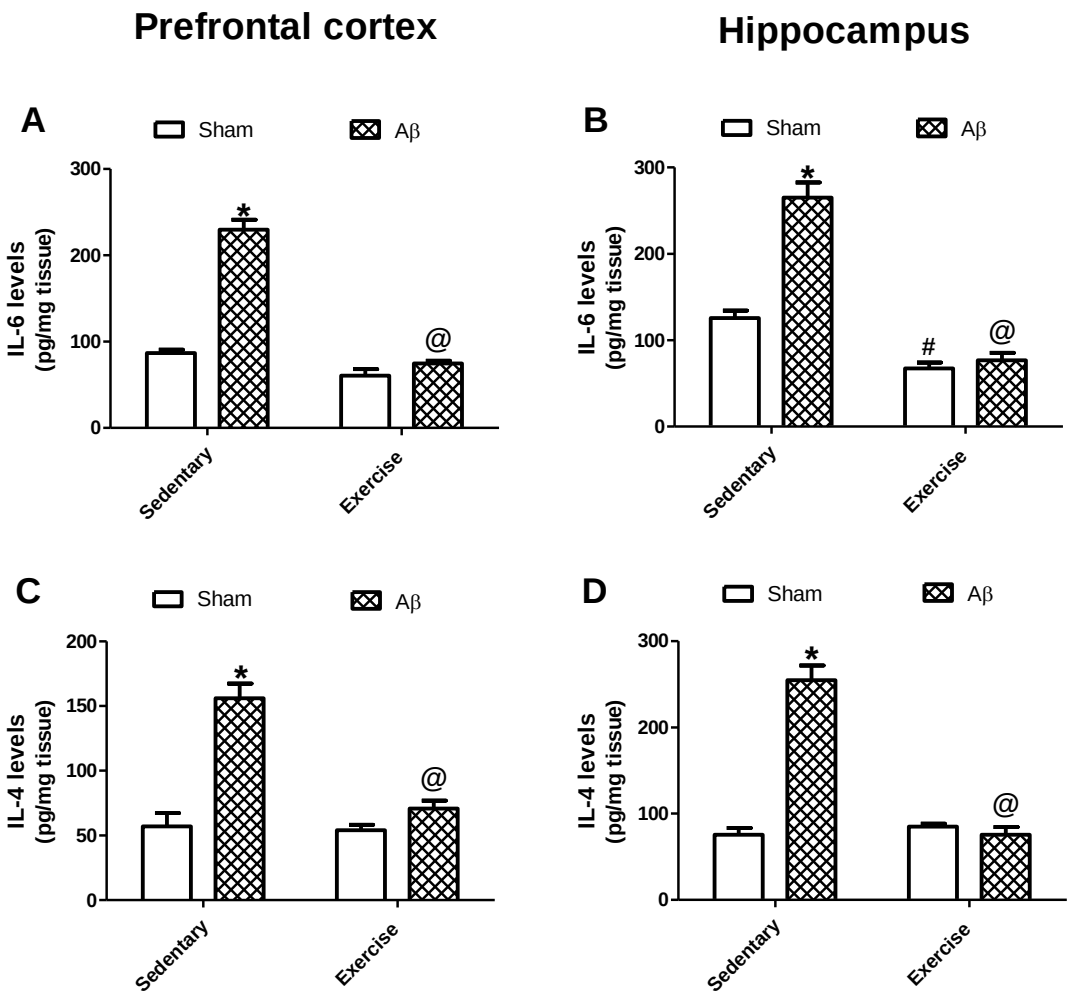
Prefrontal cortex
Hippocampus
A
B
C
D
Sham
Aβ
IL-6 levels (pg/mg tissue)
IL-4 levels (pg/mg tissue)
Sedentary
Exercise
*
#
@

Fig. 5

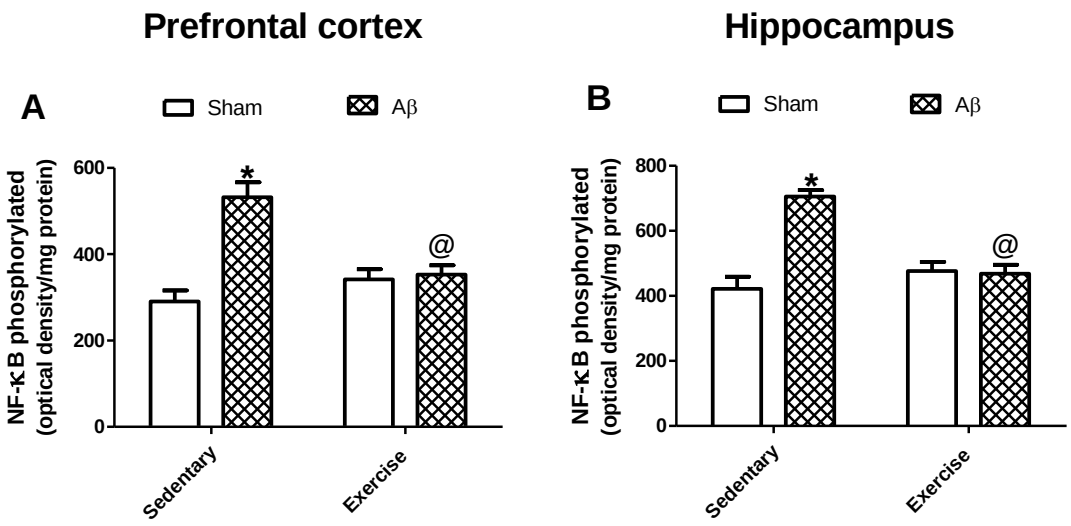
Prefrontal cortex
Hippocampus
A
B
Sham
Aβ
NF-κB phosphorylated
(optical density/mg protein)
600
400
200
0
800
*
@
Sedentary
Exercise

Fig. 6

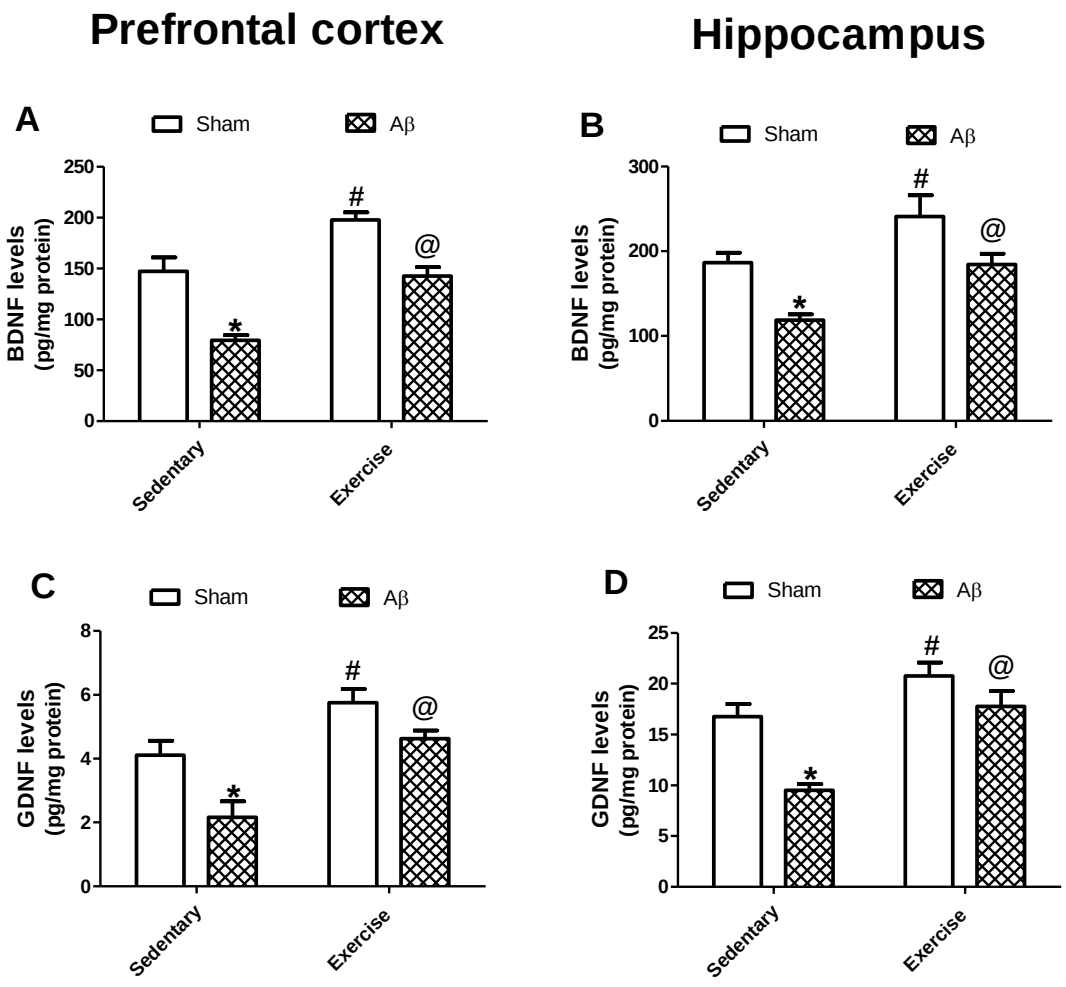
Prefrontal cortex
Hippocampus
A
B
C
D
Sham
Aβ
BDNF levels (pg/mg protein)
GDNF levels (pg/mg protein)
Sedentary
Exercise
#
@
*

Fig. 7

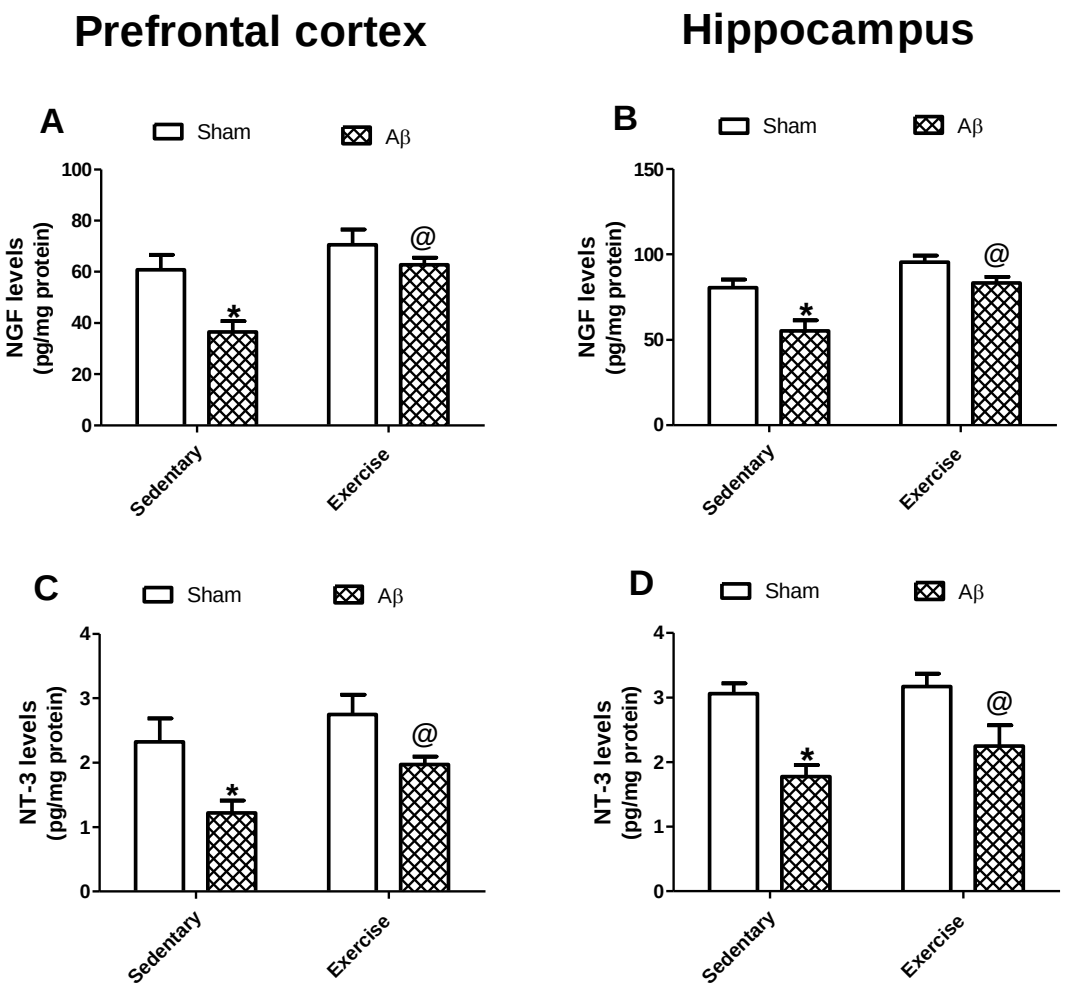
Prefrontal cortex
Hippocampus
A
B
C
D
Sham
Aβ
NGF levels (pg/mg protein)
NT-3 levels (pg/mg protein)
Sedentary
Exercise
*
@

Fig. 8

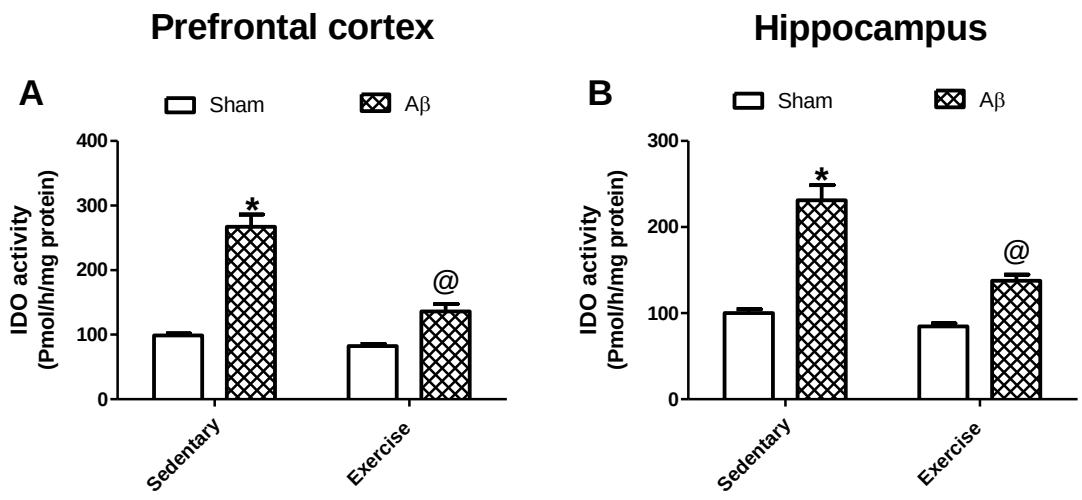
Prefrontal cortex
A
Sham
Aβ
IDO activity
(Pmol/h/mg protein)
400
300
200
100
0
*
@
Sedentary
Exercise
Hippocampus
B
Sham
Aβ
IDO activity
(Pmol/h/mg protein)
300
200
100
0
*
@
Sedentary
Exercise

Fig. 9

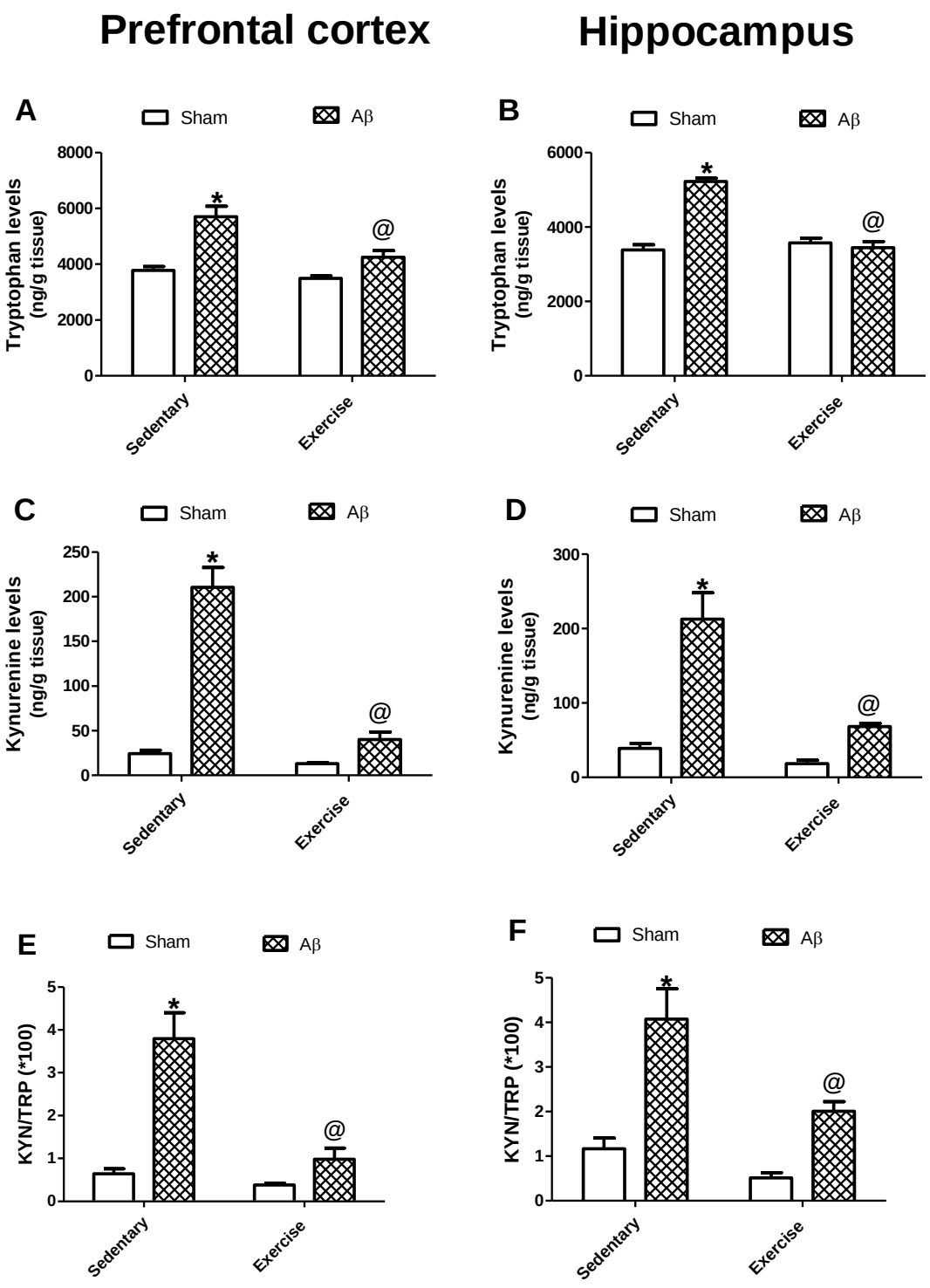
Prefrontal cortex
Hippocampus
A
B
C
D
E
F
Sham
Aβ
Tryptophan levels (ng/g tissue)
Kynurenine levels (ng/g tissue)
KYN/TRP (*100)
Sedentary
Exercise
*
@

Fig. 10

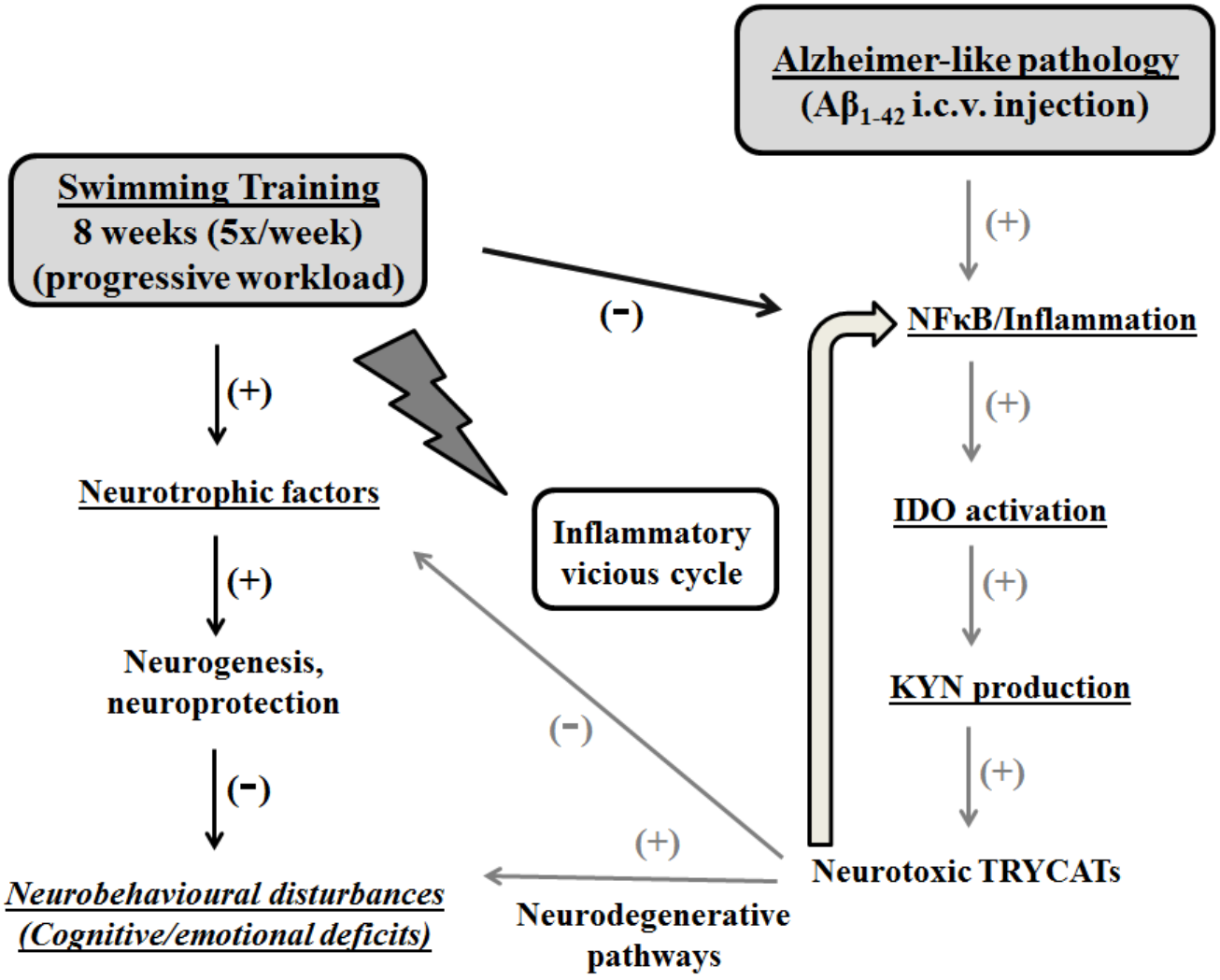
Alzheimer-like pathology
(Aβ1-42 i.c.v. injection)
Swimming Training
8 weeks (5x/week)
(progressive workload)
(+)
(-)
NFκB/Inflammation
(+)
(+)
Neurotrophic factors
Inflammatory
vicious cycle
IDO activation
(+)
(+)
Neurogenesis,
neuroprotection
KYN production
(-)
(+)
(-)
(+)
Neurotoxic TRYCATs
Neurobehavioural disturbances
(Cognitive/emotional deficits)
Neurodegenerative
pathways

Fig. 11

Table 1. Eight-week swimming training protocol

	Intensity (%)	Training time (min/day)
1st week	0%* (water depth 5 cm)	20
2nd week	0% (water depth 20cm)	30
3rd week	1%	30
4th week	1%	40
5th week	2%	50
6th-8th week	3%	60

***** Percent weight of BW attached to tail

PARTE III

DISCUSSÃO

Este estudo mostrou o efeito neuroprotetor do exercício de natação contra a neurotoxicidade induzida pelo peptídeo $A\beta_{1-42}$ em camundongos. Em um estudo prévio do nosso laboratório (Souza et al., 2013), foi demonstrado que o exercício de natação diminui a neuroinflamação e o estresse oxidativo no cérebro de camundongos que receberam infusão i.c.v. do peptídeo $A\beta_{1-40}$, com estes resultados acontecendo em paralelo à proteção da memória, analisada no teste de reconhecimento de objetos. No presente trabalho, utilizando o mesmo paradigma que mimetiza alguns déficits dos estágios iniciais da DA em roedores, porém utilizando a isoforma $A\beta_{1-42}$ que tem maior propriedade neurotóxica, nós aprofundamos e estendemos os achados prévios tanto em relação à neurotoxicidade do peptídeo Aβ quanto ao efeito neuroprotetor do exercício físico, através de três achados principais.

Primeiro, nós demonstramos que os distúrbios cognitivos e emocionais, assim como a resposta inflamatória e a redução de fatores neurotróficos, induzidos pela administração do peptídeo $A\beta_{1-42}$, são marcadamente mediados pela ativação da IDO no cérebro de camundongos. Segundo, nós constatamos que o estado inflamatório, a diminuição dos níveis de BDNF, a depleção de GSH e a diminuição da capacidade antioxidante associados ao envelhecimento, torna os camundongos idosos mais vulneráveis à perda de memória, aos sintomas de ansiedade e à desregulação da KP provocados pelo peptídeo $A\beta_{1-42}$. Finalmente, utilizando o protocolo de treinamento de natação (ST) validado pelo nosso estudo prévio (Souza et al., 2013), nós demonstramos que o ST preveniu os déficits cógnitos/emocionais e neurobiológicos $A\beta_{1-42}$-induzidos, principalmente pela supressão da resposta inflamatória/ativação da IDO e através da regulação positiva dos níveis de fatores neurotróficos, mais pronunciadamente do BDNF e do GDNF, no cérebro de camundongos.

A ativação da IDO no cérebro contribui para os distúrbios neurocomportamentais induzidos pelo peptídeo Aβ$_{1-42}$

Em um primeiro experimento, nós buscamos investigar os potenciais mecanismos que ligam a neuroinflamação às alterações cognitivas e emocionais desencadeadas pela infusão i.c.v. do peptídeo Aβ$_{1-42}$. A escolha por esta isoforma se deu em razão de sua maior neurotoxicidade, incluindo uma maior indução de respostas inflamatórias e danos por radicais livres em maior extensão, quando comparada com a isoforma Aβ$_{1-40}$ (Klein et al., 1999). Além disso, foi mostrado que, em contraste com a isoforma Aβ$_{1-40}$, a isoforma Aβ$_{1-42}$ é capaz de induzir uma ativação da IDO (Guillemin et al., 2003). Embora mais estudos sejam necessários para comprovar este achado, uma explicação razoável para esta ativação da IDO por parte da isoforma Aβ$_{1-42}$ pode ser a sua forte estimulação sobre o IFN-γ, uma potente citocina indutora desta enzima (Yamada et al., 2009).

O presente estudo demonstrou que os camundongos expostos ao peptídeo Aβ$_{1-42}$ tiveram sua performance diminuída no ORT. Este resultado possui uma importante aplicação para o campo de estudos da DA, uma vez que o ORT é um teste altamente dependente do circuito córtico-hipocampal da memória declarativa (Eichenbaum, 2000; Winters et al., 2008), a qual é um tipo de memória que se encontra prejudicada nos pacientes com DA (Antunes & Biala, 2012). A curva de tempo x resposta mostrou que o prejuízo na memória de reconhecimento de objetos no ORT ocorreu 3 e 7 dias após a administração Aβ$_{1-42}$. Tendo em vista o curto tempo de retenção usado em nosso estudo (90 min.), nós mostramos que o peptídeo Aβ$_{1-42}$ causou uma deterioração na memória de curto prazo, a qual é um sintoma inicial da DA (Antunes & Biala, 2012). Como uma possível explicação mecanística para este prejuízo, nós sugerimos que a neurotoxicidade Aβ$_{1-42}$-induzida pode ter causado efeitos detrimentais sobre os processos de codificação e consolidação das características dos objetos, refletido por uma diminuição da preferência pelo objeto novo durante a fase de teste do ORT.

Além da perda progressiva da memória, os pacientes de DA frequentemente manifestam sintomas neuropsiquiátricos associados à demência (NPSD), incluindo a depressão e a ansiedade, os quais estão

fortemente correlacionados com o grau de comprometimento funcional e cognitivo, piorando a progressão da doença (Cerejeira et al., 2012). A fim de investigar se estes comportamentos são afetados em camundongos tratados com $A\beta_{1-42}$, nós lançamos mão do teste de suspensão de cauda (TST) e do teste de labirinto em cruz elevado (EPMT), os quais são testes amplamente utilizados para a triagem de substâncias com atividade antidepressiva e ansiolítica, respectivamente (Castagné et al., 2006). Nossos resultados demonstraram que os prejuízos cognitivos nos camundongos ocorreram concomitantemente com as respostas emocionais (comportamento tipo depressivo e tipo-ansiedade) mensuradas pelos testes TST e EPMT, 7 dias após a administração do peptídeo $A\beta_{1-42}$. Portanto, os nossos achados recapitularam com êxito os sintomas cognitivos e não-cognitivos observados em pacientes (Cerejeira et al., 2012) e modelos animais da DA (García-Mesa et al., 2011; Filali et al., 2012), provendo evidências adicionais de que as disfunções cognitivas estão acompanhadas de alterações neurocomportamentais nos estágios inicias da DA (Rosenberg et al., 2015).

Evidências atuais sugerem que a neuroinflamação está envolvida nos mecanismos da neurotoxicidade do peptídeo Aβ (Piermartini et al., 2010; Souza et al., 2013; Chen et al. 2015) e da fisiopatologia da DA (Heneka et al., 2015; Heppner et al., 2015; Wang et al., 2015). Um importante componente nesse processo é o desequilíbrio entre citocinas pró e anti-inflamatórias no cérebro (Wang et al., 2015) que é apontado como um fator precipitante para o surgimento dos sintomas cognitivos e neuropsiquiátricos na DA (Wuwongse et al., 2010; Holmgren et al., 2014; Takeda et al., 2014). No presente estudo, nós detectamos níveis elevados de IL-1β, TNF-α e IFN-γ e níveis diminuídos de IL-10 no córtex pré-frontal e hipocampo dos camundongos nos tempos 1, 3 e 7 dias após a administração de $A\beta_{1-42}$. Os níveis máximos foram encontrados 7 dias após o insulto, uma característica temporal que coincidiu com o momento onde os distúrbios comportamentais foram mais evidentes. Interessantemente, nós encontramos um comportamento bifásico do IFN-γ, o qual apresentou inicialmente um aumento exponencial após 1 dia da infusão $A\beta_{1-42}$, seguida de uma diminuição nos tempos 3 e 7 dias. Em consonância com a literatura, o presente estudo suporta e estende a noção de que uma resposta inflamatória,

revelada por um desequilíbrio de citocinas no sentido pró-inflamatório, em regiões cerebrais implicadas com a regulação cognitiva e emocional, pode ser um importante mecanismo neurobiológico dos distúrbios cognitivos e emocionais provocados pelo peptídeo $A\beta_{1-42}$.

Os fatores neurotróficos são proteínas que desempenham um papel vital na regulação do desenvolvimento e da função dos circuitos neurais no cérebro de mamíferos, e.g. sobrevivência neuronal, plasticidade sináptica, neurogênese e consolidação da memória (Park & Poo, 2013). Danos a estas funções, principalmente devido à uma diminuição de fatores neurotróficos, incluindo BDNF, GDNF, NGF e NT-3, estão envolvidos com a fisiopatologia da DA (Allen et al.,2013; Sopova et al., 2014; Budni et al., 2015). Aqui, a curva de tempo x resposta mostrou que a infusão i.c.v. do peptídeo $A\beta_{1-42}$ causou uma deficiência neurotrófica revelada pela diminuição dos níveis de BDNF, GDNF e NGF no córtex pré-frontal e hipocampo dos camundongos, tendo seu efeito máximo no tempo de 7 dias após a infusão. Estudos *in vivo* e *in vitro* já tinham demonstrado uma diminuição evidente da expressão de BDNF (mRNA e níveis de proteína) após a exposição ao $A\beta_{1-42}$ (Tapia-Arancibia et al., 2008).

Foi reportado que os níveis de BDNF, GDNF, NGF e NT-3 são afetados em estágios iniciais na DA, contribuindo para o início de uma cascata de eventos que podem exacerbar a patologia e levar aos sintomas da demência, tanto em domínios cognitivos quanto neuropsiquiátricos (Allen et al., 2011, 2013; Budni et al., 2015). Visto que o NGF é vital para o suporte trófico de neurônios colinérgicos do prosencéfalo basal e o BDNF é a principal molécula efetora da plasticidade sináptica e formação da memória no hipocampo, uma desregulação no metabolismo destas moléculas resulta em prejuízos nas funções cognitivas e degeneração neuronal (Sopova et al., 2014). Em particular importância, Matrone et al. (2008) demonstraram que a privação de NGF e BDNF em uma cultura de neurônios hipocampais resultou em ativação da rota amiloidogênica e na morte celular, sugerindo que estas neurotrofinas compartilham atividades antiamiloidogênicas. O GDNF é um potente promotor de sobrevivência neuronal no CNS e no sistema nervoso periférico (Allen et al., 2011). Apesar do GDNF ter sido descrito como criticamente envolvido na neuroproteção de neurônios dopaminérgicos na DP (Allen et al., 2013), o seu

papel na fisiopatologia da DA não foi totalmente explorado. Straten et al. (2009) indicaram que os níveis de GNDF estão diminuídos no líquor de pacientes com DA, assim como Revilla et al. (2014) mostraram uma infra-regulação do GDNF no cérebro de camundongos com transgenia tripla para a DA (3xTgAD *mice*). Reforçando esta idéia, o presente estudo proveu evidências adicionais de que uma depleção dos níveis de GDNF no cérebro está diretamente envolvida nas disfunções cognitivas e emocionais $A\beta_{1-42}$-relacionadas. Assim, nós sugerimos que uma deficiência neurotrófica no córtex pré-frontal e hipocampo que ocorre em resposta à exposição ao peptídeo $A\beta_{1-42}$ está fortemente ligada à deterioração de memória de reconhecimento de objetos e aos comportamentos tipo-depressivo e tipo-ansiogênicos observados no nosso estudo.

Há um crescente corpo de evidências implicando a ativação da IDO e da KP como mediadores críticos da fisiopatologia da DA (Bonda et al., 2010; Kincses et al., 2010; Tan et al., 2012; Wu et al., 2013; Maddison & Giogini et al., 2015). Em condições inflamatórias, tem sido proposto que as citocinas pró-inflamatórias podem deflagrar a ativação da IDO no cérebro (Dantzer et al., 2008). No presente estudo, nós observamos um aumento da atividade enzimática da IDO no córtex pré-frontal e hipocampo dos camundongos 3 e 7 dias após a exposição ao peptídeo $A\beta_{1-42.}$ Esta ativação apresentou uma semelhança temporal com o aparecimento das alterações comportamentais, indicando um papel-chave para a IDO nos sintomas iniciais da DA. Aqui, é importante ressaltar que o IFN-γ demonstrou ser a principal citocina indutora da IDO corroborando o estudo *in vitro* de Yamada et al. (2009), no qual foi mostrado que o efeito principal do peptídeo $A\beta_{1-42}$ para indução da IDO é mediado por esta citocina. Assim sendo, o nosso estudo está em linha com estudos anteriores, os quais reportaram que o aumento de citocinas inflamatórias em resposta à desafios imunológicos centrais e periféricos induzidos por LPS causou comportamento tipo-depressivo e tipo-ansiedade (Salazar et al., 2012; Gibney et al., 2013; Lawson et al., 2013), assim como prejuízos na capacidade de reconhecimento de objetos (Heisler & O'Connor, 2015), sendo estes efeitos atribuídos à ativação da IDO no cérebro. Reciprocamente, um estudo prévio do nosso laboratório destacou que a ativação da IDO no hipocampo é mediada por citocinas pró-inflamatórias em

um modelo de depressão induzida pela infusão i.c.v. de estreptozotocina em camundongos (Souza et al., 2017). No que se refere à DA, foi reportado que uma maior imunorreatividade da IDO e também da TDO foi encontrada no hipocampo de pacientes com DA, localizadas proximamente às placas amilóides (Bonda et al., 2010; Wu et al., 2013). Junto com a literatura, nós demonstramos pela primeira vez que a infusão i.c.v. do peptídeo $A\beta_{1-42}$ causa uma ativação da IDO no córtex pré-frontal e hipocampo dos camundongos. Outras investigações são necessárias para elucidar o envolvimento da TDO na inflamação induzida por $A\beta_{1-42}$.

No nosso estudo, nós também observamos que o aumento da atividade da IDO induzida por $A\beta_{1-42}$ foi seguida de um aumento dos níveis de TRP no córtex pré-frontal e hipocampo. Este achado é de certa forma contraintuitivo e argumenta contra a hipótese de que a ativação da IDO causa uma depleção do TRP, interferindo na síntese de serotonina. Por exemplo, estudos clínicos anteriores reportaram que a degradação do TRP leva a uma disfunção serotoninérgica, que pode ser uma explicação para a ocorrência dos sintomas cognitivos na DA (Porter et al., 2000, 2003). Portanto, o nosso estudo sugere que as complicações neurobiológicas induzidas por $A\beta_{1-42}$ não envolvem uma degradação do TRP. Nós consideramos razoável explicar que este aumento dos níveis de TRP seja um mecanismo compensatório do cérebro em resposta à inflamação $A\beta_{1-42}$-induzida. Neste contexto, Gibney et al. (2013) mostraram que os níveis de TRP são aumentados no cérebro de camundongos em resposta a um desafio imunológico periférico induzido pelo ácido poliinosínico:policitidílico (poly I:C), um agonista de receptores tipo-toll 3 (TLR3), que é capaz de iniciar respostas inflamatórias comparáveis a uma infecção viral sistêmica. Como o nosso modelo experimental envolve uma inflamação aguda induzida por uma única infusão do peptídeo $A\beta_{1-42}$, nós especulamos que em modelos de inflamação crônica, induzida por repetidas administrações do peptídeo, a diminuição dos níveis de TRP e uma disfunção serotoninérgica possam ser identificados.

Outro importante resultado do nosso estudo foi de que a exposição ao peptídeo $A\beta_{1-42}$ precipitou os distúrbios comportamentais paralelamente ao aumento dos níveis de KYN e da razão KYN:TRP, de uma forma tempo-

dependente, suportando o papel do metabolismo da KYN em conduzir os sintomas da DA. Este achado de que os níveis de KYN aumentam, independentemente de uma depleção do TRP, aponta para a hipótese do desvio para o braço neurotóxico da KP sob condições inflamatórias (Myint & Kim, 2003), onde o aumento dos metabólitos neurotóxicos do TRP podem mediar os eventos de neurodegeneração presentes em doenças neuropsiquiátricas (Dantzer et al., 2008; Gibney et al., 2013) e também na DA (Guillemin & Brew, 2002; Kincses et al., 2010). Novos estudos utilizando modelos animais da DA são necessários para confirmar o envolvimento dos catabólitos neurotóxicos do TRP no surgimento dos sintomas da DA.

Por fim, nós conduzimos um experimento a fim de determinar com mais consistência o papel da IDO na neurotoxicidade induzida por $A\beta_{1-42}$. Para tanto, nós realizamos um pré-tratamento com o inibidor da IDO, 1-MT, antes da infusão i.c.v. de $A\beta_{1-42}$. Os mesmos testes comportamentais e análises neuroquímicas foram realizadas 7 dias após a administração de $A\beta_{1-42}$, tempo no qual as alterações se mostraram mais pronunciadas. Nós demonstramos que a inibição farmacológica com 1-MT bloqueou a ativação da IDO e preveniu as alterações comportamentais e neuroquímicas $A\beta_{1-42}$-induzidas. Considerados em conjunto, os nossos resultados nos permitem formular explicações mecanísticas para os eventos decorrentes da exposição ao peptídeo $A\beta_{1-42}$: primeiro, o aumento de citocinas pró-inflamatórias pode ter causado a ativação da IDO e, consequentemente o aumento dos níveis de KYN, os quais podem ter mediado as alterações comportamentais observadas. O acúmulo de KYN no cérebro pode ter ativado vias neurodegenerativas através dos metabólicos neurotóxicos da KP, que ultimamente podem ter desencadeado à perda de fatores neurotróficos, contribuindo para as complicações neurocomportamentais. Finalmente, uma resposta neuroinflamatória induzida diretamente pelo peptídeo $A\beta_{1-42}$, ligada à diminuição de fatores neurotróficos e à redução da IL-10, não pode ser descartada. Portanto, nós sustentamos que os distúrbios comportamentais provocados pelo peptídeo $A\beta_{1-42}$ são mediados pela atividade da IDO no córtex pré-frontal e hipocampo de camundongos.

A perda da memória e o comportamento tipo-ansiedade induzidos pelo peptídeo Aβ$_{1-42}$ são exacerbados pelo envelhecimento

Após termos estabelecido uma ligação mecanística entre a ativação da IDO e os distúrbios neurocomportamentais resultantes da inflamação Aβ$_{1-42}$-induzida, nós buscamos investigar se a idade, o principal fator de risco para a DA, está associada a uma maior sensibilidade aos déficits provocados pelo peptídeo Aβ$_{1-42}$, e se a ativação da KP está envolvida nesses efeitos. Para a avaliação dos sintomas cognitivos e emocionais, nós utilizamos a mesma bateria de testes comportamentais do primeiro estudo deste trabalho.

O envelhecimento é caracterizado por um progressivo declínio na eficiência das funções fisiológicas, aumentando a suscetibilidade a doenças e à morte (Gemma et al., 2007). Somado a isso, é bem reconhecido que a homeostase cerebral na idade avançada está prejudicada, precipitando os sintomas cognitivos e a vulnerabilidade a doenças neurodegenerativas em indivíduos saudáveis (Udeochu et al., 2016). Dentre estas alterações no microambiente cerebral, um estado inflamatório crônico (Franceschi & Campisi, 2014) e a diminuição das defesas antioxidantes (Tong et al., 2016) e de neurotrofinas (Tapia-Arancibia et al., 2008; Budni et al., 2015; Souza et al., 2015) são consideradas alterações fenotípicas que podem facilitar e exacerbar as disfunções mentais durante o envelhecimento e a DA.

Em linha com a literatura e com os nossos trabalhos anteriores (Souza et al., 2013, 2016), nós demonstramos que a infusão i.c.v. do peptídeo Aβ$_{1-42}$ causou perda da memória e comportamento tipo-depressivo e tipo-ansiogênico nos camundongos jovens (3 meses de idade) e idosos (20 meses de idade) através da ativação da IDO no córtex pré-frontal e hipocampo. O resultado mais importante, no entanto, foi de que os camundongos idosos apresentaram maiores déficits cognitivos e sintomas de ansiedade mais elevados após a administração de Aβ$_{1-42}$, em comparação com os animais jovens Aβ-tratados. Os animais idosos também responderam ao peptídeo com uma maior deficiência de BDNF, GSH e TRAP, uma maior atividade da IDO e maiores níveis de KYN e razão KYN:TRP no córtex pré-frontal e hipocampo. Estes efeitos detrimentais estavam associados a um maior estado pró-inflamatório, revelado pelo aumento da IL-6 e da diminuição da IL-10 nestas regiões

cerebrais. Assim sendo, o nosso estudo é o primeiro a evidenciar que a neuroinflamação e a perda de substâncias endógenas neuroprotetoras, como o GSH e o BDNF decorrentes do processo normal de envelhecimento, tornam os camundongos idosos mais vulneráveis à ativação da IDO, à perda de memória e aos sintomas de ansiedade induzidos pelo $A\beta_{1-42}$. Embora outros mecanismos neurotóxicos do peptídeo não possam ser descartados, aqui, nós confirmamos a hipótese de que a ativação da IDO e uma desregulação do metabolismo da KP, refletido pelo aumento dramático nos níveis de KYN, é um importante fator predisponente ao desenvolvimento dos sintomas da DA. Adicionalmente, é importante ressaltar que, até onde sabemos, a maioria dos estudos que fizeram uso de modelos animais de DA induzidos pela administração central de formas exógenas do peptídeo Aβ utilizaram animais adultos jovens, desconsiderando os efeitos detrimentais do envelhecimento como possíveis eventos adicionais ou amplificadores da neurotoxicidade Aβ. Aqui, nós defendemos que apesar de o envelhecimento exacerbar alguns déficits causados pelo insulto e mimetizar a interação entre os fatores idade x Aβ, o uso de animais jovens reproduz com bastante confiabilidade os sinais e sintomas da DA.

O exercício de natação previne os distúrbios cognitivos e emocionais induzidos pelo peptídeo $A\beta_{1-42}$ através da imunomodulação da atividade da IDO no cérebro

Depois de caracterizarmos de forma bastante consistente o envolvimento da IDO e da KP nas complicações neurocomportamentais mediadas pelo peptídeo $A\beta_{1-42}$, tanto na fase adulta quanto na idade avançada, nós procuramos examinar uma intervenção de natureza não-farmacológica que fosse capaz de neutralizar estes efeitos deletérios. Nesse sentido, nós investigamos o efeito neuroprotetor do exercício físico de natureza aeróbia, utilizando um programa de natação com duração de 8 semanas e uma metodologia de aumento progressivo de carga (Souza et al., 2013) em intensidade realizada abaixo do limiar anaeróbio, conforme descrito previamente (Gobatto et al., 2001; Almeida et al., 2011). Aqui, vale destacar que nós realizamos este experimento com animais jovens, primeiro por que os

animais idosos não apresentaram uma piora na sintomatologia depressiva e, segundo, devido à escassez de protocolos de exercício de natação validados para camundongos com idade avançada (20-24 meses de idade), tendo sido encontrados apenas protocolos com este tipo de exercício em animais com meia-idade (12-16 meses de idade).

O protocolo de ST utilizado em nosso estudo provou ser uma contramedida eficaz contra os efeitos neurotóxicos do peptídeo $A\beta_{1-42}$ ao prevenir a perda de memória no ORT e os sintomas tipo-neuropsiquiátricos observados, notavelmente os comportamentos tipo-depressivo e tipo-ansiedade, avaliados no TST e EPMT, respectivamente. Ademais, nossos resultados demonstraram que os animais exercitados sham-operados exibiram um menor tempo de imobilidade no TST em comparação com seus pares sedentários, indicando que o exercício físico, por si, exerceu um efeito antidepressivo. Esses dados experimentais vão ao encontro dos efeitos pró-cognitivos, antidepressivos e ansiolíticos do exercício físico em pacientes da DA reportados pela literatura clínica (Paillard et al., 2015; Matura et al., 2016; Ryan & Kelly, 2016). Acerca do efeito antidepressivo do exercício físico, foi indicado que ele pode ser tão efetivo quanto os tratamentos farmacológicos e psicoterápicos na melhoria dos sintomas depressivos (Bartley et al., 2013). Em acordo com os nossos resultados, foi mostrado que o exercício regular melhora as memórias espaciais e não-espaciais (Liu et al., 2011; Pietropaolo et al., 2008; Zang et al., 2016;), assim como alivia as alterações emocionais (García-Mesa et al., 2011; Pietropaolo et al., 2008; Walker et al., 2015) em vários modelos transgênicos para a DA. Nesse particular, um estudo bastante importante foi o de Garcia-Mesa et al., (2011), o qual mostrou que o exercício voluntário em rodas de correr melhorou a deterioração cognitiva e os sintomas tipo-neuropsiquiátricos em camundongos 3xTg-AD. Considerados em conjunto, os nossos resultados são complementares aos dados de estudos experimentais e clínicos, estendendo a noção de que o ST é um paradigma de exercício altamente efetivo em proteger contra os distúrbios cognitivos e emocionais provocados pelo peptídeo $A\beta_{1-42}$ ou, até mesmo, pode ser utilizado para aliviar estas desordens em pacientes com DA.

Em linha com os nossos estudos anteriores que evidenciaram o efeito pró-inflamatório do peptídeo $A\beta_{1-40}$ (Souza et., 2013) e $A\beta_{1-42}$ (Souza et al., 2016), no vigente estudo nós encontramos uma resposta neuroinflamatória nos camundongos tratados com $A\beta_{1-42}$, refletida pelos níveis elevados da citocina pró-inflamatória IL-6 e da citocina anti-inflamatória IL-4 no córtex pré-frontal e hipocampo. A IL-6 é uma citocina pleiotrópica que estimula as micróglias e os astrócitos a liberarem uma cascata de outras citocinas inflamatórias, contribuindo para o declínio cognitivo e a neurodegeneração no envelhecimento e na DA (Wang et al., 2015). A IL-4, por sua vez, possui ação inibitória sobre a expressão e liberação de citocinas pró-inflamatórias, exercendo efeitos protetores no cérebro (Wang et al., 1995). Nesse sentido, um estudo *in vitro* de Szczepanik et al. (2001) mostrou que a IL-4 tem atividade imunomodulatória no CNS suprimindo a ativação microglial e a subsequente produção de mediadores inflamatórios. Portanto, os níveis elevados de IL-4 encontrados em nosso estudo podem ser explicados como um mecanismo compensatório do cérebro para contrabalancear a supra-regulação de citocinas inflamatórias Aβ-mediadas. Em contrapartida, nós observamos que o nosso regime de ST marcadamente anulou a resposta neuroinflamatória Aβ-induzida, mantendo as quantidades de IL-6 e IL-4 aos níveis do grupo controle. Estes resultados suportam a hipótese do efeito anti-inflamatório do exercício físico (Gleeson et al., 2011; Pedersen et al., 2011; Spielman et al., 2016).

Um dos potenciais mecanismos pelos quais o exercício regula a inflamação é através do aumento da produção e liberação da citocina anti-inflamatória IL-10 (Gleeson et al., 2011). No nosso estudo prévio (Souza et al., 2013) nós demonstramos que este mesmo protocolo de ST é capaz de aumentar de forma expressiva os níveis de IL-10 no córtex pré-frontal e hipocampo. De fato, tem sido proposto que as contrações musculares causam um aumento de miocinas, que são citocinas e peptídeos com ação hormonal, capazes de cruzar a BBB e exercer efeitos anti-inflamatórios no cérebro (Pedersen et al., 2011). Dentre essas moléculas, a IL-6 é apontada como a principal ligação entre o músculo e o cérebro (Spielman et al., 2016). Embora a redução da IL-6 exercício-induzida encontrada em nosso estudo possa parecer paradoxal, é importante mencionar que o incremento dos níveis circulantes de

IL-6 após uma sessão de exercício ocorre de forma aguda, atuando na modulação do aparecimento de outras moléculas anti-inflamatórias na circulação, dentre elas a IL-10, o IL-1ra e o sTNF-α, todos capazes de atravessar a BBB (Petersen & Pedersen, 2005). Portanto, as repetidas sessões de exercício físico podem atuar de forma imunomodulatória, criando um microambiente cerebral anti-inflamatório capaz de proteger contra insultos inflamatórios, como o gerado pelo peptídeo $A\beta_{1-42}$.

No presente estudo, nós também encontramos um efeito protetor do ST sobre a ativação do NF-κB no córtex pré-frontal e hipocampo dos camundongos $A\beta_{1-42}$-tratados. Este é um importante resultado, posto que tem sido indicado que a ativação deste fator de transcrição está associada com o ciclo vicioso de respostas inflamatórias e com a neurodegeneração na DA (Rubio-Perez & Morillas-Ruiz, 2012; Wang et al., 2015; Zhang & Jiang, 2015). Por outro lado, nós observamos uma tendência de aumento nos níveis de NF-κB no cérebro dos animais sham-exercitados em comparação com seus homólogos sedentários. Embora este resultado seja aparentemente conflitante, é cabível ressaltar que a ativação do NF-κB é indicado como uma importante via de sinalização das adaptações do treinamento físico. Nesse sentido, a geração de espécies reativas exercício-induzidas é responsável por estimular vias de sinalização redox-sensíveis, como a via dependente do NF-κB, resultando no aumento da expressão de enzimas antioxidantes (Gomez-Cabrera et al., 2008). Estas regulações têm sido apontadas como fazendo parte dos efeitos horméticos do exercício físico (Radak et al., 2008). Esta propriedade antioxidante do exercício físico foi demonstrada pelo nosso estudo prévio, no qual nós mostramos que este mesmo regime de ST é eficaz em reduzir o estresse oxidativo cerebral induzido pelo peptídeo $A\beta_{1-40}$.

Em síntese, os resultados do presente estudo representam novas evidências de que o exercício físico é uma intervenção efetiva para inibir a ativação do NF-κB e, consequentemente, da resposta neuroinflamatória no cérebro de camundongos infundidos com o peptídeo $A\beta_{1-42}$, interrompendo o ciclo vicioso da neurotoxicidade deste peptídeo. A modulação exercício-induzida de vias de sinalização inflamatória pode ser um importante

mecanismo neurobiológico contra as alterações cognitivas e emocionais Aβ-induzidas.

Além do efeito anti-inflamatório, tem sido sugerido que um dos mecanismos subjacentes ao efeito neuroprotetor do exercício na DA é a sua capacidade de aumentar a síntese e a liberação de fatores neurotróficos (Paillard et al., 2015; Ma et al., 2017). Aqui, nós demonstramos que a diminuição dos níveis de BDNF, GDNF, NGF e NT-3 no córtex pré-frontal e hipocampo causados pelo peptídeo $A\beta_{1-42}$ foram prevenidos pelo ST, preservando os níveis destes fatores tróficos ao nível do grupo sham-sedentário (controle). Além disso, nós encontramos um aumento significativo dos níveis de BDNF e GDNF e uma tendência de aumento dos níveis de NGF e NT-3 nessas estruturas cerebrais dos camundongos sham-exercitados, quando comparados aos seus congêneres sedentários. Assim sendo, os nossos resultados destacam que o nosso protocolo de ST atua de forma mais pronunciada sobre a regulação do BDNF e GDNF, embora outras investigações sejam necessárias para confirmar estes achados. Portanto, os nossos resultados indicam que a supra-regulação dos fatores neurotróficos, principalmente dos níveis de BDNF e GDNF, são importantes processos mediadores da neuroproteção do exercício físico contra os distúrbios cognitivos e emocionais induzidos pelo peptídeo $A\beta_{1-42,}$ provendo novas evidências para o entendimento das bases biológicas pelas quais o exercício físico atua na prevenção da DA.

De grande interesse, o nosso protocolo de ST mitigou de forma bastante consistente a ativação da IDO e o aumento dos níveis de KYN, TRP e razão KYN:TRP no córtex pré-frontal e hipocampo dos camundongos $A\beta_{1-42}$-tratados. O nosso resultado é consistente com o estudo de Liu et al. (2013), no qual foi reportado que o exercício de natação é capaz de reduzir os níveis de citocinas inflamatórias e subsequentemente os níveis de IDO no córtex de ratos cronicamente estressados, sendo atribuído a estes efeitos regulatórios a melhoria dos sintomas depressivos. Portanto, embora outros mecanismos possam estar envolvidos, nós indicamos que, em parte, a ação anti-inflamatória do nosso protocolo de ST atenuou a ativação da IDO no cérebro, prevenindo o aparecimento dos distúrbios comportamentais induzidos pelo peptídeo $A\beta_{1-42.}$

Com este estudo nós fornecemos outra importante evidência para os estudos dos mecanismos neuroprotetores induzidos pelo exercício, indicando pela primeira vez que o exercício físico pode conferir efeitos protetores contra o insulto inflamatório $A\beta_{1-42}$-mediado, através da modulação da KP no cérebro de camundongos. Nós ressaltamos que uma maior compreensão desses mecanismos e suas relações potenciais com o exercício será de suma importância para a descoberta de novos biomarcadores da DA, assim como para a investigação de diferentes protocolos de exercício com o intuito de aliviar os sintomas da DA.

Por fim, através das evidências deste trabalho, nós indicamos que o binômio sedentarismo x envelhecimento é um fator predisponente cujos componentes podem interagir de maneira complexa e aditiva para aumentar o risco de desenvolver a DA. Estratégias não-farmacológicas e com elevado custo x benefício como o exercício físico, devem ser adotadas e incentivadas por políticas públicas desde a mais tenra idade, a fim de melhorar a saúde da população e reduzir as taxas de prevalência e incidência da DA.

CONCLUSÕES

O presente trabalho demonstrou que a ativação da IDO no cérebro desempenha um papel crítico na mediação das perturbações cognitivas e emocionais relacionadas à neuroinflamação induzida pelo peptídeo $A\beta_{1-42}$. O presente estudo é um importante passo em direção à compreensão do início do declínio cognitivo e das alterações neuropsiquiátricas associadas à DA, assim como do papel da citocinas e da ativação da KP em mediar estas complicações.

Os dados do presente trabalho também mostraram que a ativação da KP e os déficits de substâncias neuroprotetoras como o BDNF e o GSH, assim como os prejuízos na memória e os sintomas de ansiedade induzidos pelo peptídeo $A\beta_{1-42}$ são agravados pelo envelhecimento.

Além disso, nós demonstramos que o exercício de natação preveniu os déficits comportamentais e neurobiológicos $A\beta_{1-42}$-induzidos. Nós indicamos que este efeito neuroprotetor do exercício está envolvido com a imunomodulação de citocinas inflamatórias/ativação da IDO e com a regulação positiva do BDNF e do GDNF no cérebro de camundongos.

Com base nesses resultados, nós sugerimos que a KP e seus metabólitos são importantes alvos terapêuticos para o desenvolvimento de novas intervenções na DA, que possam atuar de forma adjuvante às terapias anti-inflamatórias e anti-amilóides existentes. Ademais, abordagens profiláticas que visem a diminuição da inflamação associada ao envelhecimento são importantes para prevenir a desregulação da KP e os sintomas cognitivos e neuropsiquiátricos em idosos e pacientes da DA. Por fim, nós indicamos que o exercício físico, através de seus mecanismos anti-inflamatórios e pró-neurogênicos, pode ser usado como uma estratégia não-farmacológica para reduzir as alterações associadas ao envelhecimento, a fim diminuir a incidência da DA e, até mesmo, para aliviar os sintomas cognitivos e emocionais desta doença.

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PERSPECTIVAS

Pretende-se dar continuidade a esse trabalho. Em nosso entendimento, outros biomarcadores precisam ser investigados no intuito de se compreender melhor como a via da quinurenina está envolvida na neurotoxicidade induzida pelo peptídeo $A\beta_{1-42}$, assim como entendemos que diferentes tipos de protocolos de exercício físico precisam ser testados a fim de se determinar quais tipos de métodos, intensidades e durações de exercício são mais eficazes em prevenir os sinais e sintomas da DA. Adicionalmente, entendemos que a avaliação do efeito protetor da melatonina, cujos efeitos antioxidantes e anti-inflamatórios vem sendo amplamente demonstrados, deve ser examinada em modelos animais da DA.

Com este propósito, objetiva-se investigar os níveis cerebrais dos metabólitos neurotóxicos da via da quinurenina, 3-HK e QUIN, e a expressão das enzimas limitantes KAT e KMO em resposta à infusão i.c.v. do peptídeo $A\beta_{1-42}$. Objetivamos também validar um protocolo de treinamento de natação para camundongos idosos, para viabilizar o estudo da interação exercício X envelhecimento X peptídeo $A\beta_{1-42}$ em um mesmo experimento. Além disso, pretendemos estudar o efeito protetor do treinamento de natação, realizado com o método de treinamento intervalado de alta intensidade, no modelo de DA induzido pela infusão i.c.v. do peptídeo $A\beta_{1-42.}$ Finalmente, utilizando este modelo, nós objetivamos investigar o efeito protetor da melatonina sob parâmetros comportamentais e marcadores da via da quinurenina.

ANEXO I

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

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PROTOCOLO Nº 012/2012

Título: Estudo do efeito neuroprotetor do exercício aeróbio no hipocampo e córtex pré-frontal de camundongos infundidos com beta-amilóide1-40

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Após a análise detalhada do projeto de pesquisa a relatoria do CEUA-Unipampa emite parecer **FAVORÁVEL** para o cadastro do protocolo e execução do referido projeto.

Atenciosamente

Luiz E. Henkes
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Coordenador da CEUA/Unipampa