Universidade de Lisboa

Faculdade de Medicina de Lisboa

Neuroprotective action of a Kyotorphin derivative in an animal model of Alzheimer's disease

Margarida Leonor Ferreira Martins

Orientadores:

Prof. Doutora Ana Maria Ferreira de Sousa Sebastião

Prof. Doutor Miguel Augusto Rico Botas Castanho

Dissertação especialmente elaborada para obtenção do grau de Mestre em Neurociências

2016

Universidade de Lisboa

Faculdade de Medicina de Lisboa

Neuroprotective action of a Kyotorphin derivative in an animal model of Alzheimer's disease

Margarida Leonor Ferreira Martins

Orientadores:

Prof. Doutora Ana Maria Ferreira de Sousa Sebastião

Prof. Doutor Miguel Augusto Rico Botas Castanho

Dissertação especialmente elaborada para obtenção do grau de Mestre em Neurociências

2016

A impressão desta dissertação foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 16 de fevereiro de 2016.

Acknowledgments

Tal como já dizia António Gedeão 'Sempre que um homem sonha, o mundo pula e avança' e, de facto, é preciso haver sonhos para surgirem criações, progressos, descobertas... Esta dissertação de mestrado resultou disso mesmo, de um sonho, de uma ideia nova e, sem dúvida, que com a realização da mesma o meu mundo mudou! Durante esta jornada, tive oportunidade de conhecer pessoas incríveis, de fortalecer amizades e, acima de tudo, de aprender imenso. Desde já estou eternamente grata por tudo isso.

Este trabalho não teria sido possível sem o apoio dos meus orientadores: *Professora Ana Sebastião* e *Professor Miguel Castanho*. Duas mentes brilhantes, pessoas inspiradoras e excelentes líderes, aos quais começo por prestar o meu profundo agradecimento. Agradeço a ambos pela confiança depositada ao terem-me possibilitado desenvolver este trabalho à luz dos meus 'sonhos' e pelo sentido de responsabilidade que me incutiram em todas as fases do trabalho.

Um especial agradecimento à *Professora Ana Sebastião*, que me acompanha desde o início do mestrado em Neurociências, pela oportunidade que me deu de integrar o seu grupo de investigação, por toda a partilha de conhecimentos, entusiasmo contagiante, disponibilidade, críticas construtivas e pelas preciosas contribuições nesta dissertação. A sua sabedoria foi fundamental para que chegasse ao fim deste trabalho com um enorme sentimento de satisfação. É, sem dúvida, uma das pessoas mais positivas que conheço, procurando sempre realçar os pontos fortes dos trabalhos dos seus alunos e a incentivá-los a serem ainda melhores.

De igual modo, não posso deixar de particularizar o meu agradecimento ao *Professor Miguel Castanho* pela sua enorme simpatia, boa disposição, total colaboração no solucionamento de dúvidas que foram surgindo, pelo apoio financeiro determinante na elaboração deste trabalho e por me ter apresentado a investigadores de excelência, o que se tornou uma mais valia para o meu futuro académico/profissional.

À *Cláudia Valente*, o meu sincero obrigada! É como tivesse sido uma terceira orientadora neste trabalho. Toda a parte molecular desta tese contou com o seu contributo. Obrigada por todos os ensinamentos, disponibilidade, paciência, correção de algumas partes desta dissertação, simpatia e pelos sábios conselhos que me deu.

Agradeço também à *Sara Xapelli*, principalmente por todo o auxílio prestado nas cirurgias, pelas valiosas discussões que tivemos sobre o meu trabalho e pela sua amabilidade.

Ao *Francisco Mouro*, o meu obrigada por todas as ajudas e dicas com os testes comportamentais e com a análise dos resultados relacionados com os mesmos.

À *Professora Maria José Diógenes (Mizé)*, agradeço por todos os proveitosos conselhos que me deu desde o início deste trabalho e pelo auxílio nas disseções.

Ao *Armando Cruz*, *Mariana Oliveira* e *Sandra Vaz*, agradeço também pelo apoio prestado com as disseções.

À *Rita Ramalho*, *André Santos*, *Sara Tanqueiro* e *João Gomes,* agradeço por todo o auxílio relacionado com as culturas primárias de neurónios que, apesar de não constarem nesta dissertação, não podia deixar de expressar o meu sincero obrigada a todos vocês.

À *Filipa Ribeiro*, o meu obrigada pelos ensinamentos, preocupação e discussões preciosas que tivemos.

Também não poderia deixar de agradecer ao grupo liderado pela *Doutora Luísa Lopes*. *Diana Ferreira*, *Mariana Temido*, *Vânia Batalha* e *Inês Morgado*, muito obrigada a todas pelo apoio e dicas durante este trabalho. E um especial agradecimento à *Joana Coelho*, por todos os conhecimentos transmitidos, colaboração, preocupação, afabilidade e pela total disponibilidade que sempre revelou para comigo.

Agradeço igualmente ao grupo de investigação liderado pelo *Professor Miguel Castanho*, nomeadamente à *Juliana Perazzo* e *Antónia Pinto* por toda a disponibilidade e ajudas prestadas durante este trabalho.

A equipa do Biotério de Roedores do Instituto de Medicina Molecular (iMM) também sempre se mostrou disponível para me ajudar e instruir, pelo que também deixo aqui o meu agradecimento, especialmente à *Dolores Bonaparte*, *Joana Marques* e *Iolanda Moreira*.

Quero agradecer também ao *Professor Doutor Alexandre Mendonça*, pelo convite para apresentar o meu projeto da dissertação à sua equipa de trabalho, pelas valiosas discussões e conselhos que me deu e pela revisão de parte da introdução desta tese relacionada com a Doença de Alzheimer.

À *Doutora Paula Canas* do Centro de Neurociências e Biologia Celular (CNC), em Coimbra, agradeço a disponibilidade em esclarecer todas as minhas dúvidas e pelas dicas fulcrais que deu de modo a garantir o sucesso deste trabalho.

A todos os meus colegas de laboratório que direta ou indiretamente me ajudaram em algum momento, o meu eterno obrigada! Gostaria de salientar a *Catarina Lourenço*, *João Gomes*, *Tatiana Morais* e *Rui Rodrigues*, agradecendo a amizade que desenvolvemos. Obrigada pelo companheirismo, simpatia e palavras de incentivo. É sempre mais fácil trabalhar com pessoas genuinamente bem-dispostas, permitindo que cada dia fosse encarado com particular motivação. Também uma referência especial à *Sara Tanqueiro*, pela forte amizade que criámos. Agradeço-lhe a partilha de bons momentos, a ajuda e os estímulos nas alturas de desânimo.

Agradeço também à *Alexandra Botelho* e *Cristina Varandas* por toda a amabilidade e apoio administrativo e ao *Sr. João Baião* por toda a ajuda técnica com os animais quando era necessário.

E um enorme obrigada à *Liana Shvachiy*. Agradeço por se ter interessado verdadeiramente no meu projeto, pela sua vontade de aprender e de querer aperfeiçoar todas as técnicas que lhe ensinei, por ter colaborado ativamente neste trabalho e, claro, pela sua amizade e apoio. Trabalhar com ela tornou tudo mais simples e recompensador. Fico feliz por saber que todos os conhecimentos que lhe transmiti contribuíram para a sua formação académica.

Não podia também deixar de agradecer aos meus verdadeiros amigos. Agradeço de um modo geral a todos pelo carinho, preocupação, apoio, desabafos e vivências partilhadas. Em especial à *Rita Rosa*, pela cuidada revisão do manuscrito desta dissertação e pelas críticas construtivas que me deu, para além de toda a amizade.

Ao *David Veríssimo*, *Raquel Leal*, *Lala Policarpo*, *Luís Caldeira* e *Leticia Fleig*, os meus companheiros de ginásio, também conhecidos por *KTC team*, agradeço pela amizade, transmissão de confiança e de força e por me proporcionarem momentos saudáveis de descontração depois de um longo dia de trabalho no laboratório.

Por fim, agradeço aos meus maiores 'fãs': a minha família, particularmente os meus pais, tios e avó. Muito obrigada pelo vosso amor incondicional, por acreditarem sempre em mim, pela valorização sempre tão entusiasta do meu trabalho e por todos os ensinamentos de vida. É por eles que faço sempre mais e melhor na tentativa de, um dia, poder retribuir e compensá-los por tudo aquilo que têm feito por mim! A eles dedico este trabalho.

Margarida Martins

Index

List of Figures

List of Tables

List of Abbreviations

Abbreviations used more than once are listed.

2VO Two-vessel occlusion

Ab Antibody

ACh Acetylcholine

AD Alzheimer's disease

Ag Antigen

ANOVA Analysis of variance

APOE Apolipoprotein E

APP Amyloid precursor protein

Aβ Amyloid-β

$Aβ_{1–42}$ The 42-amino acid form of Amyloid-β

BBB Blood-brain barrier

BSA Bovine serum albumin

CA Cornu ammonis

Ca^{2+} Calcium ion

CNS Central nervous system

CSF Cerebrospinal fluid

CTL Control

DG Dentate gyrus

EC Entorhinal cortex

ECL Enhanced chemiluminescence

FAD Familial Alzheimer's disease

FBS Fetal bovine serum

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

GFAP Glial fibrillary acidic protein

HRP Horseradish peroxidase

I.c.v. Intracerebroventricular

I.p. Intraperitoneal

I.t. Intrathecal

Iba-1 Ionized calcium binding adaptor molecule 1

IbKTP-NH_2 Amidated Kyotorphin linked to ibuprofen

IF Immunofluorescent

IHC Immunohistochemistry

iMM Instituto de Medicina Molecular

IR immunoreactivity

KTP Kyotorphin

KTP-NH_2 Amidated Kyotorphin

KTPr Kyotorphin specific receptor

L-VDCCs L-type voltage-gated calcium channel

MAPT Microtubule (MT)-associated protein tau

Met-enk Methionine-enkephalin

MRI Magnetic resonance imaging

NeuN Neuronal nuclei marker

NMDA N-Methyl-D-aspartate

NO Nitric oxide

NOR Novel Object Recognition

NSAID Non-steroidal anti-inflammatory drug

O/N Overnight

OF Open Field

P-tau Phosphorylated tau protein

PBS Phosphate-buffered saline

PBSTw PBS with 0.1% Tween

PFA Paraformaldehyde

PSEN Presenilin

PVDF Polyvinylidene difluoride

RI Retention interval

RT Room temperature

SAD Sporadic Alzheimer's disease

SC Subcutaneous

SDS Sodium dodecyl sulphate

SEM Standard error of the mean

TBST Tris-buffered saline with Tween-20

USA United States of America

VGCC Voltage-gated calcium channel

WB Western Blotting

Abstract

Alzheimer's disease (AD) is the most common form of dementia. It is characterized by early memory deficits, followed by the gradual erosion of other cognitive functions. A hallmark of this disease is the accumulation of amyloid-beta (Aβ) peptide in the brain. Despite decades of effort and a massive investment, AD still represents a challenge for drug discovery, since an effective treatment is still needed. Kyotorphin (KTP), apart from being an endogenous analgesic peptide, has been pointed out as a putative neuroprotective molecule, although this has never been studied in the context of AD.

Consequently, the aim of this work was to investigate the neuroprotective effect of prolonged treatment with KTP–NH_2 (Amidated KTP, which can cross the blood-brain barrier) in a non-transgenic animal model of AD.

Male Wistar rats (8-10 weeks) were intracerebroventricularly injected with 5 µL of Amyloid-β 1-42 ($A\beta_{1–42}$) (2.25 mg/mL) at day zero. Then, starting at day 2, KTP-NH_2 (100 µmol/kg) was intraperitoneally (i.p.) injected every day. Animals were tested in several behavioural paradigms, between days 15 and 20 and sacrificed at day 21 for immunohistochemistry and western blot analysis of the hippocampus. Values of $P \leq 0.05$ (one-way ANOVA followed by Holm-Sidak's multiple comparisons test, or Student's t-test) were considered to represent statistically significant differences.

Results showed that $A\beta_{1–42}$-injected rats displayed spatial working memory deficits assessed by the percentage of spontaneous alternation in the Y-Maze test as compared with controls (CTL, sham-operated rats) ($A\beta_{1-42}$: 55 ± 3.6 *vs* CTL: 69 ± 2.0; n= 6-7 animals per group; $P \leq 0.05$). However, when the animals were treated with KTP-NH_2 the percentage of spontaneous alternation in the Y-Maze was similar to CTL group ($A\beta_{1-42}$ KTP-NH_2: 67 ± 1.3; n=6; $P > 0.05$). Moreover, $A\beta_{1–42}$-injected rats demonstrated episodic long-term memory deficits assessed by both object preference and object recognition indexes in the Novel Object Recognition (NOR) test ($P \leq 0.05$ as compared with CTL; n=6-7). $A\beta_{1–42}$-injected rats receiving KTP-NH_2 scored in the NOR as CTL ($P > 0.05$ as compared with CTL; $P \leq 0.05$ as compared with $A\beta_{1–42}$-injected rats not treated with KTP-NH_2; n=6-7). None of the groups displayed impairments of locomotor performance (evaluated by the total number of arm entries in the Y-Maze and by Open Field (OF) test) or differences in the exploratory behaviour (evaluated by OF test) ($P > 0.05$; n=6-7). Neuronal damage at the hippocampal *cornus ammonis* 1 and dentate gyrus, evaluated by immunohistochemistry and quantitative analysis of NeuN (neuronal marker) fluorescence, was evident in $A\beta_{1–42}$-injected rats that did not receive KTP-NH_2 ($P \leq 0.05$ as compared with CTL; n=3), but virtually absent in the $A\beta_{1–42}$ group treated with KTP-NH_2 ($P > 0.05$ as compared with CTL; n=3). No signs of astrogliosis (evaluated by GFAP immunoreactivity) or microgliosis (evaluated by Iba-1 immunoreactivity) were detected using both immunohistochemistry and western blotting techniques ($P > 0.05$; $n \geq 3$).

The results suggest that KTP-NH_2 reduces neurodegeneration as well as memory impairments induced by $A\beta_{1-42}$ peptide, being potentially useful in the treatment of AD.

Key-words: Aβ peptide; Alzheimer's disease; Kyotorphin derivative; memory impairments; neuroprotection.

Resumo

A Doença de Alzheimer (*Alzheimer's disease* – AD) é a doença neurodegenerativa mais comum em idosos e a forma mais frequente de demência. Com base no constante aumento da esperança média de vida nas sociedades ocidentais, estima-se que os casos de AD tripliquem até 2050. Os mais recentes dados epidemiológicos (2009) apontam para a existência de 153 000 pessoas com demência em Portugal, das quais 90 000 têm AD, ou seja, cerca de 1% do total da população nacional sofre desta patologia.

Atualmente, sabe-se que a AD é caracterizada por uma deterioração progressiva das funções cognitivas, pela formação de placas senis e emaranhados neurofibrilares, bem como por uma profunda alteração nas respostas gliais, desregulação neuronal da homeostase do cálcio, disfunção colinérgica e extensa perda sináptica e neuronal. De igual modo, está descrito que o primeiro sinal de desenvolvimento de AD é a perda de memória. Assim sendo, é muito importante criar e utilizar modelos animais que exibam dificuldades de memória. Um exemplo de tal modelo animal é obtido pela injeção intracerebroventricular (i.c.v.) de β-amiloide (Aβ), que é o principal componente das placas senis.

É também importante salientar que esta doença neurodegenerativa representa um dos maiores desafios no que diz respeito à descoberta de fármacos eficazes para a sua cura e, apesar de nos últimos 30 anos se ter tentado desenvolver um tratamento capaz de travar ou reverter a AD, infelizmente ainda não existe disponível um medicamento com estas potencialidades.

Posto isto, torna-se evidente a necessidade de se investigar novas moléculas que se considerem promissoras no contexto da terapêutica de AD.

Uma dessas moléculas é a Quiotorfina (*Kyotorphin* – KTP) – dipéptido endógeno (constituído por L-tirosina e L-arginina). Este dipéptido foi encontrado pela primeira vez, em 1979, num encéfalo bovino e desde então foi também isolado a partir de encéfalos de várias espécies, incluindo o líquido cefalorraquidiano (*cerebrospinal fluid* - CSF) de humanos. Estudos anteriores sugerem que a KTP tem propriedades neuroprotetoras nomeadamente no hipocampo e no cerebelo, para além de desempenhar um papel primordial na regulação da dor no sistema nervoso central. Contudo, falta ainda saber se esta molécula tem uma ação neuroprotetora num modelo animal de AD. Sabe-se que a sua ação protetora poderá estar relacionada com a capacidade de inibir correntes de cálcio na membrana pós-sináptica.. Por outro lado, o seu efeito analgésico é 4,2 vezes superior ao da metionina-encefalina (*Methionine-enkephalin* – met-enk), um pentapéptido opioide endógeno. Embora apresente uma atividade opioide, pois a sua ação é prevenida por antagonistas de recetores de opioides, provou-se que a KTP não se liga, *in vitro*, aos subtipos mu-, delta- ou kappa dos recetores de opioides. Este facto levou à hipótese de um mecanismo de ação indireto, através da ligação a um recetor específico. Como

este recetor ainda não foi identificado, os detalhes do mecanismo de ação da KTP são desconhecidos, tendo sido, no entanto, sugeridas duas hipóteses: a ativação do recetor pela KTP leva à libertação de met-enk, que leva à ativação de um recetor opioide; ou a KTP é degradada rapidamente e a L-arginina formada é utilizada pelo enzima óxido nítrico sintetase que leva à formação de óxido nítrico, que por sua vez induz a libertação de met-enk.

Devido à reduzida capacidade de atravessar a barreira hematoencefálica (*Blood-brain barrier* – BBB), a atividade analgésica da KTP em modelos animais foi apenas observada quando a molécula era injetada diretamente no cérebro. De modo a ultrapassar tal limitação, sintetizaram-se derivados da KTP. Um desses derivados resultou da substituição do grupo carboxilo terminal por um grupo amida (KTP-NH_2). Esta modificação, ao aumentar a carga global do péptido e a sua hidrofobicidade, melhora a sua interação para com as membranas biológicas, permitindo assim atravessar a BBB.

Estudos clínicos recentes têm vindo também a reforçar a hipótese de uma ligação entre a AD, dor e a KTP em seres humanos. Alguns estudos apontam que a dor é subestimada em doentes com Alzheimer e que estes doentes apresentam níveis diminuídos de KTP no CSF. Adicionalmente, verificou-se também uma correlação inversa entre os níveis de proteína fosforilada-tau (um marcador molecular de progressão de AD) e de KTP.

Assim sendo, uma dupla ação, analgésica e neuroprotetora, num único fármaco seria de extrema importância, no contexto da dor e neurodegeneração na AD. Para além disso, como os níveis deste dipéptido endógeno neuroprotetor estão diminuídos em pacientes com AD, torna-se interessante avaliar se a sua aplicação exógena (ou dos seus derivados) pode travar algumas das disfunções associadas à AD.

Consequentemente, o objetivo deste trabalho foi investigar as potencialidades da KTP-NH_2, de que existem vários indícios que atravessa a BBB, no combate à progressão da AD. Avaliei então a capacidade desta molécula para reduzir alguns dos sintomas característicos da AD, nomeadamente o defeito de memória e a morte neuronal.

Para tal, usei um modelo animal de AD – ratos machos Wistar (8-10 semanas de idade) injetados i.c.v. com 5 µL do péptido $A\beta_{1-42}$ (2,25 mg/mL) no dia zero. A partir do dia 2, KTP-NH_2 (100 µmol/ kg) foi intraperitonealmente (i.p.) injetada todos os dias. Os animais foram testados em diversos paradigmas comportamentais a partir do dia 15 até ao dia 20. No dia 21 foram sacrificados para análises imuno-histoquímicas e de *western blot* dos hipocampos. Valores de $P \leq 0,05$ (Análise de Variância com um fator (*one-way ANOVA*) seguida do teste Holm-Sidak para múltiplas comparações, ou Teste t de Student) foram considerados para representar diferenças estatisticamente significativas.

Os resultados obtidos demonstraram que os ratos injetados i.c.v. com $A\beta_{1-42}$ (ratos $A\beta_{1-42}$) exibiram défices na memória de trabalho espacial avaliada no teste do Labirinto em Y (*Y-Maze)* – ao compará-los com os ratos controlo (CTL, ratos *sham-operated*) ($A\beta_{1-42}$: 55 ± 3,6 *vs* CTL: 69 ± 2,0; n= 6-7

animais por grupo; P≤0,05). Contudo, quando os animais foram tratados com KTP-NH_2 a percentagem de alteração espontânea no *Y-Maze* foi semelhante ao grupo controlo ($A\beta_{1-42}$ KTP-NH_2: 67 ± 1,3; n=6; P>0,05). Adicionalmente, os ratos $A\beta_{1-42}$ revelaram défices na memória episódica a longo prazo que foi avaliada tanto pelo índice de preferência do objeto como pelo índice de reconhecimento do objeto no teste de Reconhecimento do Novo Objeto – *Novel Object Recognition test* (NOR) (P≤0,05 comparando com os ratos CTL, n=6-7). Os ratos $A\beta_{1-42}$ que foram tratados com KTP-NH_2 tiveram uma prestação semelhante à dos ratos CTL no teste NOR (P>0,05 comparando com os ratos CTL; P≤0,05 comparando com os ratos $A\beta_{1–42}$ que não foram tratados com KTP-NH_2; n=6-7). Nenhum dos grupos demonstrou disfunção locomotora (avaliado pelo número total de entradas nos braços do *Y-Maze* e pelo teste do Campo Aberto – *Open Field* (OF) *test*), nem perturbações na atividade exploratória (avaliado pelo teste OF) (P>0,05; n=6-7). O dano neuronal no *cornus ammonis* 1 e corpo tufado (*dentate gyrus*) do hipocampo, avaliado pela técnica de imuno-histoquímica e análise quantitativa da fluorescência do NeuN (marcador neuronal), foi evidente nos ratos $A\beta_{1-42}$ que não foram tratados com KTP-NH_2 (P≤0,05 comparando com ratos controlo; n=3), mas virtualmente ausente nos ratos $A\beta_{1-42}$ tratados com KTP-NH_2 (P>0,05 comparando com os ratos CTL; n=3); Não foram detetados, em nenhum dos grupos experimentais, sinais de astrogliose (avaliado pela imunoreatividade do GFAP) nem de microgliose (avaliado pela imunoreatividade do Iba-1), usando tanto a técnica de imuno-histoquímica com a de western blot (P>0,05; n≥3).

Estes resultados sugerem que a KTP-NH_2 reduz a neurodegeneração no hipocampo assim como défices de memória induzidos pelo péptido $A\beta_{1–42}$, podendo ser potencialmente útil no tratamento da AD.

Estudos futuros de farmacocinética e farmacodinâmica são necessários para descobrir quais os alvos moleculares da KTP e dos seus derivados, de modo a otimizar ao máximo o potencial terapêutico desta molécula.

Palavras-chave: péptido Aβ; doença de Alzheimer; derivado da Quiotorfina; défices de memória; neuroprotecção.

‘It is timely to interrogate the term Alzheimer’s disease.

The term Alzheimer’s syndrome seems more appropriate.’

Richards and Brayne, 2010 [1’]

1. Introduction

1.1. Alzheimer's Disease

First described in 1906 by Dr. Aloysius "Alois" Alzheimer, Alzheimer's disease (AD) is the most common type of dementia and the largest unmet medical need in neurology.[1–4]

Clinically, AD is characterized by a progressive cognitive decline, where memory of recent facts, spatial orientation, attention and executive functions are ones of the first affected.[4–9] This is followed by behavioural symptoms such as apathy and depression, which form an integral part of the disease process.[4–9] At later stages, AD patients are completely dependent on others, since motor signs appears such as difficulty speaking, swallowing and walking, with death occurring, on average, 9 years after diagnosis, usually resulting from general inanition, malnutrition and pneumonia.[4–9] Thus, it is reasonable to think that this neurodegenerative disease has also a significant impact on families and caregivers of the patients.

Concerning epidemiological aspects of this disease, it is known that AD affects 5% of individuals over the age of 65; 20% over the age of 80 and more than a third of those over the age of 90.[8] In Portugal, the most recent epidemiologic data (October, 2009) point to the existence of 153 000 patients with dementia, of which 90 000 have AD, which means that about 1% of the total national population suffers from this condition.[10] Its prevalence will therefore continue to escalate over the next decades as life expectancy increases, with current best estimates indicating that the number of dementia cases in Europe will reach almost 14 million in 2050, i.e. the equivalent of about 150% of the current Portuguese population.[8,11,12] This projection definitely indicates the importance of facing the problem, since neither healthcare nor financial systems are prepared to face the magnitude of the situation.[8,11,12] Moreover, AD incurs an enormous personal cost to those affected and the worldwide financial cost in 2010 was estimated at US$604 billion.[8,13]

In conclusion, AD has been identified as a research priority and represents a major and rising public health concern.[8,13,14] Therefore, the development of more effective therapies to treat and delay the onset of the disease is desirable for both social and economic reasons.[8,13,14]

Before elucidating what are the current therapies available and reinforce the importance of finding new treatments with a proven disease-modifying effect for AD, I will discuss about other issues of this disease, starting with the genetics and neuropathological findings in AD.

1.1.1. Genetics

AD can be classified as familial (FAD, usually with onset <65 years) or sporadic (SAD, onset >65 years).[15]

FAD , also known as early-onset autosomal dominant form of AD, is due to mutations in three genes — amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) and *PSEN2* (reviewed in Ref.[16])— and duplication of the *APP* gene.[4,16–18] From a therapeutic perspective, targeting the mechanisms of FAD, which accounts for less than 1% of the total number of AD cases, makes the implicit assumption that this disease is essentially similar to SAD.[4,15,19]

The genetics of SAD is an active area of investigation. The most widely known risk factor for late-onset AD is apolipoprotein E (APOE), since as many as 40 % of all AD patients are carriers of the APOE ε4 allele (APOE4).[4,19] Exactly how the mutated genes or different isoforms increase the risk of disease risk remains unclear and, at least in the case of *APOE4*, a consensus mechanism of pathogenesis has not emerged in more than a decade after the discovery of its role in AD. In fact, *APOE4* is neither necessary nor sufficient for development of amyloid pathology or incident AD, suggesting that other contributing factors remain to be discovered.[4,19]

1.1.2. Neuropathology

Post-mortem analysis of human AD brains provided the first clues about the mechanisms of the disease and potential interventions.[4] This led to the description of the disease a century ago and the identification of the hallmark lesions of AD — senile plaques composed of extracellular deposits of amyloid-β (Aβ) (Figure 1) and neurofibrillary tangles formed by accumulation of abnormal filaments of tau (Figure 2) — in brain regions that serve memory and cognition.[3,4,17,20] Note that these neuropathological findings are the ones that still provide the definitive diagnosis of AD following post-mortem analysis of brain parenchyma.[9,14]

Besides these hallmarks, prominent activation of inflammatory processes and the innate immune response are observed, plus neuronal dysregulation of calcium (Ca^{2+}) homeostasis (increased Ca^{2+} influx induced by Aβ), cholinergic dysfunction and extensive synaptic and neuronal loss at specific sites.[4,20–23]

However, determining whether a given pathological structure drives the disease is a neutral bystander or just represents an unsuccessful repair attempt remains an unsolved issue.[4]

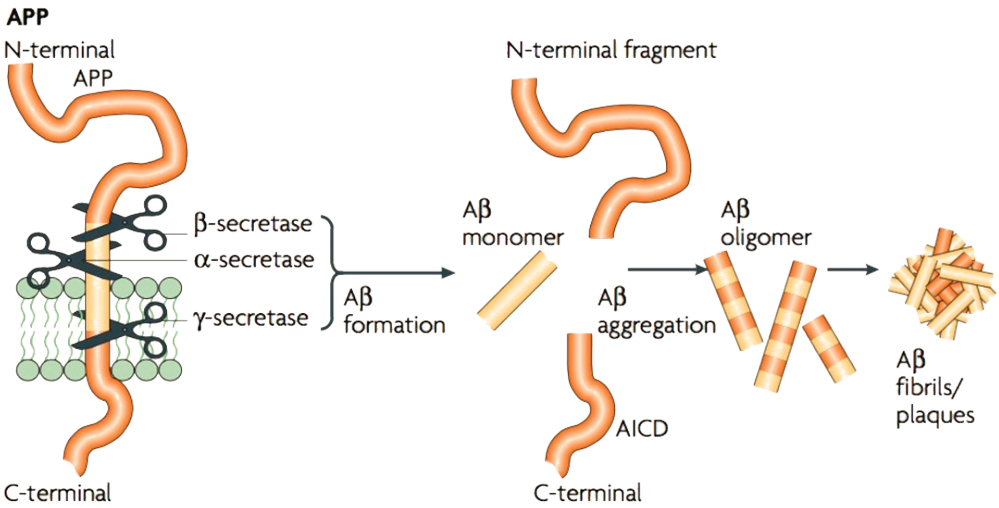


Figure 1. β-Amyloid plaques are hallmark lesions of Alzheimer's disease.

Aβ is a 4 kDa protein that exhibits microheterogeneity in amino acid sequence and in a variety of biophysical states. The major protein component of the plaques is a 40–42 amino acid polypeptide termed Aβ ($Aβ_{1-40}$ and $Aβ_{1-42}$) that is derived by proteolytic cleavage from the amyloid precursor protein, APP. While β-secretase generates the amino terminus of Aβ, γ-secretase dictates its length, with $Aβ_{1-40}$ being the more common and $Aβ_{1-42}$ the more fibrillogenic and neurotoxic species. Aβ forms toxic oligomeric aggregates and eventually deposits as plaques, if they cannot be cleared from the brain. Additional products of APP processing are an N-terminal fragment that is released by shedding and the Aβ intracellular cytoplasmic domain (AICD). β-secretase activity has been attributed to a single protein, BACE, whereas γ-secretase activity depends on four molecules: presenilin, nicastrin, anterior pharynx-defective 1 (APH1) and presenilin enhancer 2 (PEN-2). α-secretase is involved in the non-amyloidogenic pathway by cleaving APP within the Aβ domain, consequently preventing Aβ formation. Thus, the pathological accumulation of Aβ is a result of the increased proteolytic cleavage of APP by β- and γ-secretase and/or reduced clearance due to decreased expression of e.g. enzyme responsible for its removal, the insulin-degrading enzyme. Although it has been traditionally thought that extracellular Aβ aggregates in the form of senile plaques are the main pathogenic species, recent literature data recognizes that intraneuronal accumulation of the oligomeric non-fibrillar Aβ form precedes and contributes to the extracellular pathology.[17,24] *(Figure adapted from Gotz et al., 2008)*[17]

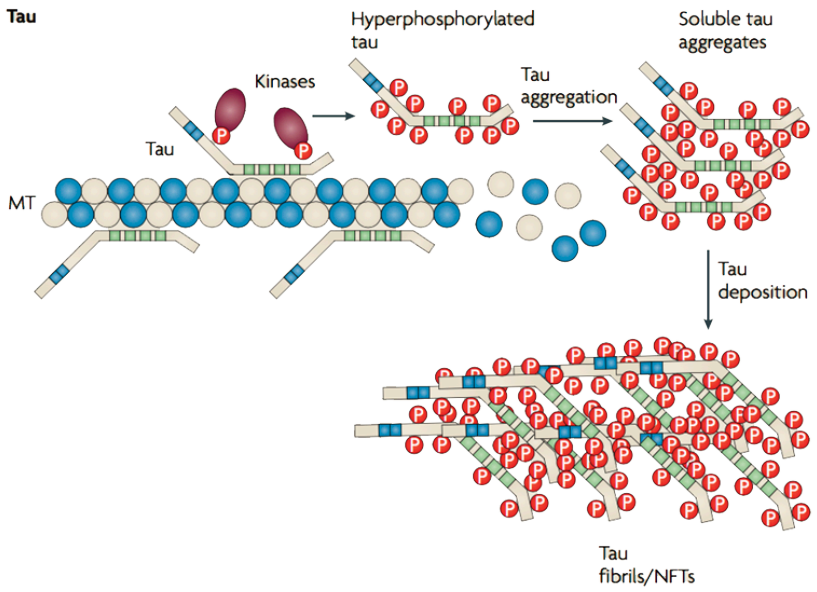


Figure 2. Neurofibrillary tangles (NFTs) are most commonly known as a primary marker of Alzheimer's disease.

The neurofibrillary lesions contain aggregates of the microtubule (MT)-associated protein tau. Under physiological conditions tau, which is synthesized and produced in all neurons, is mainly localized in the axon for binding to tubulin and stabilize microtubules. Tau is a phosphoprotein owing to its high numbers of serine and threonine residues and is therefore a substrate of many kinases. Under pathological conditions, tau is hyperphosphorylated, which means that it is phosphorylated to a higher degree at physiological sites, as well as at additional "pathological" sites. On the other hand, hypophosphorylated tau dissociates from MTs, causing them to depolymerize, while tau is deposited in aggregates such as NFTs.[17] *(Figure adapted from Gotz et al., 2008)* [17]

To sum up, it is reasonable to accept that AD is a multifactorial disease, but the role of each player still deserves further clarification.[18]

Next, I will summarize and discuss current knowledge about AD, focusing on the amyloid cascade hypothesis – the dominant hypothesis in the field of AD pathogenesis.[5]

1.1.3. The Amyloid Cascade Hypothesis

The amyloid cascade hypothesis , which was first established by Selkoe and his research group in 1991, has been very influential in the research conducted both in academia and the pharmaceutical industry in the context of AD.[25,26]

Regardless the primary cause and clinical form of AD, this hypothesis integrates histopathological and genetic information and suggests that the deposition of the Aβ peptide in the brain parenchyma initiates a sequence of events that ends with the dementia we know as AD (Figure 3).[5,24,26,27]

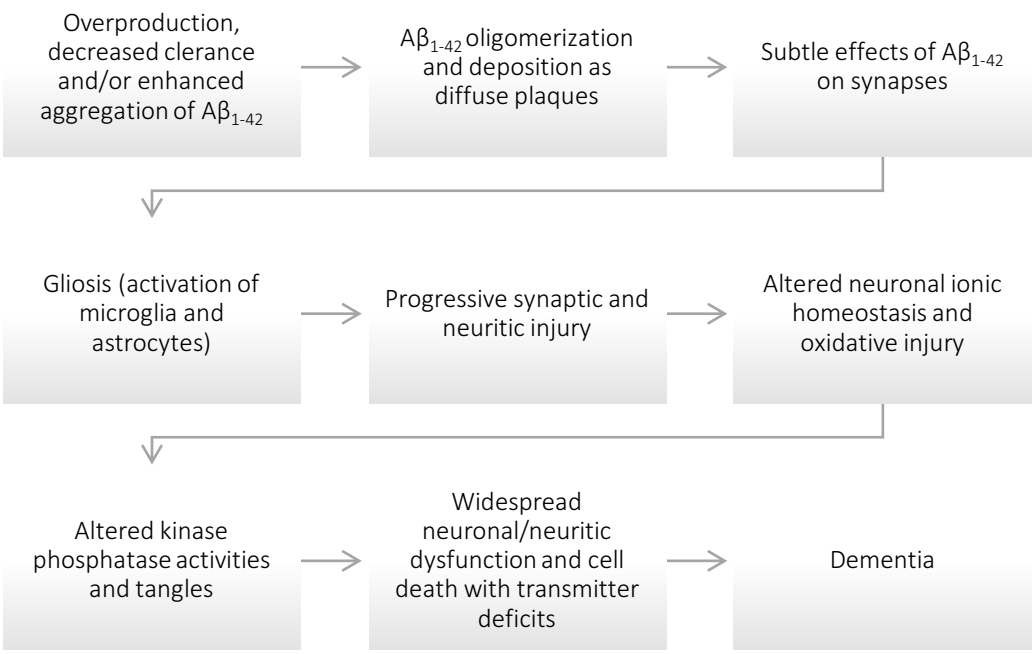


Figure 3. The amyloid cascade hypothesis.

The pathogenic events shown can eventually lead to Alzheimer's disease. The cascade starts with the generation of the 42-amino acid form of Amyloid-β ($A\beta_{1-42}$).[27] *(Figure adapted from Hardy et al., 2002)*[27]

Since, in the past 20 years, the amyloid cascade hypothesis has become the focus of AD research, it is imperative to critically re-examine this hypothesis and address its strengths and weaknesses, both real and perceived, which are summarized in Table 1.

Table 1. Strengths and weaknesses of the amyloid cascade hypothesis.

(Table adapted from Herrup, 2015)[5]

	STRENGTHS	WEAKNESSES
GENETICS	• FAD: APP and PSEN genes are the only genes identified • SAD: APOE variants affect AD risk and also Aβ clearance • Rare A673T *APP* mutation lowers Aβ production and protects against AD	• FAD: No α-secretase (ADAM10) or β-secretase (BACE) mutations yet found • SAD: APP, PSEN, BACE and MAPT (tau) polymorphisms show little association • *MAPT* mutations associate with frontotemporal dementia
BIOCHEMISTRY	• Amyloid comes from APP after cleavage by γ-secretase (PSEN) • Conditions that favour γ-secretase cleavage to the longer $A\beta_{1\text{-}42}$ favour aggregation and AD • APOE4 increases risk of AD and slows clearance of Aβ	• Transgenic mice expressing only Aβ suggest amyloid alone is not sufficient • Other biochemical deficits are present in AD and are sufficient to create dementia
ANIMAL MODELS	• Overexpression of human APP in mouse produces plaques • Mouse transgenics for human APP show memory deficits • Aβ is toxic to neurons in culture • Overexpression of human APP in fruit flies produces neurodegeneration	• Overexpression of human APP in mouse does not produce tangles, neurodegeneration or AD-like dementia • PSEN transgenics show neither plaques nor tangles nor neurodegeneration • Memory deficits in transgenics correct quickly and completely
PATHOLOGY	• Amyloid plaques are more frequent in AD-affected brains	• Tangles correlate better with neurodegeneration than plaques do • Individuals with substantial plaque burdens can have normal cognition
CLINICAL FINDINGS	• Presence of plaques on imaging associated with increased AD risk • In some subjects with amyloid burdens and early dementia, anti-amyloid therapy improves cognition	• By definition, there is no AD without plaques and plaque deposits without dementia is preclinical AD • Inhibition of γ-secretase increases AD symptoms
EPIDEMIOLOGY		• Certain nonsteroidal anti-inflammatory drugs reduce AD risk by half

In spite of the fragilities of this hypothesis, it is important to stress that they do not contest that Aβ is involved in AD. Along with APP and the secretases, Aβ remains a central part of the current thinking on the pathophysiology of the disease. Moreover, even if Aβ proves to be only a correlate of AD and nothing more, the correlation is still robust. Therefore, instead of rejecting the amyloid cascade hypothesis, there is an urge to redefine the disease. Nevertheless, rejecting it is not a defeat or an admission of failure.[5] It is important to keep in mind that AD is perhaps one of the most complex systematic malfunctions of the nervous system that exists.[5,28] Indeed, for a disease with the prevalence and complexity of AD, the real surprise would be if there was in fact a single, linear pathway that led from healthy brain aging to AD.[5] Consequently, several perspectives on the limitations of the hypothesis have been put forward (see, for example, Ref. [5,28,29]), but an alternative hypothesis explaining the cause and early pathogenesis of AD that has as much experimental support as the Aβ hypothesis has not yet emerged.[27]

In conclusion, the amyloid hypothesis has been formally articulated and varieties of studies, from many laboratories worldwide, have supported its broad outlines. Importantly, overall findings were consistent with the notion that cerebral Aβ accumulation is one of the major influence in AD, the disease progression resulting from an imbalance between Aβ production and Aβ clearance.[27]

As we can perceive from Table 1, much of the data that support the amyloid hypothesis were obtained using animal models of AD. Therefore, it is important to analyse and give some examples of animal models that are being used to investigate AD.

1.1.4. Animal models of Alzheimer's Disease

Animal models of a disease are a cornerstone of the drug development process, since they can offer precious tools for evaluating new therapeutic strategies for the treatment of human diseases.[7,30] Additionally, they are crucial for studying the pathological mechanisms involved in the disease processes.[7,30]

Since aetiology of AD remains an unsolved issue, all the available models have limitations, which have to be cautiously considered when using them. There are no natural models of AD, so most of the research is performed using models simulating the disease phenotypes by active manipulation of the animals or, more recently, using transgenic animal models.[7] Therefore, these models might not accurately reproduce the anatomical distribution of the lesions in human brain, but biochemically they are very similar to the human condition.[17]

Numerous animal species have been used to model different aspects of AD, including fruit flies (*Drosophila melanogaster*), nematodes (*Caenorhabditis elegans*) and zebrafish.[17,31] However, rats and mice are the ones that are widely used.[17] Although the rat has been the animal of choice for drug

development and fundamental research for decades, it progressively faded away in favour of mice. This happened mainly due to increasing knowledge of advanced genetic techniques developed in the mouse, in addition to the discovery of gene mutations causative of familial forms of AD, which allowed the generation of a growing number of transgenic mouse models.[7,30] A validly complete list of transgenic mouse models relevant for AD is continuously updated on the *Alzheimer Research Forum* homepage (http://www.alzforum.org/res/com/tra/default.asp).

Nonetheless, currently, the rat has been making a comeback as an AD model. In fact, rats are a better animal model comparing to mice for numerous reasons. First, the rat is genetically, physiologically and morphologically closer to humans than mice.[32–34] Its larger body and brain size facilitates intrathecal (i.t.) administration of drugs, microdialysis, multiple sampling of CSF, *in vivo* electrophysiology, as well as neurosurgical/stereotaxic and neuroimaging procedures.[7,35] Second, compared to the rat, the mouse exhibits a simpler behavioural repertoire and much less flexibility in dealing with novel situations.[7] Consequently, the mouse poses a problem for neurobehavioral research as it is a species functioning at a low level of complexity, relative to the rat.[7] These behavioural differences may be accounted by the fact that rats, like humans and opposed to mice, have a post-natal brain development that would lead to a greater number of synapses and a more complex synaptic organization.[35] Consequently, rat models of AD permit a more sophisticated characterization at the behavioural level and thus enable a more accurate assessment of the impact of the pathology on cognitive outcomes.[7,35] They also allow a better assessment of the effects of potential therapeutics on cognition in longitudinal studies.[35] And finally, regarding transgenic models, with recent developments in technologies to manipulate the rat genome, some transgenic rat models (reviewed in Ref. [7,35]) offer a more accurate representation of the human disease compared to mice bearing the same transgene.[35]

Therefore, most scientists prefer to use rats over mice as an AD animal model. Additionally, since all the drug candidates that failed in clinical trials showed anti-AD activity in various transgenic animal models and because transgenic models of AD address only the familial form of the disease, which barely represents 1% of AD cases, currently, most of the research in AD involves non-transgenic rat models.[30]

As a matter of fact, there are a great variety of non-transgenic rat models of AD. For example, chemical and lesion-induced rat models have been used particularly to test the cholinergic hypothesis of AD.[35] This hypothesis states that central nervous system (CNS) cholinergic deficits in elderly adults and demented patients are the main factors responsible for their cognitive impairments and has led to the well-established, symptomatic anticholinesterase therapies (reviewed in Ref.[36]). Another example is the ferrous amyloid buthionine rat model, in which the AD phenotype was induced by administering a solution containing the human 42-amino acid form of Amyloid-β ($A\beta_{1-42}$), ferrous sulfate and buthionine sulfoximine via the intracerebroventricular (i.c.v) route over a period of 4 weeks, with the aim of creating an animal model of AD that corresponds to the sporadic form of the disease.[30]

Other rat models were developed to determine if the Aβ injection result in sufficient neural degeneration to cause measurable impairment of learning and memory.[35]For instance, i.c.v. injection of $A\beta_{1-42}$ is a well-established model of AD, since this model can recapitulate some key features of human AD including neuron loss and behaviour deficits in naïve rats.[35]

Besides the animal model i.c.v. injected with $A\beta_{1-42}$, there are other variants of it, but the general principle is the same: inject a form of amyloid peptide into the rat brain so the animal develops one or several of the AD pathological features documented in clinics.[30] For example, Aβ can be injected directly in the hippocampus.[37–41] A disadvantage of this model compared to i.c.v. $A\beta_{1-42}$-injected rats is the fact that the first one is a more aggressive and an acute model of AD, while the second tries to minimize possible acute neuronal damage caused by the direct injection of the peptide.

In summary, non-transgenic animal models provide an important alternative strategy for the discovery of new therapeutic substances with potential antidementia activity based on protection against the amyloid pathogenic pathway. As WJ Hadlow once wrote 'Even though finding an animal model embodying the total picture of senile brain disease with dementia is unlikely, efforts should be made to identify in some animal each of the several aspects of the aging process and dementia', it appears that i.c.v. $A\beta_{1-42}$-injected rats can recapitulate some key-aspects of AD and because of that are a valuable model.[42] Moreover, the rat is, in many aspects, closer to humans than mice. Because of its predictable and multi-faceted behavioural display, this species is of great value for accurate cognitive assessment.[35] However, it is important to keep in mind that the extrapolation of conclusions from rat to humans require a measure of caution and any compound in a pre-clinical level should be also tested in multiple animals models before being tested on humans.[35]

Since memory impairments is one of the most desirable phenotypes in animal models of AD, the next section will be dedicated to the region of the brain primarily associated with the memory – the hippocampus.

1.1.5. The Hippocampus

The hippocampus, named for its structural resemblance to a seahorse (Figure 4), is a paired structure located on the medial temporal lobe of the cerebral cortex in close contact with the lateral ventricles and is a crucial component of the limbic system.[43,44]

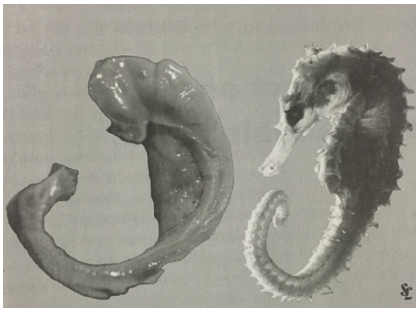


Figure 4. Human hippocampus dissected free (left) and compared to a specimen of *Hippocampus leria* (right). (*Figure adapted from Andersen et al., 2006*) [44]

Although there is a lack of consensus relating to terms describing the hippocampus and its adjacent cortex, the term hippocampus or hippocampal formation usually applies to the dentate gyrus (DG), the hippocampus proper - composed of *Cornu Ammonis* 1 (CA1), CA2 and CA3 fields -, the subiculum, presubiculum, parasubiculum and the entorhinal cortex (EC).[43–45]

The organization of the hippocampal circuitry has been traditionally characterized as a unidirectional, trisynaptic excitatory pathway (reviewed in Ref.[46]), in which glutamate is the main neurotransmitter.[43–46] Briefly, the EC provides the main source of input to the hippocampus through connections to the DG. Information flow then proceeds from DG to CA3 to CA1. In turn, CA1 projects to the subiculum and sends the hippocampal output back to the deep layers of EC. There is also a monosynaptic circuit, in which sparse axons from the EC directly project to the CA1 or CA3 subregion.[43–46]

It is important to mention that the basic layout of cells and fibber pathways is almost the same in all mammals.[44] However, in terms of comparison between the humans and the rat hippocampus (Figure 5), for instance, in volume is about 100 times larger in humans than in rats.[44] Nevertheless, since the basic hippocampal architecture is shared in mammalian species, the study of hippocampal functions in rat models allows a correlation to human hippocampal processes.[44]

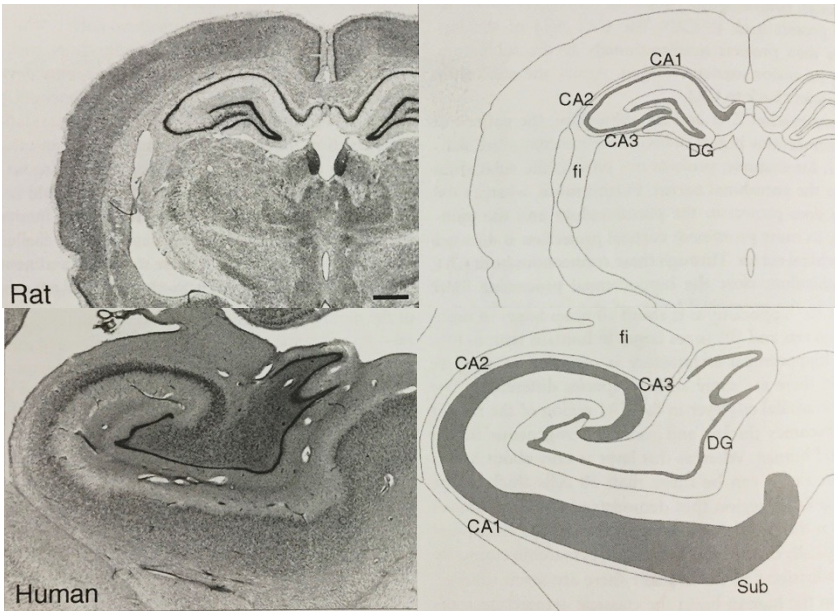


Figure 5. Nissl-stained sections and line drawings illustrating the general organization and similarities of the subdivisions of the hippocampal formation in the rat and human.

Note the differences in the relative position of the fimbria (fi) in the rat (located lateroventrally) and in the human (located mediodorsally).[44] Scale Bar = 1mm applied to all images. (*Figure adapted from Andersen et al., 2006*)[44]

In fact, the hippocampus has played a central role in neuroscience, since this structure is commonly regarded as being in the centre of a brain network supporting encoding, consolidation and retrieval of memory.[47] Regarding the study of human memory, the hippocampus has been implicated in episodic and semantic long-term memory, novelty detection, sleep-dependent memory consolidation, pattern discrimination, spatial navigation and the binding of temporally and spatially distributed representations.[47]

Besides these cognitive functions, the hippocampus is also involved in the regulation of emotion, fear, stress and anxiety.[47] Moreover, the hippocampus plays a crucial role in converting short-term memories to long-term memories by processing new memories and temporarily storing them prior to permanent storage in the cortex.[43] Lesion studies have suggested that the hippocampus is important for this temporary storage and the retrieval of contextual fear memory for up to 2-3 weeks after learning.[43]

Interestingly, the hippocampus is one of the brain areas most susceptible to external insults, being one of the most and primarily affected brain areas upon normal ageing and pathologies, such as AD.[47] Actually, the hippocampus is the first brain region to exhibit neurodegeneration in AD and determination of AD-related alterations in hippocampal structure and function is central to AD diagnosis.[44,45,47]

For instance, the use of volumetric Magnetic Resonance Imaging (MRI) measures of hippocampal atrophy as surrogate markers of disease in AD is validated by the demonstration of a strong correlation between MRI-determined hippocampal volumes and neuronal numbers in the hippocampus in AD.[44] Thus, a number of studies have demonstrated that AD is associated with significant hippocampal volume loss.[44] In addition, many studies suggest that in AD there is a dysregulated adult hippocampal neurogenesis.[43,45] Note that increased adult neurogenesis in the DG of the hippocampus has been shown to improve memory acquisition, memory formation and maintenance.[43] Therefore, a decline in neurogenesis may underlie the cognitive impairments associated with AD.[45]

Since the hippocampus has a pivotal role in different types of memory and memory loss is a prominent aspect of AD, it is reasonable to hypothesized that drugs firstly used to improve memory of AD patients should exert their effect in the hippocampus.[44] This is the case of cholinesterase inhibitors, as well as the other treatment options in AD, which will be discussed more in detail in the next section.

1.1.6. Treatment options in Alzheimer's Disease

The history of drug development for AD is not a successful story.

As the loss of cholinergic neurons in the frontal cortex and the hippocampus is a prominent histopathological feature of AD, initial drug developers focused on restoring central cholinergic transmission, which is essential to recognition memory.[48,49] Acetylcholinesterase inhibitors, which include donepezil, rivastigmine and galantamine, are indicated for the treatment of patients with mild to moderately severe AD.[48,50] This strategy is based on the hypothesis that inhibiting the enzyme that degrades acetylcholine (ACh) would restore physiological concentrations of Ach in the synaptic cleft and the functionality of cholinergic neurotransmission, resulting in therapeutic benefit.[30] However, this therapy is only symptomatic and does not halt disease progression.[48,50,51] It has thus limited long-term efficacy.

In 2002, a new drug called memantine received European marketing approval for the treatment of patients with moderate to severe AD.[52] This drug is a non-competitive N-methyl-D-aspartate (NMDA) antagonist, which reduces glutamatergic excitotoxicity, since cognitive decline in AD patients has also been linked to neuronal damage as a result of excitotoxicity caused by the persistent overactivation of NMDA receptor by the amino acid glutamate.[30,53] Although the toxicological profile of memantine is excellent, the beneficial effects of this drug are modest and the treatment is largely palliative.[30,53]

Since then, the AD pipeline has suffered numerous setbacks due to failed clinical trials. A recent example is the vaccine AN-1792, the amyloid peptide ligand/plaque formation inhibitor tramiprozate, the γ-secretase modulator tarenflurbil, the γ-secretase inhibitor LY540139 and the anti-histamine

latrepirdine.[30] In addition, more recently, several disease-modifying therapeutic approaches targeting amyloid peptides have been proposed.[54–56] A number of drug candidates (including bapineuzumab) not only directed to remove insoluble Aβ but also to prevent the formation of Aβ plaque have entered clinical development.[48,50,54–57] Similarly, soluble Aβ monomers and oligomers had also been targeted, using the humanized monoclonal antibody solanezumab.[58] Unfortunately, all these studies have failed at significantly improving AD symptoms.[50]

Presently, the exact causes for the failure of these compounds remains unclear. However, it still needs to be determined whether these treatment approaches are indeed ineffective or whether the therapies have just been administered too late in the disease process, when there is already a significant and presumably permanent neuronal loss.[57]

In summarize, AD is challenging to treat and virtually all of the available drugs have less-than-desirable therapeutic profiles.[59] It is a fact that more is known about AD and other dementias than a decade before, but newfound knowledge and new drugs currently being studied also pose new questions that pharmaceutical industry and the broader scientific community have to be able to intelligibly answer: What are the reasons for the past failures of investigational drugs? Which class/classes of molecules may be more suitable for delaying AD pathology? How can effective neuroprotection be achieved?[59] The answer to these questions is not going to be simple as it is becoming increasingly evident that AD is a multifactorial and heterogeneous disorder involving several different etiopathogenic mechanisms.[60]

To conclude, the AD drug-development pipeline is relatively small considering the magnitude of the problem and the rate of success of AD clinical trials is limited.[61] An urgent need exists to increase the number of novel molecules entering the pipeline and progressing effectively toward new therapy for AD patients.[61]

1.2. Potential of Peptides as Drugs

Currently, the pharmaceutical industry is putting peptides in a spotlight, meaning there has been a rapid expansion in the use of peptides as drug candidates over the last decade. They are already being used therapeutically in diverse areas such as neurology, endocrinology and hematology.[62]

In fact, peptides are involved in a variety of physiological and pathological processes and play very important roles in modulating various cell functions, including immunity, stress, growth, homeostasis and reproduction.[62–64] In terms of chemical complexity, peptides fill a niche between typical small molecule chemicals and the larger proteins.[65]

One of the principal reasons that makes peptides appealing for drug discovery and development is that they can bind with great specificity to their *in vivo* targets, resulting in remarkably high potencies of action and relatively few off-target side effects, since they do not accumulate in specific organs.[64,66] Additionally, this type of molecule do not cause serious immune responses, plus the cost of synthetizing peptides is dropping as production scale and efficiency is rising due to progresses in synthesis and purification strategies.[64,67]

However, there are some disadvantages of using peptides as drugs, namely: their poor oral availability because peptides can be readily degraded and pass poorly through the intestinal mucosa and also, for good or bad, most peptides are unable to cross the blood-brain barrier (BBB).[64,67–69] In addition, their activity can be impaired by pH value and salt concentrations existent in physiological conditions.[62]

Nevertheless, there are some optional administration routes such as injection delivery, nasal delivery, sublingual and pulmonary delivery.[64] Furthermore, there are also other strategies to develop peptide drugs that can be administered orally, such as: optimization of the pharmacokinetic properties of the peptides (e.g. enhancing lipophilicity and decreasing enzymatic susceptibility); conjugation with molecules recognized by membrane transport systems and the use of a delivery carry system based, for example, on hydrogels, microspheres and nanoparticles.[62,69] In Table 2 are summarized some of the pros and cons of peptides as drugs, compared to small molecule drugs.

Table 2. Advantages and disadvantages of peptides as drugs.

(*Table adapted from Craik et al., 2013)*[66]

Advantages	Disadvantages
High potency	Poor metabolic stability
High selectivity	Poor membrane permeability
Broad range of targets	Poor oral bioavailability
Potentially lower toxicity than small molecules	High production costs
Low accumulation in tissues	Rapid clearance
High chemical and biological diversity	Sometimes poor solubility

Due to the number of recent technological breakthroughs and advances, which facilitate the discovery and identification of a wealth of novel peptides with pharmaceutical potential, the proportion of peptides in pharma is anticipated to increase.[62,65,70] This proportion is estimated to grow faster (9.4% annual growth in 2012–2018) than the global industry (3–6% annual growth in 2012–2016).[65] Thus, not

only is the number of approved peptide drugs expected to increase, but also the diversity of their potential therapeutic applications.[65]

Presently, there are approximately 60-70 approved peptide drugs in the global market, with 100-200 more in clinical trials, 400-600 more in pre-clinical studies and maybe hundreds to thousands more on the laboratory bench.[67,70] For instance, the year 2012 has proven to be a landmark for the peptide pharmaceutical sector, with 5 peptides meeting market approval in Europe and 6 peptides approved in United States of America (USA).[71]

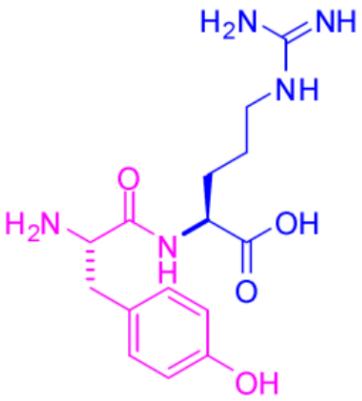
This was the highest number of approvals ever achieved for new biological entities in one year, which brings some optimism to the sector. This optimism is confirmed by the statistics, since the regulatory approval rate for peptides is around 20%, in contrast to 10% for small molecules.[67,71] Furthermore, starting from the year 1996 the number of patent applications per year has invariably overcome 10 000, a very high number, reflecting a very dynamic development of the peptide market.[65]

In conclusion, with the growth of approved peptide-based drugs and the progress in peptide-associated technologies, there is a new avenue for novel peptides with biomedical applications, capable to fight common diseases. Therefore, it is reasonable to anticipate a bright future for therapeutic (as well as diagnostic) peptides, since the pharmaceutical peptide pipeline is strong and stable, with increasingly new candidates approaching drug approval status, and the commercial value of the therapeutic peptide market is well established.

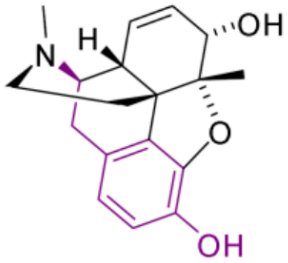
1.3. Kyotorphin

Kyotorphin (KTP) is an endogenous peptide, with only two amino acid residues in its structure: L-tyrosyl-L-arginine, which has been proposed in the literature as a promising drug, namely due to its potent analgesic action.[72–74]

This dipeptide was first isolated from bovine brain in 1979 by Hiroshi Takagi and his group at the University of Kyoto in Japan.[72,73] Thus, KTP owes its name because of the city when it was discovered and due the fact that it has properties similar to morphine.[72] The resemblances with opioid molecules go much further regarding the activity: both display a phenolic ring, considered a crucial structure for the interaction of morphine with receptors (Figure 6).[75]



Kyotorphin



Morphine

Figure 6. Chemical structures of Kyotorphin (KTP) and Morphine.

The structural similarities are evident: the phenolic hydroxyl group in close proximity to a positive charge (coloured in pink and purple for KTP and morphine, respectively).[75] *(Figure adapted from Santos et al., 2014)*[75]

Later, in 1980 and 1981, Takagi and his group found that KTP was also present in the brain of rats, mice, guinea pigs, rabbits and humans, with the highest levels being detected in the lower brain stem and the spinal cord, particularly in the dorsal half, correlating with the areas most sensitive to morphine or electrical stimulation-induced analgesia.[76–79] However, when the results are expressed as a percentage of the total brain KTP found in different sections, the cortex seems to contain around 50%, an area where the contents of opiate receptors and enkephalins is low, which also suggests that KTP non-opioid actions are important in its neurochemical action.[78] Moreover, this dipeptide can also be detected in the cerebrospinal fluid (CSF) in humans and a study using human CSF samples revealed that in patients with persistent pain, KTP content is lower.[80]

There are two pathways for the synthesis of KTP: it can be formed either by biosynthesis from the amino acids tyrosine and arginine, in the nerve terminals, by an ATP-dependent synthetase, or by processing precursor proteins , via membrane-bound, leupeptin-sensitive 'KTP converting enzymes'.[81–83]

KTP degradation occurs rapidly through membrane-bound aminopeptidases, which, together with the fact that it was found in areas of the brain related with pain inhibition and it could be synthesized in the nerve terminals, led to its classification as a neuropeptide. Further studies have solidified the inclusion of KTP in the neuropeptide family, when the L-tyrosyl-L-arginine sequence was found to be a common motif in other analgesic peptides and when it was found that KTP was released in response to depolarizing stimuli.[72,81–85]

Regarding the analgesic action of KTP, when it was first isolated, its analgesic effect was measured and an intracisternal administration revealed that KTP was 4.2 times more potent than methionine-enkephalin (met-enk), an endogenous opioid pentapeptide that acts as a neurotransmitter regulating pain transmission in the CNS.[72,73,86]

Concerning KTP receptor, despite the similarity between KTP and opioid molecules there is evidence proposing that KTP does not bind to opioid receptors, which lead to the assumption that its effect is mediated by enkephalins.[73] Indeed, the ability of KTP to produce potent naloxone-reversible and long-lasting analgesia suggests that KTP activity is indirectly mediated by opioid receptors.[87,88] Nevertheless, some authors argue that KTP binds to a specific receptor (KTPr), triggering a cascade of events that leads to strong analgesia in the brain.[82,89] This receptor, which was never isolated, seems to be functionally coupled to protein G and is antagonized by the dipeptide L-Leucine-L-Arginine, that is a KTP antagonist.[90] Curiously, conformational studies on KTP revealed that there might be strong resemblances between the putative KTPr and the structural family of opioid receptors.[91]

Disappointedly, the exact mechanism of action of KTP remains undetermined. On the one hand, some authors support that KTP binding to its specific receptor induces met-enk release followed by activation of G-protein and Phospholipase C.[82] On the other hand, there are other authors who defend that KTP experiences a fast degradation, originating L-Arginine, a substrate of nitric oxide (NO) synthase.[92,93] This would induce the formation of NO, leading to the release of met-enk, which then acts as the first mechanism described above.[92,93] Regardless of the mechanism, it might involve the release of met-enk. Indeed, a specific radioimmunoassay performed in slices of isolated guinea pig striatum and spinal cord showed that KTP induces met-enk release.[94] In addition to its higher analgesic potential, KTP exhibits a relatively long lasting effect, which has been attributed to a stabilizing effect on the released met-enk by a weak inhibition of the enkephalin degrading enzymes.[72,73]

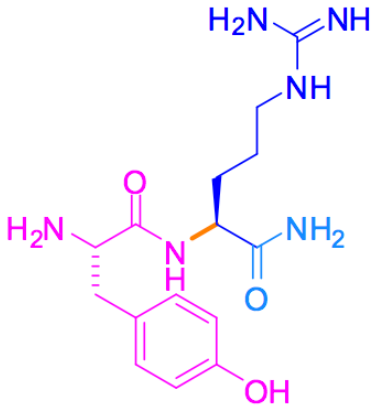
Besides its anti-nociceptive activity, KTP was also reported to have other activities, such as anti-hibernating regulation, thermoregulatory activity, inhibition of cells proliferation and even an epilepsy seizure protection effect.[95–99] Other authors described that KTP attenuates vasopressin release but, when in excess, mimics the stress response with an increase of oxytocin and activation of sympathetic nervous system, with a consequent increase of blood pressure.[100] Moreover, it has been suggested that KTP has neuromodulating and neuroprotective properties.[79,101–105] This suggestion has been based on the observation that a single intranasal 0.05 mg/kg dose of KTP given 30 min after the beginning of resuscitation from arterial occlusion in rats, increased survival, accelerated restoration of neurological status, normalized emotional reactivity, orientation and search behaviour and actually prevented death of neurons in the hippocampus and cerebellum.[103] In addition to this data, a complex of behavioural tests revealed diminished anxiety, increased locomotor and exploratory activities and changes in different learning tests in resuscitated animals treated with an antioxidant in combination with KTP. [104]

Moreover, it has been pointed out that KTP can block Ca^{2+} influx currents, as demonstrated in myocardium from cold-blooded (frogs) and warm-blooded (rats, ground squirrels) species and by alterations in the behaviour of goldfish.[101,106,107] In fact, data from a number of experiments in goldfish, where KTP was applied onto the Mauthner neurons of the medulla oblongata (a double-cell motor centre responsible for standardized forms of behaviour in fish and amphibian), revealed that KTP can block voltage-dependent Ca^{2+} channels on the postsynaptic membrane.[102] In addition, KTP can function both as an electron acceptor and as an electron donor which, according to previous data, can affect the ability of this neuropeptide to block that type of channels.[108]

With all of these different activities of KTP it is clear that this molecule has a strong biological potential and has appealing characteristics for pharmaceutical pipelines.

Therefore, researchers started to study the pharmacological potential of KTP following systemic administration (intraperitoneal injection (i.p.), intravenous or oral). Undesirably, KTP only showed a brief activity and at a high dose of 200 mg/kg when systemically administered to rodent animals.[109] The clear difference in activity when different administration routes were used (systemic *vs* direct) was thought to be due to the most usual reason that prevent drugs to reach the CNS: limited capacity to cross the BBB and susceptibility to various clearance mechanisms, such as lytic enzymes. Additionally, the diffusion of KTP in the CNS is still questionable. Peptide transporter 2 (PEPT2) is a low capacity peptide transporter that perhaps acts clearing the CSF of di- and tri-peptides, some of which might be neurotransmitters. These transporters can potentially act on KTP but this hypothesis has not been validated.[74]

Thus, KTP can only be converted into a valuable and marketable drug if modified to have an enhanced BBB-crossing ability, with its structure and chemistry preserved so that the end outcome can continue effective and nontoxic. Subsequently, a strategy to overcome this limitation is chemical derivatization. Since lipophilicity is one of the key factors that influences BBB-crossing, a derivative with improved lipophilicity was synthesized based on amidation of the C terminal, which consists on a substitution of the carboxyl group for an amide, resulting in Amidated Kyotorphin (KTP-NH_2) (Figure 7). Thus, this modification increased cationicity and lipophilicity and, consequently, enhanced peptide-anionic membrane interaction. Following systemic administration, KTP-NH_2 had a strong analgesic effect, but whit short duration, suggesting that it was able to cross the BBB.[74] Although KTP-NH_2 analgesia is inhibited by naloxone administration (either i.p. or i.t.), direct binding of KTP-NH_2 to opioid receptors is virtually absent, similarly to the original, nonamidated, dipeptide.[110]



KTP-NH_2

Figure 7. Chemical structure of Amidated Kyotorphin (KTP-NH_2).

(*Figure adapted from Santos et al., 2014*)[75]

1.4. Kyotorphin in Alzheimer's Disease

As mentioned previously, in addiction to analgesic activity, it has been pointed out that KTP has neuromodulating and neuroprotective properties in hippocampus and cerebellum.[79,101–105] Therefore, this molecule can interfere with cellular pathways that are common to analgesia and AD progression. Actually, a dual analgesic and neuroprotective action in a single drug would be of upmost importance, since recent clinical studies have demonstrated a correlation between AD, pain and KTP in humans.[79,111]

It is difficult to evaluate pain in AD patients because of the motor and cognitive deterioration that occur in the course of the disease, which is accompanied by a reduction in the ability to communicate.[79,112] By failing to receive adequate pain treatment, structural and irreversible changes may occur in CNS structures involved in the transmission/modulation of nociceptive information, which accounts to chronic pain installation.[79,112]

In humans, a study demonstrated that KTP levels are decreased in the CSF samples of AD patients.[79] This can be explained by a disease-specific cortical thinning and hippocampal volume loss, with an acceleration phase during the early stages of the AD, meaning less cortical mass, which possibly results in less KTP production capability and KTP dropping levels in the CSF of these patients.[79,80,113] More important, it was demonstrated that CSF samples of AD patients show high phosphorylated tau protein (p-tau) levels compared to normal subjects.[44] It is also known that there is an inverse correlation between p-tau and KTP in CSF in AD patients.[79] p-Tau in CSF acts as a marker of neurodegeneration, being released from senescent neurons.[115] KTP is also produced in neuronal cells, so its levels naturally

falling as neurons die.[79] As more neuronal cells are destroyed in the course of the disease, p-tau is released and consequently KTP production is impaired.[79]

So it seems that when neural death occurs there is a loss of KTP production that can worsen neurodegeneration (with concomitant alteration of pain threshold) in AD patients. Then, it is natural that one asks: if we manage to increase the levels of KTP would it result in neuroprotection?

If so, hope arises from the possible use of KTP-related drugs in the context of neurodegeneration in AD.

2. Aim of the work

As stated in 'Introduction', AD is the most devastating neurodegenerative disorder in the elderly and the treatment options are severely limited. Therefore, there is an urgent need to find new molecules with a true therapeutic value, especially those that could act in early stages of the disease and halt or slow its progression.

KTP, apart from being an endogenous analgesic peptide, appears to satisfy these requirements, since it has been pointed out as a putative neuroprotective molecule.[79,102–104] Moreover, recent work headed by Professor Miguel Castanho, showed that two derivatives of KTP (including KTP-NH_2) prevented memory impairment and neuronal damage in the hippocampal CA1 subfield caused by two-vessel occlusion (2VO) in rats.[105] The 2VO animal model causes a progressive neurodegeneration in the hippocampus, learning deficits and memory loss as it occurs in AD, but it is closer to a model of cerebral hypoperfusion dementia than of AD. Nevertheless, the study demonstrates the neuroprotector potential of KTP-NH_2 and prompted the interest in further evaluating its therapeutic potential in an AD animal model.

Therefore, the aim of this work was to investigate the neuroprotective effect of prolonged treatment with KTP–NH_2 in an animal model of AD, namely, Wistar adult rats i.c.v. injected with $A\beta_{1-42}$.

To answer this question, the following steps were accomplished:

I. Evaluate spontaneous locomotor activity, exploratory behaviour, spatial working memory and episodic long-term memory of $A\beta_{1-42}$ injected rats, as compared with sham-operated controls.

II. Assess $A\beta_{1-42}$-induced neuronal damage by immunohistochemistry and gliosis by immunohistochemistry plus western blotting, at the rat hippocampus.

III. Test whether KTP–NH_2 could revert any $A\beta_{1-42}$-induced behavioural impairments and/or neuronal damage.

3. Materials and Methods

3.1. Ethics Statement

All described procedures were conducted in compliance with the European Community legislation (Directive 2010/63/EU) and approved by the Ethical Committee of the Faculty of Medicine, University of Lisbon and by the Animal Ethics Committee of Instituto de Medicina Molecular (iMM). Furthermore, the project was submitted to license by *Direção Geral de Alimentação e Veterinária* (DGAV), the Portuguese competent authority for animal protection.

3.2. Compounds

The 42-amino acid form of Amyloid-β ($A\beta_{1-42}$) was purchased from Bachem (Bubendorf, Switzerland, ref. H-1368). This form was chosen since it is more prone do aggregation and assumed to be more neurotoxic than $A\beta_{1-40}$ and other Aβ variants.[12]

The peptide KTP–NH_2 was synthesized as described elsewhere.[116]

Anaesthetic, analgesic and anti-septic drugs used during- and post-operative surgical procedures are referred in Table 3 and for terminal transcardial perfusion in Table 4.

Table 3. Compounds used in surgical procedures.

ACTIVE SUBSTANCE	ROUTE/SITE	BRAND NAME	COMPANY
Bupivacaine Hydrochloride 0.25%	subcutaneous (SC)	Bupivacaine Hydrochloride 0.25%	B. Braun, Berlin, Germany
Buprenorphine 0.3 mg/mL	SC	Bupaq®	Richter Pharma AG, Wels, Austria
Carbomer 974P 0.3%	local	Lacryvisc®	Alcon, Hünenberg, Switzerland
Dexpanthenol 50 mg/g and Chlorhexidini Dihydrochloridum 5 mg/g	local	Bepanthen® Plus 50 mg/g + 5 mg/g Cream	Bayer, Leverkusen, Germany
Isoflurane	inhalation	Isoflo®	Esteve, Barcelona, Spain
Lidocaine 2.5% and Prilocane 2.5%	local	EMLA® cream	Astrazeneca, London, UK
Povidone-Iodine	local	Betadine®	Meda Pharmaceuticals, New Jersey, USA

Table 4. Compounds used in transcardial perfusion.

ACTIVE SUBSTANCE	ROUTE/SITE	NAME OF THE PRODUCT	COMPANY
Ketamine 100 mg/mL	intraperitoneal (i.p.)	Imalgene® 1000	Merial, Lyon, France
Xylazine 2%	i.p.	Rompun® 2%	Bayer

3.3. Animals and Housing

Male Wistar rats were purchased from Charles River Laboratories (Lyon, France). Animals had a minimum period of 5 days for acclimatization, following arrival at the iMM's rodent facility, before they were used in experimental procedures. They were housed together in groups of 2 per cage with unrestricted access to water and food, and under controlled temperature and light conditions (20 ± 2°C; lights on between 7 a.m. and 9 p.m.).

Surgical procedures were performed when animals reached 230-320 g (8-10 weeks).

All procedures (surgery and behavioural experiments) were conducted during the light period of the 14/10 h light/dark cycle.

3.4. Surgery: intracerebroventricular injection of $A\beta_{1-42}$

The animal model of AD was created using the $A\beta_{1-42}$ i.c.v. injection method, as previously described.[117,118]

For the surgical procedures, animals were anaesthetized with isoflurane (2-3% in O_2) using a RC^2 Rodent Anaesthesia System (VetEquip Inc., California, USA). Anaesthesia was first induced in a plexiglas chamber and thereafter maintained via facial mask. In addition to this, and as described below, local anaesthetics were subcutaneously applied.

During all the procedures, the animals were breathing normally and body temperature was kept constant with the help of a 37°C heating pad.

Firstly, the heads of the rats were shaved. Afterwards, EMLA® cream was applied in the ear canal of the animals. This cream is a local anaesthetic, which works by blocking nerves from transmitting painful impulses to the brain.[119] Therefore, it was used to prevent rats to feel pain during the use of the ear bars in the stereotaxic apparatus. Secondly, Buprenorphine (0.05 mg/Kg, SC), which is a narcotic analgesic with general action, since it works in the brain and nervous system to decrease pain, was

administered pre-emptively, on the back of the animal, so that it would be already in action when the animals recover from the anaesthesia.[120] Bupivacaine Hydrochloride 0.25% (8 mg/kg, SC) was also administered at the site of the incision for local anaesthesia of subcutaneous tissues and skull. After that, Lacryvisc® was applied on the eyes of the animals, which is an ophthalmic gel to protect eyes from dehydration.

Next, the animals were mounted on a stereotaxic apparatus (Stoelting®, Wisconsin, USA). The skin over the skulls of the rats was first disinfected with Betadine® and then it was made an incision along the midline with a scalpel. Subsequently, a drill (FOREDOM® ELECTRIC CO., Connecticut, USA) was used to make a small hole in the skull on the side of the corresponding position to allow i.c.v. injection of $A\beta_{1-42}$ in the right lateral ventricle (anteroposterior: -0.84 mm from Bregma, medial/lateral: 1.5 mm and dorsal/ventral: -3.5 mm) (Figure 8). Coordinates were tested in a preliminary experiment by injecting Trypan Blue Solution 0.4% (Sigma-Aldrich®, Missouri, USA) into the rat right lateral ventricle.

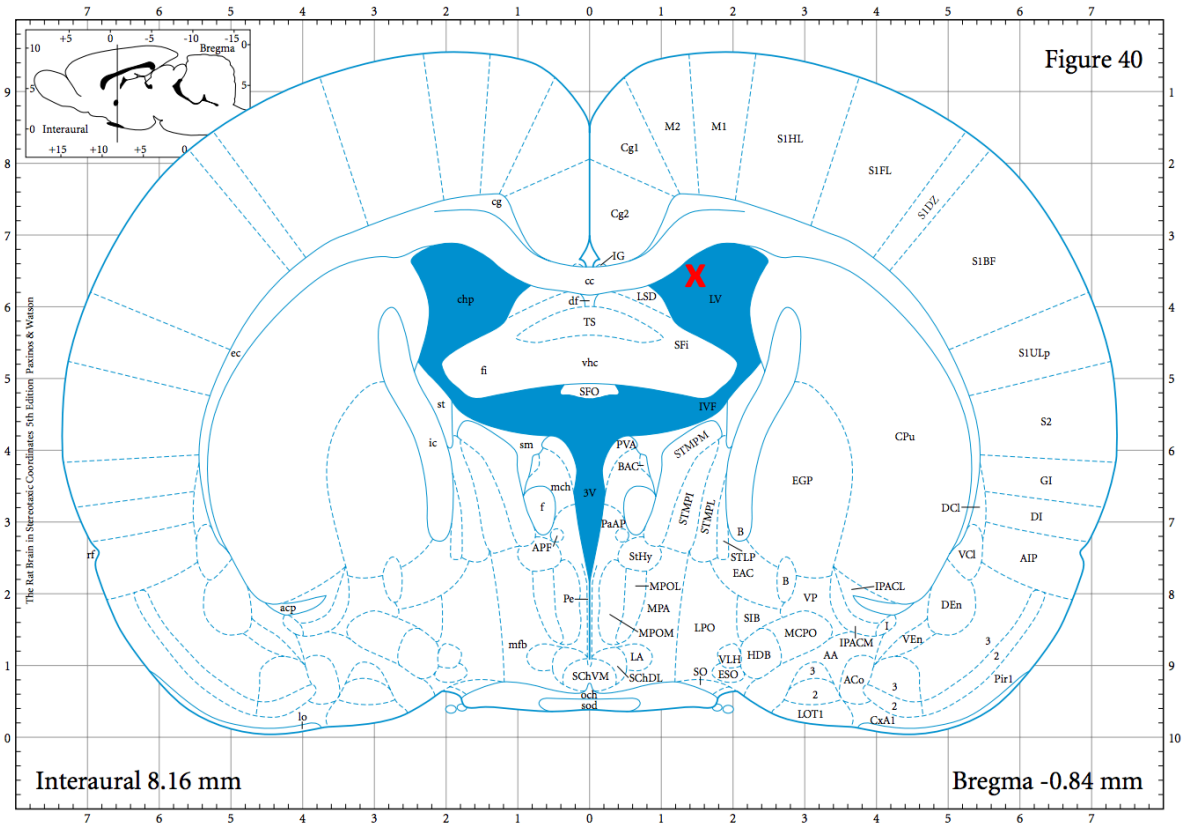


Figure 8. Representation with an "x" in red of the stereotaxic coordinates used to perform the intracerebroventricular injection of the 42-amino acid form of Amyloid-β ($A\beta_{1-42}$).

LV, lateral ventricle. (*Figure adapted from Paxinos et al., 2005*)[121]

For the injection, $A\beta_{1-42}$ was dispersed in water at a concentration of 2.25 mg/mL and 5 µL was administered i.c.v.. Sham-rats were injected with the same volume of water. I.c.v. injection was made

with a 33-gauge Hamilton microsyringe (Hamilton Company, Nevada, USA) using a microinjection pump (World Precision Instruments, Inc., Florida, USA) with a rate of 500 nL/min. Thus, the injection lasted 10 min and the needle with the syringe was left in place for 2 min after the injection to ensure complete infusion of $A\beta_{1-42}$. When the injection was finished, the animal was removed from the stereotaxic frame to another heating pad and the skin overlying the skull was closed with 4/0 Silk sutures (Silkam®, B Braun). Bepanthene Plus® was applied in the suture immediately after finishing the surgical procedure.

After that, the rats were housed in pairs and had free access to food and water. Animals were closely monitored during the post-operative recovery for general appearance, activity, feeding behaviour and body weight. Body weight was measured before the surgery and controlled whenever the animals were handled for i.p. injection. All animals survived to this surgical procedure, with no signs of distress or discomfort.

3.5. KTP-NH_2 administration schedule

KTP–NH_2 was dissolved in physiological saline solution (0.9 % NaCl) at a concentration of 100 mM prior to i.p. injection. The volume injected in each animal was kept constant at 1 mL/kg body weight, the dose injected being 32.3 mg / kg, ≅ 100 µmol / kg. This dose corresponds to a schedule of ≅10mg per day per animal. The selected dose of the KTP derivative was based on previous results concerning their analgesic action profile.[116,122]

This KTP derivative was administrated as a chronic treatment regimen until the sacrifice of animals (single i.p. dose/day), since the purpose was to evaluate the prolonged effect of this molecule and not its acute effect. This drug administration schedule was chosen taking into account conditions that could better mimic therapeutic interventions in chronic diseases. It is also important to mention that the treatment started at the second day after $A\beta_{1-42}$ administration, because I did not want to interfere in the acute lesion caused by $A\beta_{1-42}$ i.c.v. injection.

Experimental groups (Figure 9) were as follows: (i) control group: sham-operated (CTL rats); (ii) test group: i.c.v. injection of $A\beta_{1-42}$ ($A\beta_{1-42}$ rats). Each of these groups were divided into subgroups, with 6-7 animals each, as follows: (a) subgroup 1 where test drug (KTP-NH_2) was administered i.p.; (b) subgroup 2: where no drug was administered but the animals received daily i.p. injections of the vehicle in the same volume used to inject the test drug. Therefore, number of animals used: 4 subgroups of animals (2 control and 2 test), with 6-7 animals per subgroup, total: 26 rats (Figure 9).

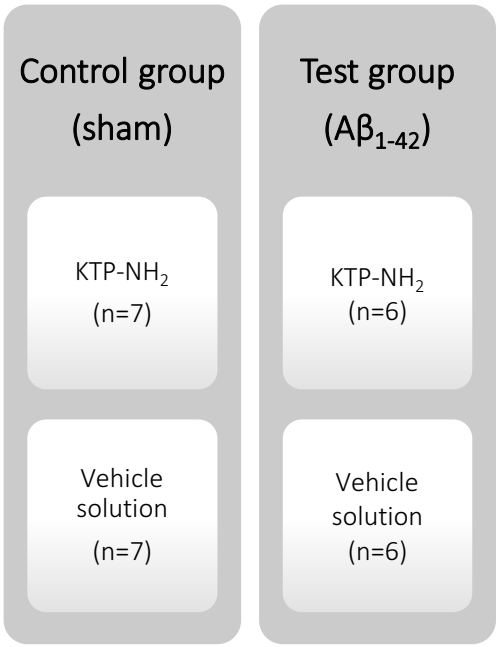


Figure 9. Experimental groups used.

$Aβ_{1-42}$, the 42-amino acid form of Amyloid-β; KTP-NH_2, Amidated Kyotorphin; n, number of animals per group.

In addition, and for the validation of the animal model of AD, it was used 14 rats (7 sham-operated and 7 i.c.v. injected $Aβ_{1-42}$ rats). These rats were used before those referred in Figure 9.

A timeline of experimental events is depicted in Figure 10.

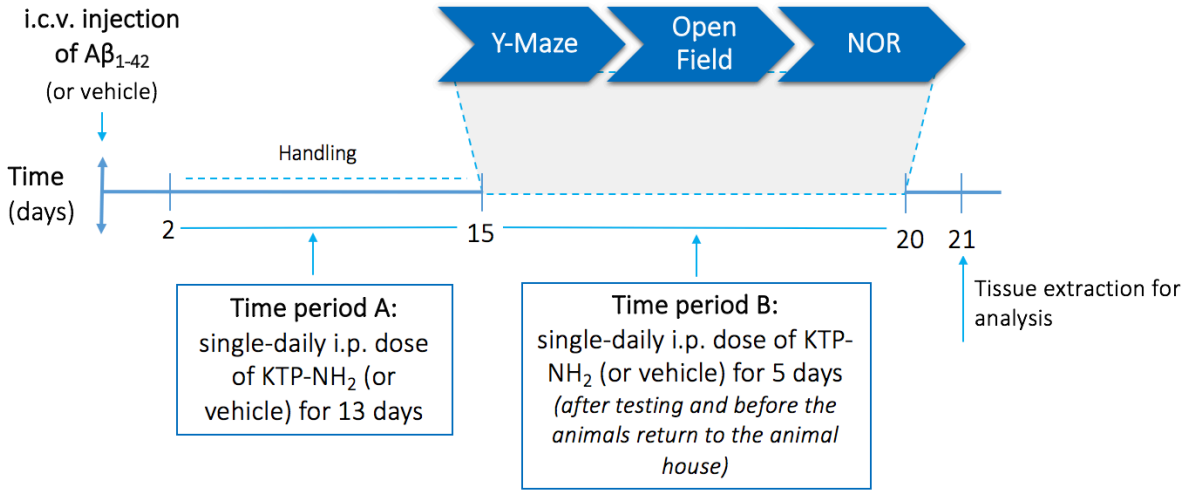


Figure 10. Timeline of the experiments.

Animals were i.c.v injected with $A\beta_{1-42}$ (2.25 mg/mL) or water (vehicle) at day zero and i.p. injected with KTP–NH_2 (32.3 mg/kg ≈ 100 µmol/kg) or saline solution (vehicle) as indicated. During time period A, the animals were also handled for a few minutes before the i.p. injection so that they became used to the experimenter previously to behavioural testing. During time period B, the animals were tested in several behaviour paradigms, being i.p. injected with the KTP derivative or with the vehicle (according to the group they belong to) after testing and before return to the animal house. After the last behavioural test, half of the animals in each group were perfused transcardially for tissue fixation for immunohistochemistry assessment of the hippocampus; the other half was used for fresh hippocampi dissection for western blot analysis.

i.c.v., intracerebroventricular; $A\beta_{1-42}$, the 42-amino acid form of Amyloid-β; i.p., intraperitoneal; KTP-NH_2, Amidated Kyotorphin; NOR, Novel Object Recognition test.

3.6. Behavioural Tests Procedures

Since it has been reported[117,123] that at day fifteen after surgery the memory deficits induced by $A\beta_{1-42}$ become evident, the behavioural tests were performed at that time point (Figure 10).

Rats were tested in standard behavioural paradigms, such as Open Field (OF) test, to examine spontaneous locomotor activity and exploratory behaviour; Y-Maze test, to evaluate spatial working memory and Novel Object Recognition (NOR) test, to assess episodic long-term memory.

The Y-Maze was performed before the NOR test because memory should be evaluated first in less complex paradigms and then in tests that are cognitively more stimulating.

All behavioural studies were carried out between 9 a.m. and 6 p.m. in animals accustomed to the testing room and to researchers performing the trials (handling period).

At the day of experiments, animals were brought into the testing room for at least 1 h prior to the start of the behavioural session. All behaviour apparatus used were cleaned with 70% ethanol between animals, even if it was not dirty prior to the initial trial, so that any residual smell of the disinfectant was experienced equally by every animal.

After placing the animal in the behavioural apparatus, the experimenter immediately left the room, to avoid serving as a cue for the animal or to introduce an unintentional bias into the study.

3.6.1. Y-Maze

Y-Maze Spontaneous Alternation is a behavioural test taking advantage of the willingness of rodents to explore new environments, since rodents typically prefer to investigate a new arm of the maze rather than returning to one that was previously visited.[124] The Spontaneous Alternation Behaviour measured in this test reflects the operation of spatial working memory, i.e., the caching of behaviourally relevant spatial cues on a timescale of seconds.[125] Consequently, many parts of the brain - including the hippocampus - are involved in this task.[124–126] It is, therefore, not surprising that, in recent years, this test has been enthusiastically embraced by behavioural pharmacologists and others as a quick and relatively simple test of memory.[126]

The testing protocol for Y-Maze test has been described in detail previously.[117,127] The maze used is composed of 3 arms (each with 30 cm long, 20 cm height and 10 cm wide) converging to an equal angle and is made of wood, with the interior painted in red (Figure 11). Visual cues were placed on the walls of the maze, for rats to remember which arms have already been visited. Briefly, each rat was placed at the end of one arm and allowed to move freely through the maze during 8 min, without prior habituation.

The series of arm entries were recorded visually. An entry occurred when all four limbs were within the arm and an alternation was defined as entries in all three arms on consecutive occasions. The number of maximum possible alternations for each animal was therefore the total number of arm entries minus two. The percentage of spontaneous alternation was calculated as (actual alternations/maximum alternations) x 100. For instance, if the arms were called A, B, C and the rat performed ABCACBACCAB, the number of arm entries would be 11, and the successive alternations: ABC, BCA, ACB, CBA, BAC, CAB. Thus, the percent of alternation would be [6/(11 - 2)] x 100 = 66.7%. In addition, for each animal, the total number of arm entries was used as a measure of locomotor activity.

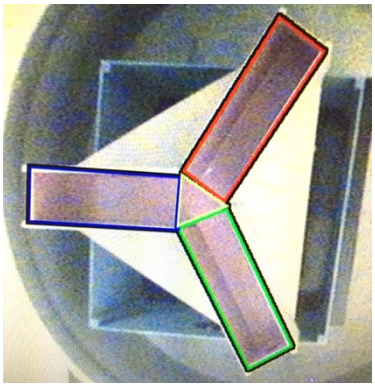


Figure 11. Photograph of the maze used in this work.

Each arm of the maze was virtually delimited with a colour (green, red or blue) to facilitate the recording of spontaneous alterations.

3.6.2. Open Field Test

The OF test is widely used, since it provides a unique opportunity to systematically assess general locomotor activity and novel environment exploration. Moreover, this test provides an initial screen for anxiety-related behaviour in rodents.[128,129] Regarding anxiety behaviour, it has been suggested that it is triggered in rats due to two main factors: (i) social isolation resulting from the physical separation from cage mates when performing the test and (ii) agoraphobia, since the arena is very large relative to the usual environment. Thus, it is easy to understand that these two factors trigger anxiety behaviour in gregarious species and/or in species that show fear of open spaces into which they are forced. This is precisely the case of rats, since wild rats live in social groups and in small tunnels.[128,129]

Normally, a 5 min test session is sufficient to capture the critical components of general exploratory locomotion and it is known that rats typically spend an appreciably greater amount of time exploring the periphery of the arena, usually in contact with the walls (thigmotaxis), than in the unprotected centre area.[128–130]

The open field apparatus consisted of an empty square box (67 x 67 x 51 cm height). For exploratory behaviour analysis, the arena was "virtually" divided in three concentric squares: borders (near the walls), periphery and centre (Figure 12).

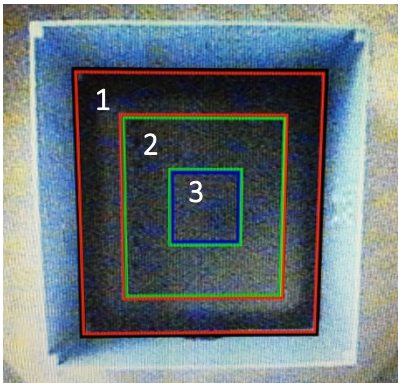


Figure 12. Photograph of the Open Field arena used in this work.

The different concentric zones of the open field arena were "virtually" divided.

1 – border zone (67x67 cm); 2 – peripheral zone (43x43 cm); 3 – centre zone (19x19 cm).

The OF test was performed in a quiet room with dimmed light, without prior habituation.

The testing protocol has been described in detail previously.[122,130] Briefly, rats were placed individually in the centre of the arena box and allowed to explore the apparatus for 5 min. Their behaviour was video-recorded during the testing period. Animal tracking along the different areas on the open field arena was analysed using a specific software (Smart version 2.5; Panlab, Barcelona, Spain).

Results are shown as average velocity (cm/s), time spent resting (s), total distance travelled (cm) and % of time spent in the periphery of the arena. Average velocity is the mean velocity with the resting time excluded. Each animal was considered to be resting if the mean velocity was < 3 cm/s. All animals were tested only once.

3.6.3. Novel Object Recognition

The NOR test has raised major interest on memory studies, since it is very useful to evaluate different sorts of memory through manipulation of the retention interval (RI). The RI corresponds to the amount of time animals must retain the information of the sample objects presented during the familiarization phase, before to the test phase. [131] At the test phase one of the familiar objects is replaced by a novel one.[131] So, this test can evaluate short-term memory (2 min RI), intermediate-term memory (4 h RI) and long-term memory (24 h RI).[132] In the present work, the NOR test was designed to assess episodic long-term memory, which is the predominant cognitive deficit in AD, thus a RI interval of 24 h was used. Note that this inability to acquire, encode and retrieve memories is characteristic of earliest stages of the disease and progresses with disease severity.

The testing protocol thus consists of three phases: habituation, familiarization and the test phase. In the habituation phase (3 consecutive days), each animal was allowed to explore freely the OF arena for 15 min in the absence of objects (the same arena used in the OF test). Animal behaviour in the initial 5 min of the first day of habituation was quantified as a measure of locomotor activity (OF test). During the familiarization phase, that occurred in the fourth day, the animal was presented with the two to-be-familiarized objects (commonly referred to as 'sample objects') for 5 min. Following sample-objects exposure, the animal returned to the home cage for 24 h. During the test phase, the animal returned to the arena and was presented with two objects: one previously experienced ('sample object') and a novel object, for 5 min.

In both familiarization and test phases, to prevent coercion to explore the objects, the animals were released in the middle of the opposite wall and with its back to the objects. The objects used in this test were a bottle of sparkling water and a bottle of beer, both without label, as illustrated in Figure 13. To choose the objects, the following rules were taken in consideration: the objects should not be totally dissimilar form each other, but must be different enough so that the animal can discriminate between them, the objects should not be easily gnawed by animals, should be easily cleaned and should be heavy enough that animals cannot move it, as well as height enough to unable animals climbing or resting on it during the trial. [131,133]

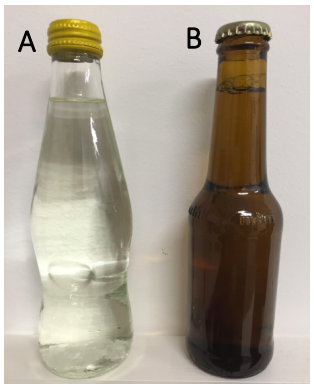


Figure 13. Objects used in Novel Object Recognition test.

(A) bottle of sparkling water; (B) bottle of beer.

In the test phase, the novel object was placed in 50% trials in the right side and 50% in the left side of the arena for eliminate possible confound variable due to any preference for a specific side of the arena. Moreover, in this phase, the location of objects did not change, but one of them was replaced by a novel one. Note that in half of the trials the novel object was the familiar object used in the other half. These modifications were made to reduce any object preference effect. To allow these alterations and to facilitate the objects cleaning between trials, they were fixed to the floor of the arena only with Velcro tape.

Therefore, this object-recognition procedure takes advantage of the tendency to approach and explore novelty, does not require exposure to aversive stimuli, does not required food or water restriction and has been replicated in many laboratories using a variety of apparatus designs and objects.[131]

There are two indexes that can be calculated in order to evaluate both preference and recognition of novel object. The Object Preference Index is a ratio of the amount of time spent exploring any one of the two objects in the training phase or the novel one in test phase over the total time spent exploring both objects, i.e., A or B/(A + B), where '1' represents the total time exploring both objects in each phase. On the other hand, we can calculate the Object Recognition Index, which is similar to Object Preference Index, but just takes into account the test phase and focus on the novel object, i.e., it is calculated by the ratio between the time spent exploring the novel object over to the total time spent exploring both objects and it is the main index of retention.[131]

Exploration was scored when the rat touched an object with its forepaws or snout, bit, licked, or sniffed the object from a distance of no more than 1.5 cm, while running around the object or climbing on it was not recorded as exploration.

3.7. Immunohistochemistry & Fluorescence Microscopy

3.7.1. General considerations on the technique

In 1941, Coons et al.[134] described an immunofluorescence technique for detecting cellular antigens in tissue sections. This publication marked the beginning of immunohistochemistry (IHC). Since then, IHC has become a valuable tool in diverse areas, such as neuroscience, allowing, for instance, structural studies of biologically relevant neuronal circuits.[135]

The basis of IHC is very simple and bridges three scientific disciplines: immunology, histology and chemistry.[136] Moreover, ICH is not a merely descriptive method. In fact, this method allows to obtain images of processes that reflect what occurs *in vivo* at a specific time point and therefore constitute one of the pillars of biomedical research.[137]

The fundamental concept behind IHC is the identification of antigens (Ag) located in a specific protein by using specific antibodies (Abs).[136,138,139]

Abs are made by immunizing animals (mouse, rabbit, goat, donkey, etc.) with purified Ag and they can be defined as monoclonal or polyclonal Abs (Figure 14).[136,140] Polyclonal Abs are produced in numerous animal species, mostly rabbit, horse, goat and chicken and have higher affinity and extensive reactivity, but lower specificity when compared with monoclonal Abs.[136,140]

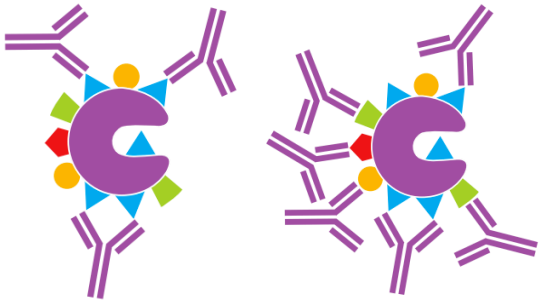


Figure 14. Schematic diagram of monoclonal (left) and polyclonal (right) antibodies.

Note that a monoclonal antibody (Ab) reacts with a specific epitope on an antigen, whereas a polyclonal Ab can bind to various epitopes.[140] *(Figure adapted from Key, 2009)*[140]

The Ag-Ab reaction cannot be seen with the light microscope except if it is labelled.[136] Therefore, labels (reporter molecules) are attached to primary or secondary Abs to allow the visualization of Ag-Ab reactions.[136] A diversity of labels have been used, including fluorescent compounds, enzymes and metals.[136] I used fluorescent compounds, thus an immunofluorescent approach.

The two main methods of immunofluorescent (IF) labelling are direct and indirect. In direct IF the Ab is chemically conjugated with a fluorescent (fluorochrome) while in indirect IF, the specific Ab (called the primary Ab) is unlabelled and a second anti-immunoglobulin Ab directed toward the constant portion of the first Ab (called the secondary Ab) is tagged with the fluorescent dye (Figure 15).[136,138,139]

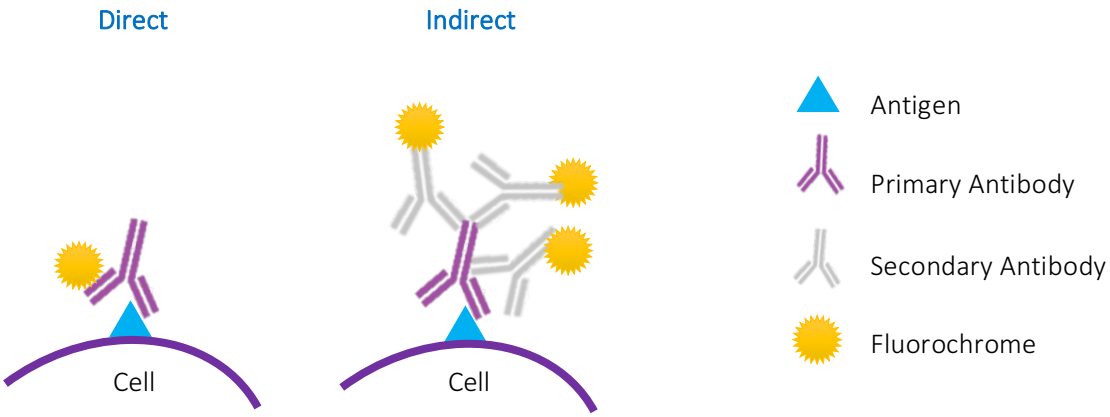


Figure 15. Schematic of direct and indirect immunofluorescence.

In this work, the indirect method was used, since it has greater sensitivity than the direct IF, mainly due to two reasons. First, the primary Ab is not labelled, it retains activity and results in a strong signal, and second, more than one secondary Ab can attach to each primary, which allows an amplification of the signal.[136] Additionally, commercially produced secondary Abs are relatively inexpensive, they are available in an array of colours and their quality is controlled.[139] Nevertheless, this method has some disadvantages that should be considered. For instance, the potential for cross-reactivity and the need to find primary Abs that are not raised in the same species, plus samples with endogenous immunoglobulin may exhibit a high background.[139]

The principles of IF are based on the luminescent properties of some molecules that absorb light and then emit light of a different wavelength.[139] Each fluorochrome dye is excited at a different efficiency and, consequently, the resulting emission will be at different intensities for equivalent fluorochrome concentrations.[139]

Fluorescent signals are detected through a fluorescence microscope. Its basic function is to deliver excitation energy to the fluorochrome(s) in the specimen to be investigated, to separate the much weaker emitted fluorescence light from the excitation light and, finally, to send it to the detector, where a high-contrast image is generated (Figure 16).[141]

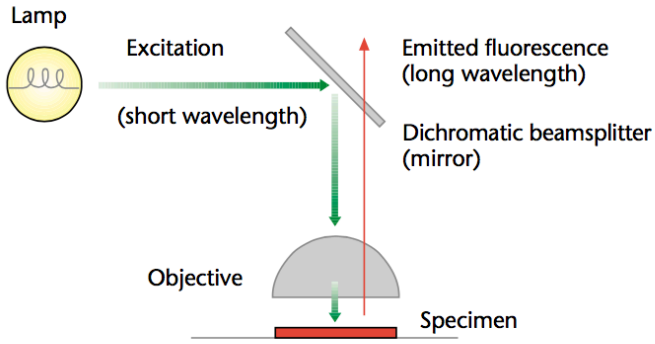


Figure 16. Epifluorescence microscopy.

The (relatively strong) excitation light is directed to the specimen by reflection on the dichromatic beam splitting mirror and focusing through the objective. The (relatively weak) emission light, with a longer wavelength, is separated as it passes the mirror and reaches the photodetector.[141] *(Figure adapted from Fritschy et al., 2001)*[141]

Excitation light sources used in microscopes are usually mercury and xenon lamps. The former emit peaks of energy at discrete wavelengths (e.g., 365, 400, 440, 546 and 580 nm), whereas the latter have a uniform intensity profile from the ultraviolet to the far red. The appropriate excitation and emission wavelengths are selected with the corresponding bandpass filters.[141]

To conclude, it is important to refer that a number of factors can affect the quality of a fluorescence image. The quality and concentration of the labelled Ab are important to achieve a high signal-to-noise ratio.[138] Too much nonspecific Ab binding from either a poor-quality Ab or a high concentration may not allow accurate localization of immune complexes.[138] Alternatively, a dilute Ab may not provide sufficient signal.[138] Biological autofluorescence in mammalian cells due to flavin coenzymes and reduced pyridine nucleotides can also be problematic in the detection of fluorescence probes in tissues and cells.[139] Moreover, fixation with aldehydes can result in high levels of autofluorescence.[139] Other inconvenient is the signal from fluorescent probes, which is lost over time with exposure to light. This phenomenon is called photobleaching, which is a photochemical destruction of a fluorophore due to the generation of reactive oxygen species in the specimen as a byproduct of fluorescence excitation.[138,139] Therefore, it is important to keep samples in the dark when not in use, use only as much light as necessary from the microscope and use an antifade reagent in the mounting medium.[138] Finally, the performance of the detection instrument (i.e. how well the microscope has been calibrated and set), the specificity of the Abs and the specimen preparation are other factors that limit IF and should be taken into account.[139]

3.7.2. Immunohistochemistry protocol

After the last behavioural test, 12 weeks-old rats were deeply anesthetized with ketamine/xylazine mixture (120 mg/kg / 16 mg/kg) at 1 mL/kg body weight. After anaesthesia the animals were laid on their back on a plane surface and their paws were secured with tape. The toe pinch-response method was used to assess the depth of anaesthesia.

After reaching the deep anaesthesia state, the rats were perfused transcardially with 0.9% saline solution, followed by 4% paraformaldehyde (PFA) in phosphate buffer (pH 7.4), as previously described (Figure 17).[142] Briefly, incisions were made in the chest area and then in the diaphragm in order to expose the heart. After that, the perfusion needle passed through the left ventricle and a hole was snipped in the right atrium to drain fluids as the peristaltic pump (MINIPLUS®3, Gilson, Middleton, USA) started at a constant flow rate of 10 mL/min.

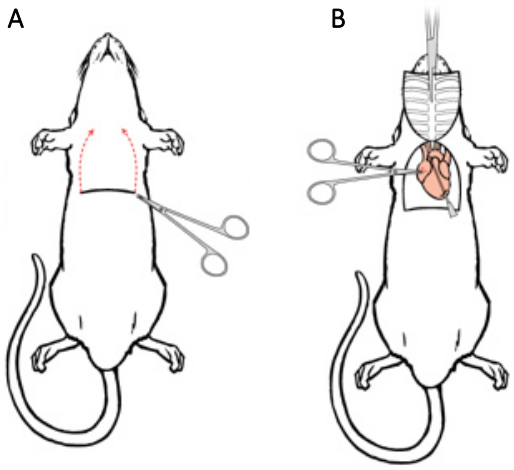


Figure 17. Representative scheme of Perfusion Surgery.

A) A lateral incision was made just below the sternum. B) The xiphoid process was hold with forceps and pulled up to allow the visualization of the diaphragm. Next, scissors were used to poke a hole in the diaphragm close to the xiphoid process. The diaphragm was cut away from the ribs, to allow visualization of the thoracic cavity. After that, a scissor was used to make parallel cuts on either side of the ribs up to the collarbone. Then, the tip of the sternum was clamped and the clamp was placed over the head. The perfusion needle was inserted into the left ventricle and a small cut was done in the right atrium to drain fluids as the peristaltic pump started to work.[142] *(Adapted from Gage, et al., 2012)*[142]

The animals were perfused with about 200 mL of saline solution to clear the blood from the circulatory system. When the extremities (paws, tail and nose) of the rats looked pale, the liver looked cleared of blood and the solution exiting in the heart was clear, the perfusion of approximately 500 mL of 4% PFA (warmed at 37°C) started. The perfusion stopped when the body of the animal was stiff, tail was holding resistance when moved and the paws and neck were rigid.

Following decapitation, brains were carefully removed and maintained for post-fixation in the same fixative solution (4% PFA) at 4°C overnight (O/N). After that, brains were washed twice with Phosphate-buffered saline (PBS containing in mM: NaCl 140, KCl 3,Na_2HPO_4 20, KH_2PO_4 1.5) and then cryoprotected (4°C) by immersion in increasing concentrations of sucrose, namely 15% and 30%.

Subsequently, brains were gelatine-embedded (7.5% gelatine in 15% sucrose) and then sectioned at a thickness of 12 µm on a cryostat (LEICA CM 3050S, Wetzlar, Germany), by iMM's Histology and Comparative Pathology Laboratory. Only the coronal sections located at the level of hippocampus (around - 2.92 mm and - 5.04 mm from Bregma) were collected, mounted on SuperFrost® Plus slides (Menzel-Glaser, Braunschweig, Germany) and stored at -20°C for further use.

For immunohistochemical analyses, hippocampal sections were stained for different markers: (i) neuronal nuclei marker (NeuN), in order to evaluate neuronal damage; (ii) glial fibrillary acidic protein marker (GFAP), to evaluate changes in the morphology of astrocytes; and (iii) ionized calcium binding adaptor molecule 1 (Iba-1), to assess modifications in the morphology of microglia (Table 5). NeuN is an antigen that is consistently detected by the corresponding monoclonal Ab anti-NeuN in the nucleus of most types of neurons in a wide range of vertebrates.[143,144] Therefore, anti-NeuN is widely used as a tool for detecting neuronal cells from the central and peripheral nervous systems. GFAP is an intermediate filament protein, which, in the CNS, stains mainly reactive astrocytes and some groups of ependymal cells.[145–147] Iba-1 is often used as a marker for microglia both in resting and active state.[148] For this reason, Iba-1, also known as Allograft Inflammatory factor 1 (AIF-1), is useful to evaluate microglia proliferation and to study their morphological changes.[148]

Table 5. List of primary antibodies used in immunohistochemistry.

	ANTIGEN	ANTIBODY	HOST	DILUTION	SUPPLIER
NEURONAL MARKER	neuronal nuclei	NeuN	Rabbit Polyclonal	1:1000	Cell Signalling Technology, Massachusetts, USA
ASTROCYTE MARKER	glial fibrillary acidic protein	GFAP	Mouse Monoclonal	1:1000	abcam®, Cambridge, UK
MICROGLIA MARKER	ionized calcium binding adaptor molecule 1	Iba-1	Goat Polyclonal	1:250	abcam®

Succinctly, slides were placed in PBS for 10 min at 37°C to remove gelatine from brain tissue. Then, each slice was surrounded with a DAKO pen (Dako, Glostrup, Denmark) to protect staining areas from drying out and from mixing with each other and also to reduce the amount of reagents.

After an incubation in 0.1 M of glycine for 10 min, which was used as a quenching solution that allows the removal of toxic small aldehydes originated from PFA degradation, sections were subsequently treated with 0.1% Triton X-100 in PBS (10 min) for membrane permeabilization, washed twice (10 min each time) with PBS in the presence of the non-ionic detergent Tween-20 (PBSTw) and then blocked at room temperature (RT). Different blocking procedures, summarized in Table 6, were used according to the primary Ab.

Table 6. Blocking procedures used in the immunofluorescence protocol.

PRIMARY ANTIBODY	BLOCKING SOLUTION	BLOCKING TIME
GFAP	B1: 10% FBS in PBSTw	1h
NeuN	B2: 10% FBS, 6% BSA in PBSTw	1h
Iba-1	B3: 10% FBS, 10% BSA in PBSTw	3h

FBS, Fetal Bovine Serum; BSA, Bovine Serum Albumin; PBSTw, PBS with 0.1% Tween

Next, slices were incubated at 4°C O/N with the primary Ab, properly diluted in the blocking solution. In the following day, sections were washed three times with PBSTw (10 min each time) and incubated with the secondary Ab (from Invitrogen, Massachusetts, USA) (1:500 dilution in blocking solution) for 2 h at RT in a humified dark chamber (Table 7).

Table 7. List of secondary antibodies used in immunohistochemistry.

PRIMARY ANTIBODY	SECONDARY ANTIBODY
GFAP	Donkey anti-mouse IgG Alexa 568
NeuN	Goat anti-rabbit IgG Alexa 568
Iba-1	Donkey anti-goat IgG Alexa 488

For nuclei staining, sections were incubated with Hoechst 33342 (1:100 dilution in PBS; Invitrogen) for 10 min at RT. Slices were washed again three times, for 10 min each time, with PBSTw, followed by one last wash in PBS. At the end, sections were mounted with Mowiol (100 µL per slice), a non-absorbing compound without autofluorescence and light scattering.

3.7.3. Visualization

Images were acquired on an inverted widefield fluorescence microscope (Zeiss Axiovert 200, Oberkochen, Germany), using a monochrome digital camera (AxioCamMR3, Zeiss), with a 40X objective (Zeiss). The software AxioVision 4.7.1 (Carl Zeiss Imaging Systems) was used for image acquisition.

Immunofluorescence images were acquired in two areas of the hippocampus: CA1 and DG (Figure 18). CA1 and DG images were taken from both hemispheres in each rat.

Images used for the quantitative assessment of neuronal damage were acquired with a 10x objective and an exposure time of 700 ms.

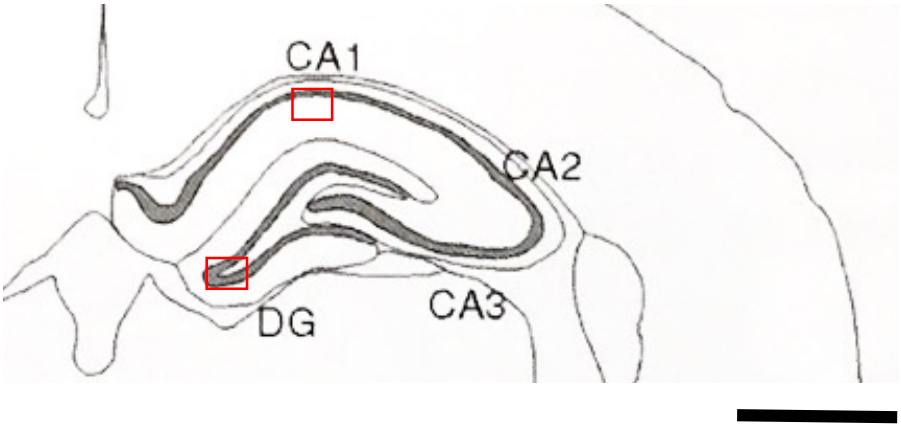


Figure 18. Representation of the hippocampus.

The areas enclosed by the red boxes show the regions where the images were taken, namely Dentate Gyrus (DG) and *Cornus Ammonis* 1 (CA1). Bar=1mm. *(Adapted from Andersen et al. 2007)*[44]

The CA1 pyramidal cell layer was chosen since it is the main output area of the hippocampus and it is closely linked to memory of temporal order of visual objects and especially over long intervals.[149] Thus, CA1 is essential for context-dependent retrieval.[150,151] Moreover, the CA1 area is particularly sensitive to neurodegeneration in AD patients, being one of the first affected areas, compared with the other hippocampal regions.[152–154]

Concerning DG, it is known that this area receives the majority of the input pathways to the hippocampus, subserving three main functions: (i) conjunctive encoding of multiple sensory inputs, (ii) spatial pattern separation and (iii) facilitation of encoding of spatial information based on its outputs to CA3.[155]

3.7.4. Quantification of the immunofluorescence images

For quantitative assessment of neuronal damage (NeuN fluorescence signal), the hippocampal regions of interest (CA1 and DG) were delineated using the software program Image-J 1.45 (Maryland, USA). The intensity value of each analysed region was obtained and corrected with a fluorescence background value. Measurements were performed bilaterally from 3 rats per group, rendering 6 data points per group.

3.8. Western Blotting

Western Blotting (WB), also sometimes referred to as immunoblotting or protein blotting, was first reported in the literature over 30 years ago[156,157] and it is the most widely used and accepted methodology for identifying proteins and semi-quantifying protein amounts (Figure 19).[158–163]

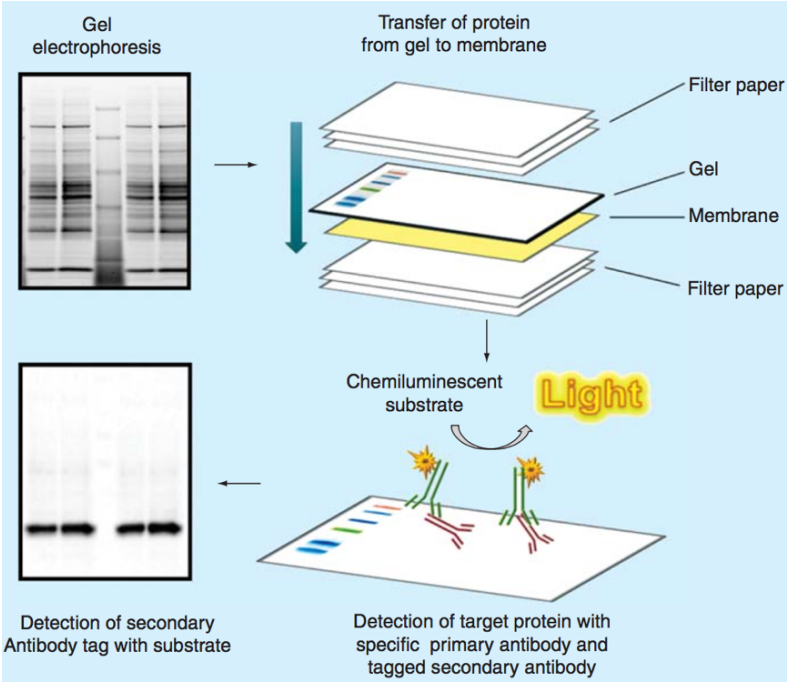


Figure 19. Schematic diagram of a typical Western Blot (WB).

The typical WB requires gel electrophoresis to separate proteins based on their molecular weight with subsequent protein transfer from the gel to a protein binding membrane. The membrane is then incubated with the primary and secondary antibodies. The tagged secondary antibody catalyses an enzymatic reaction with the substrate, which can be detected by a digital imager.[163] *(Figure adapted from Gosh et al., 2014)*[163]

The first step of this technique is dissection and tissue collection, followed by sample preparation. Since tissues display a higher degree of structure, homogenization or sonication is required to prepare protein extracts. This procedure is done at a cold temperature (under ice) with protease inhibitors to prevent protein denaturation. After quantification of the total protein, samples are diluted in a loading buffer, which contains glycerol so that the samples sink easily into the wells of the gel. A tracking dye (bromophenol blue) is also present in this buffer allowing the researcher to see how far the separation has progressed. Usually, samples are also heated in order to denature the higher order structures, while retaining sulphide bridges. Denaturing also ensures that the negative charge of amino acids is not neutralized, enabling the protein to move in an electric field.[158]

It is also essential to have a 'loading control' protein, also known as internal standard, in order to compare protein levels between samples. The loading controls are generally derived from ubiquitously expressed 'housekeeping' genes and have been widely used due to their presumed consistent level of expression across a diverse range of samples.[164]

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) uses two types of gel: stacking and separating gel. The stacking gel is slightly acidic (pH 6.8) and has a low acrylamide concentration making a porous gel, which separates protein poorly but allows them to form thin, sharply defined bands. The separating, or resolving gel is basic (pH 8.8) and has a higher polyacrylamide content, making the gel's pores narrower. Proteins are thus separated by their size. The smaller proteins travel more easily throughout the pores and hence more rapidly than larger proteins. The proteins, when loaded on the gel, have a negative charge (due to the SDS present in the loading buffer) and they will travel toward the positive electrode when voltage is applied.[158]

After separation by SDS-PAGE, proteins are transferred to a membrane. The transfer is done using an electric field oriented perpendicular to the surface of the gel, causing proteins to move out of the gel and onto the membrane. The membrane is placed between the gel surface and the positive electrode in a sandwich. This type of transfer is called electrophoretic transfer. [158]

Next, it is necessary to block, wash and incubate the membranes with the proper Abs. Blocking can help mask any potential nonspecific binding sites on the membrane itself and can promote renaturation of antigenic sites. However, it has been reported that prolonged blocking times (>24 h) can actually remove Ags.[161] Washing is also a crucial step of WB, as it minimizes background and removes unbounded Ab.[158] Detection of the tagged secondary Ab is commonly performed by an enhanced chemiluminescence (ECL) assay.[161] An advantage of using an ECL substrate is that the membranes can be successfully stripped off the reagents and reprobed with a different Ab to detect another protein.[163] Thus, the secondary Ab is tagged with the enzyme horseradish peroxidase (HRP), which catalyses the oxidation of luminol (substrate) in the presence of peroxide to 3-aminophthalate, leading to the emission of light at 428 nm, which is detected by digital imagers.[163]

Finally, quantification of the immunoreactive bands is carried out. Note that WB is typically considered to be semi-quantitative, since it only provides a relative comparison of protein levels.

To sum up, WB is a powerful and indispensable scientific technique that can be used to accurately quantify relative protein levels. This technique has a lot of advantages, such as: (i) wet membranes are pliable and easy to handle, (ii) the proteins immobilized on the membrane are readily and equally accessible to different ligands, (iii) small amount of reagents is required for transfer analysis, (iv) prolonged storage of transferred patterns, prior to use, becomes possible and (v) the same protein transfer can be used for multiple successive analyses.[162]

3.8.1. Western Blotting Protocol

3.8.1.1. Dissection and tissue collection

Rats were deeply anesthetized under isoflurane atmosphere before decapitation and tissue preparation. For WB analysis, as described for immunofluorescence studies, it was used the hippocampus, a brain area severely affected in patients with AD and crucial for memory encoding.[45]

After decapitation the brain was rapidly removed and the two hippocampi were dissected in ice-cold artificial cerebrospinal fluid (aCSF, containing in mM: NaCl 124; KCl 3; NaH_2PO_4 1.25; $NaHCO_3$ 26; $MgSO_4$ 1; $CaCl_2$ 2 and glucose 10, pH 7.4) previously gassed with 95% O_2 and 5% CO_2. Then, the left and right hippocampi were rapidly frozen in liquid nitrogen and stored at -80°C until further analysis. The two hippocampi (left and right) were analysed separately to allow the separate quantification of the hippocampus of the side of the i.c.v. injection (right) and contralateral to it. Since both hippocampi could be differently exposed to $A\beta_{1-42}$, they could display different degrees of neurodegeneration.

3.8.1.2. Sample preparation

Tissue homogenates were prepared from frozen samples. Briefly, samples were homogenized and solubilised in a Radio-Immunoprecipitation Assay (RIPA) buffer containing: 50 mM Tris-HCl (pH 7.5), 150mM NaCl, 5mM Ethylenediaminetetraacetic Acid (EDTA), 0.1% *Sodium Dodecyl Sulfate* (SDS), 1% Triton X-100 and phosphatase inhibitors: 10nM NaF; 5mM Na_3VO_4 and protease inhibitors cocktail (Roche, Penzberg, Germany). The samples were incubated at 4°C with slow agitation for 15 min and centrifuged at 13000 rpm for 10 min (4°C). The supernatant was collected and stored at -20°C.

3.8.1.3. Protein Quantification and Preparation

Protein concentration was determined by the Bradford method using the Bio-Rad DC™ Protein Assay kit (Bio-Rad Laboratories®, California, USA) and all measurements were performed in a 96-wells flat-bottom plate. Absorbance at 750 nm was acquired and plotted. A BSA calibration curve, absorbance at 750 nm *vs* BSA concentration (mg/mL), was obtained in all assays and used to calculate the concentration of each sample.

Protein samples were prepared using 1x sample buffer (350 mM Tris at pH 6.8; 10% SDS; 30% glycerol; 600 mM Dithiothreitol (DTT) and 0.06% bromophenol blue). Denaturation of proteins was performed for 10 min at 95°C.

3.8.1.4. Gel electrophoresis and Chemiluminescent detection

A 12% SDS-PAGE was used to separate the samples (35 µg of protein per lane) and the molecular weight marker (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa, ThermoFisher Scientific, Massachusetts, USA). Subsequently, proteins were transferred, at 400 mA for 1h30, from the gel to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, UK), previously activated by methanol. Membranes were stained with Ponceau S solution (Sigma-Aldrich®) to check for transference efficacy. After blocking with a 3% BSA in TBST (Tris-Buffered Saline with Tween-20 containing in mM: Tris base 20; NaCl 137 and 0.1% Tween-20) during 1 h at RT to avoid non-specific binding, the membranes were probed, O/N at 4°C, with the appropriately diluted primary Abs in 3% BSA in TBST, in order to detect changes in the proliferation of astrocytes (GFAP immunostaining) and microglia (Iba-1 immunostaining) (Table 8).

Membranes were washed with TBST for 30 min and incubated with horseradish peroxidase (HRP, EC 1.11.1.7) conjugated secondary Abs (3% BSA in TBS-T) for 1 h at RT. HRP–conjugated secondary Abs anti-rabbit IgG, anti-goat IgG and anti-mouse IgG (1:10000, Santa Cruz) were used according to the host species of the primary Ab. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control (Table 8).

Table 8. List of the primary antibodies and the loading control used in western blotting.

	ANTIBODY	HOST	DILUTION	SUPPLIER
ASTROCYTE MARKER	GFAP	Rabbit Polyclonal	1:5000	abcam®
MICROGLIA MARKER	Iba-1	Goat Polyclonal	1:1000	abcam®
LOADING CONTROL	GAPDH	Mouse monoclonal	1:1000	abcam®

Chemiluminescent detection was performed with ECL Plus Western Blotting Detection Reagent (GE Healthcare) and the band's intensity was visualized with the ChemiDoc™ XRS+ System from Bio-rad. The levels of relative expression of the protein bands were determined with Image-J 1.45 software and normalized to the loading control.

3.9. Statistical analysis

Data are represented as the mean ± SEM (standard error of the mean) for each group of animals, where *n* is the number of animals per group. All independence tests were two-tailed. The significance of differences between the means of two conditions was evaluated by Student's t-test. The significance of differences between groups was analysed with one-way ANOVA (Analyses of Variance) followed by Holm-Sidak's multiple comparisons test when indicated.

Values of $P \leq 0.05$ were considered to represent statistically significant differences.

All statistical analyses were calculated with Prism Software (GraphPad Prism®, version 6, California, USA).

4. Results

4.1. Validation of the animal model of AD

Before starting the treatment with KTP-NH_2 it was necessary to validate the animal model of AD under our experimental conditions. This model was based on other studies already described in literature.[117,165] As mentioned in 'Materials and Methods', male Wistar rats (8-10 weeks old) i.c.v. injected with 5 µL of $A\beta_{1-42}$ (2.25 mg/mL) and tested 2 weeks after injection were used as the animal model of AD.

4.1.1. $A\beta_{1-42}$ induced impairments in spatial working memory without affecting locomotor performance

Although deficits in episodic memory characterize better AD, there is increasing evidence that working memory is also impaired at the earliest stages of the disease.[166] Therefore, it was important to evaluate if i.c.v. injection of $A\beta_{1-42}$ could cause impairments in rats memory, more precisely in spatial working memory, which was evaluated by the performance of the rats in the Y-Maze test.

As stated in 'Materials and Methods', the Y-Maze spontaneous alternation paradigm is based on the natural tendency of rodents to explore a novel environment. When placed in the Y-Maze, during 8 min, rats explore the least recently visited arm and thus tend to alternate visits between the three arms. Note that specific motifs are placed on the walls of each arm allowing visual discrimination. For efficient alternation, rats need to use working memory and, consequently, they should maintain an ongoing record of most recently visited arms and continuously update such a record. Therefore, a rat with an impaired spatial working memory cannot remember which arm has just visited showing a decreased percentage of spontaneous alternation.

Figure 20 demonstrates the result of the performance of CTL and $A\beta_{1-42}$ rats in the Y-Maze test, where the percentage of spontaneous alternation behaviour was assessed. The $A\beta_{1-42}$ rats had a lower percentage of spontaneous alternation (57 ± 3.1) as compared with CTL rats (71 ± 2.8) (n=7; $P \leq 0.05$, unpaired t-test). This means that $A\beta_{1-42}$ rats failed to successively alternate between the three arms of the maze, thus suggesting a reduction in spatial working memory.

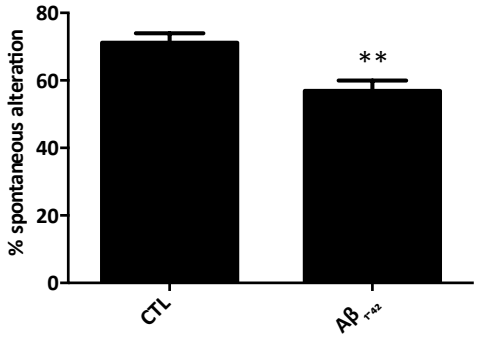


Figure 20. Quantification of the % of spontaneous alternation in the Y-Maze test of Control (CTL) and $A\beta_{1-42}$ rats.

A higher percentage of spontaneous alternation (see 'Materials and Methods') is indicative that the animal remembers the arm entered immediately before, thus does not re-enter it. Note that spatial working memory of $A\beta_{1-42}$ rats was impaired compared with CTL rats (n=7 animals per group; **P≤ 0.01, unpaired t-test). Values are mean ± SEM.

In addition, it was considered important to test if the i.c.v. injection with $A\beta_{1-42}$ influenced general motor function of rats, since it could compromise their performance in the memory tests. To do so, the total number of arm entries in the Y-Maze was analysed. No statistically significant differences were found between experimental groups in terms of total number of entries in Y-Maze arms (CTL: 26 ± 1.2 *vs* $A\beta_{1-42}$: 26 ± 1.2; n=7; P>0.05, unpaired t-test, Figure 21), therefore the differences found in memory tests cannot be attributed to motor impairments.

Note that no animal jumped out of the maze arena during the testing period.

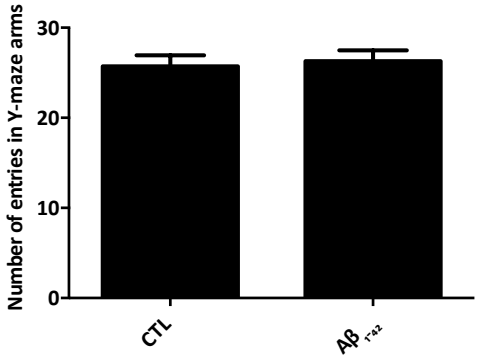


Figure 21. Number of entries in Y-Maze arms as a measure of locomotor performance of Control (CTL) and $A\beta_{1-42}$ rats.

There were no statistically significant differences between the total number of arm entries of both groups, indicating that locomotor activity remains virtually unaffected (n=7 animals per group; NS P>0.05, unpaired t-test). Values are mean ± SEM.

4.1.2. $A\beta_{1-42}$ did not affect locomotor and exploratory behaviour

Though the Y-Maze data indicated absence of locomotor impairment, it was considered important to evaluate this issue through the most commonly used test to evaluate locomotion, the OF test. The evaluation of motor performance using the OF test is important since, in rodents, one of the most imperative components of exploration, a prominent activity of the animal's repertoire of spontaneous activity, is locomotion. Moreover, this test allows evaluating anxiety-related behaviours in rodents. Indeed, alterations of locomotor activity and exploration can have an important impact in paradigms that aim to study more specific processes, such as learning, memory and others. Thus, it was crucial to further verify if the i.c.v. injection of $A\beta_{1-42}$ induced or not locomotor and exploratory impairments in the rats.

As presented in Figure 22, no statistically significant differences were found between CTL and $A\beta_{1-42}$ rats regarding average velocity (Graph A – CTL: 35 ± 0.7 *vs* $A\beta_{1-42}$: 35 ± 0.6) ; time spent resting (Graph B – CTL: 188 ± 13 *vs* $A\beta_{1-42}$: 185 ± 13) and total of travelled distance (Graph C – CTL: 4047 ± 500 *vs* $A\beta_{1-42}$: 4123 ± 477) (n=7; P>0.05, unpaired t-test). This suggests that $A\beta_{1-42}$ administration did not induce impairment in spontaneous motor activity. Concerning the exploratory behaviour, quantified as percentage of time in the periphery of the arena, no statistically significant differences were found between CTL and $A\beta_{1-42}$ (CTL: 79 ± 3.4 *vs* $A\beta_{1-42}$: 78 ± 1.6, n=7; P>0.05, unpaired t-test). The time spent in the periphery of the OF arena also gives an indication of anxiety-related behaviour, since anxious animals tend to stay much more time at periphery of the OF arena. With these results, is possible to conclude that the exploratory habits of each group of animals was similar, so any change in memory tests cannot be attributed to differences in anxiety or exploration drive.

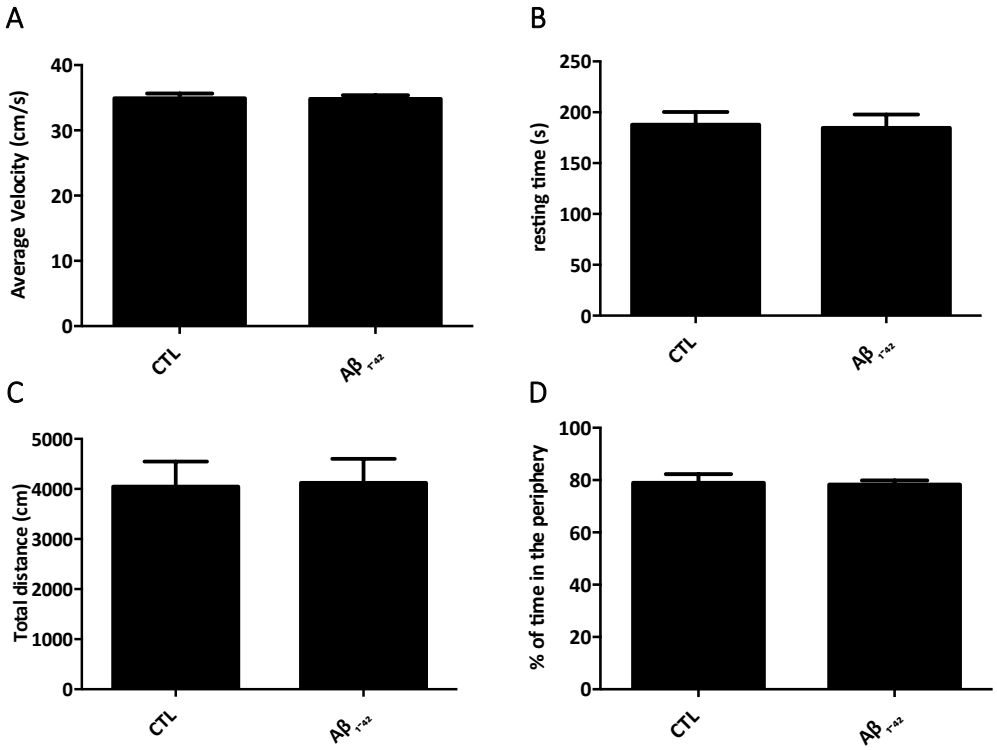


Figure 22. Locomotor performance and exploratory behaviour in the Open Field (OF) test of Control (CTL) and $A\beta_{1-42}$ rats.

Behaviour was video-recorded for a 5 min time period and data are quantified as **(A)** average velocity, **(B)** resting time, **(C)** total distance travelled and **(D)** % of time spent in the periphery of the arena.

No statistically significant differences were found regarding locomotor activity (A, B and C) and exploratory behaviour (D), between the two groups (n=7 animals per group; NS P>0.05, unpaired t-test). Values are mean ± SEM.

4.1.3. $A\beta_{1-42}$ caused episodic long-term memory failure

Since episodic memory impairment is one of the defining features of AD and, in the overwhelming majority of cases, it is the first cognitive deficit to appear, it was considered relevant to test if the i.c.v. injection of $A\beta_{1-42}$ could or not compromise the performance of the animals in the NOR test, which was designed to evaluate that type of memory.

As stated in 'Materials and Methods', NOR test is based on the natural tendency of rodents to choose to explore a novel object instead of a familiar one as well as the reactivation of exploration after object displacement, which reflects the use of learning and recognition memory processes.[131]

In the test phase, $A\beta_{1-42}$ rats failed to discriminate between the familiar object and the novel one (Figure 23), which revealed an impairment on episodic long-term memory of these rats. Indeed, the Object Recognition Index was significantly lower in $A\beta_{1-42}$ rats than in the CTL rats ($A\beta_{1-42}$: 0.5 ± 0.01 *vs* CTL: 0.7 ± 0.02; n=7; P≤0.05, unpaired t-test). Moreover, it is important to mention that in the training

phase (24 h before the test phase), animals did not show any preference for one of the two equal objects (F1 and F2), regardless which pair of objects were used as familiar, since no statistically significant differences were found between the two groups concerning the Object Preference Index ($A\beta_{1-42}$: F1 - 0.50 ± 0.04 *vs* F2: 0.50 ± 0.04 |CTL: F1 – 0.54 ± 0.05 *vs* F2 – 0.46 ± 0.05; n=7; P>0.05, unpaired t-test) and also concerning the exploration time (in seconds) for each object in the training phase ($A\beta_{1-42}$: F1 - 19 ± 1.6 *vs* F2 - 19 ± 1.3 | CTL: F1- 20 ± 1.8 *vs* F2 - 17 ± 2.5; n=7; P>0.05, unpaired t-test).

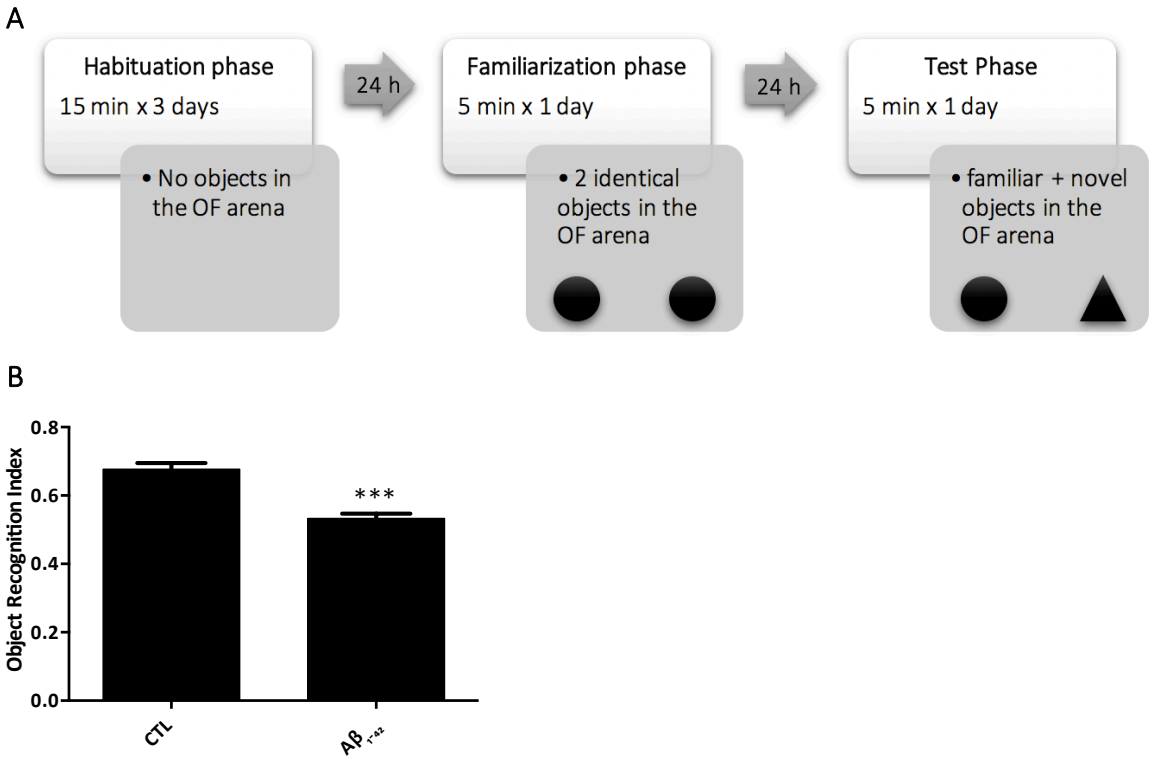


Figure 23. Performance in the Novel Object Recognition (NOR) test of Control (CTL) and $A\beta_{1-42}$ rats.

(A) Schematic representation of the protocol used in NOR test to assess episodic long-term memory. This test consisted in two 5 min phases (24 h after 3 days of a Habituation phase – 15 min each day): the first with two identical objects (Familiarization phase) and the second (Test phase, 24 h after) with two dissimilar objects (a familiar and a novel one). The NOR test was performed in the same Open Field (OF) arena used in the OF test. **(B)** Object Recognition Index was calculated by the ratio of the time spent exploring a novel object over the total exploration time of both objects (novel + familiar). A higher index value indicates higher memory performance. For each animal, the sum of the exploration time for each object is normalized to 1. $A\beta_{1-42}$ rats showed impaired episodic-like memory, which indicates their incapacity to discriminate between familiar and novel objects (n=7 animals per group; ***P≤0.001, unpaired t-test). Values are mean ± SEM.

4.1.4. $A\beta_{1-42}$ induced neuronal damage in the rat hippocampus

Since soluble forms of $A\beta_{1-42}$ can trigger a cascade of events leading to neuronal death[167], the immunohistochemical staining for NeuN was performed, in order to evaluate if $A\beta_{1-42}$ could indeed lead to neuronal damage in rat hippocampus.

As described in 'Materials and Methods', adult rat brain slices (12 µm) were labelled with an antibody against NeuN, which served as a marker for neurons, together with the nuclear marker Hoechst 33342.

As presented in Figure 24, the pattern of NeuN staining for rats injected with $A\beta_{1-42}$ was altered when compared with CTL rats. In samples from $A\beta_{1-42}$ rats, regions deprived of Hoechst and NeuN staining (indicated by arrows on the figure) can be noticed, pointing towards the occurrence of neuronal injury in CA1 and DG hippocampal areas.

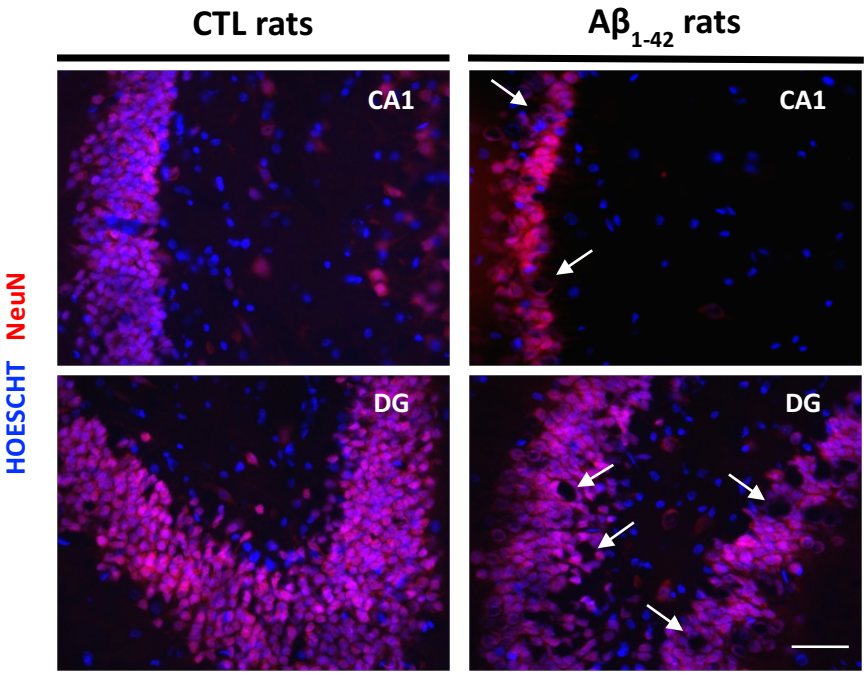


Figure 24. Detection of NeuN in CA1 and DG hippocampal areas of Control (CTL) and $A\beta_{1-42}$ rats.

Representative images of Hoechst stained nucleus (blue) with NeuN stained neurons (red) in CTL and $A\beta_{1-42}$ rats. Arrows indicate regions devoid of Hoescht and NeuN staining in $A\beta_{1-42}$ rats, which points to neuronal loss in these regions. Images were acquired on an inverted widefield fluorescence microscope (Zeiss Axiovert 200), with a 40x objective. Images representative of n=3 animals per group. Scale bar applies to all images, 50 µm.

NeuN, neuronal nuclear antigen; CA1, cornu ammonis *1; DG, dentate gyrus.*

Quantitative analysis of NeuN fluorescence (Figure 25) confirms a significant decrease in NeuN content in $A\beta_{1-42}$ rats compared with CTL rats, both in CA1 and DG hippocampal areas (**CA1** - $A\beta_{1-42}$: 171 ± 17 *vs* CTL: 379 ± 17| **DG** - $A\beta_{1-42}$: 268 ± 15 *vs* CTL: 466 ± 32; n=3; P≤0.05, unpaired t-test).

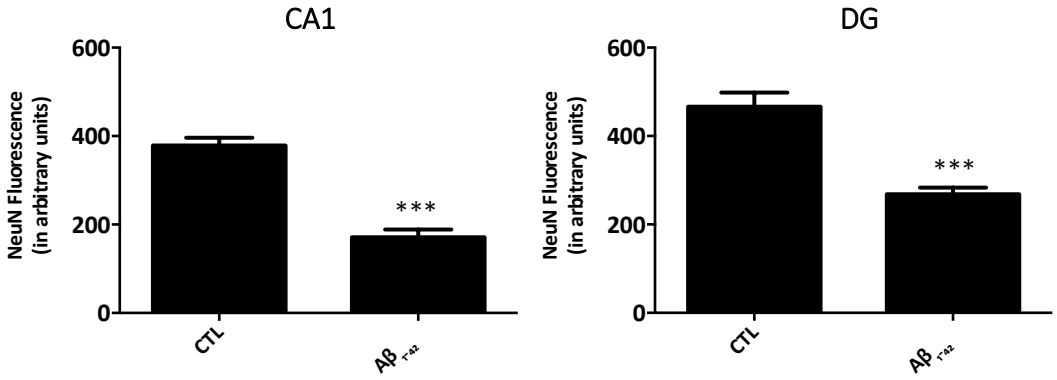


Figure 25. Quantification of the NeuN fluorescence signal in CA1 and DG hippocampal areas of Control (CTL) and $A\beta_{1-42}$ rats.

The quantification of NeuN signal in each hippocampal region is expressed in arbitrary units of fluorescence intensity. There was a significant decrease in NeuN signal in $A\beta_{1-42}$ rats compared with CTL rats, both in CA1 and DG areas, which corroborates the occurrence of neuronal damage induced by $A\beta_{1-42}$ (n=3 animals per group; ***P≤ 0.001, unpaired t-test). Values are mean ± SEM.

NeuN, neuronal nuclear antigen; CA1, cornu ammonis *1; DG, dentate gyrus.*

4.1.5. $A\beta_{1-42}$ did not induce gliosis at the rat hippocampus

In some studies, $A\beta_{1-42}$-injected animal models commonly manifest considerable gliosis, which has been linked to neuronal damage.[168] Thus, since neuronal death was detected, we decided to determine if astrogliosis and microgliosis had also occurred, i.e., if $A\beta_{1-42}$ injection into rat brain elicited also a proinflammatory reactivity.

In order to assess if $A\beta_{1-42}$ could lead to an increase in the proliferation of astrocytes and microglia, which is a sign of gliosis, a western blot assay was performed with protein extracts obtained from hippocampal homogenates. Astrocytes and microglia expression levels were identified using specific antibodies, GFAP and Iba-1 respectively. GAPDH served as internal control.

The representative immunoblots and expression pattern of GFAP and Iba-1 are depicted in Figure 26. The antibodies used detected a single band (Figure 26, A), thus indicating high specificity. Densitometry analyses revealed no significant changes (n=3-4; P>0.05, one-way ANOVA) in samples from $A\beta_{1-42}$ rats when compared with CTL rats (Figure 26, B), indicating absence of proliferation of astrocytes and microglia.

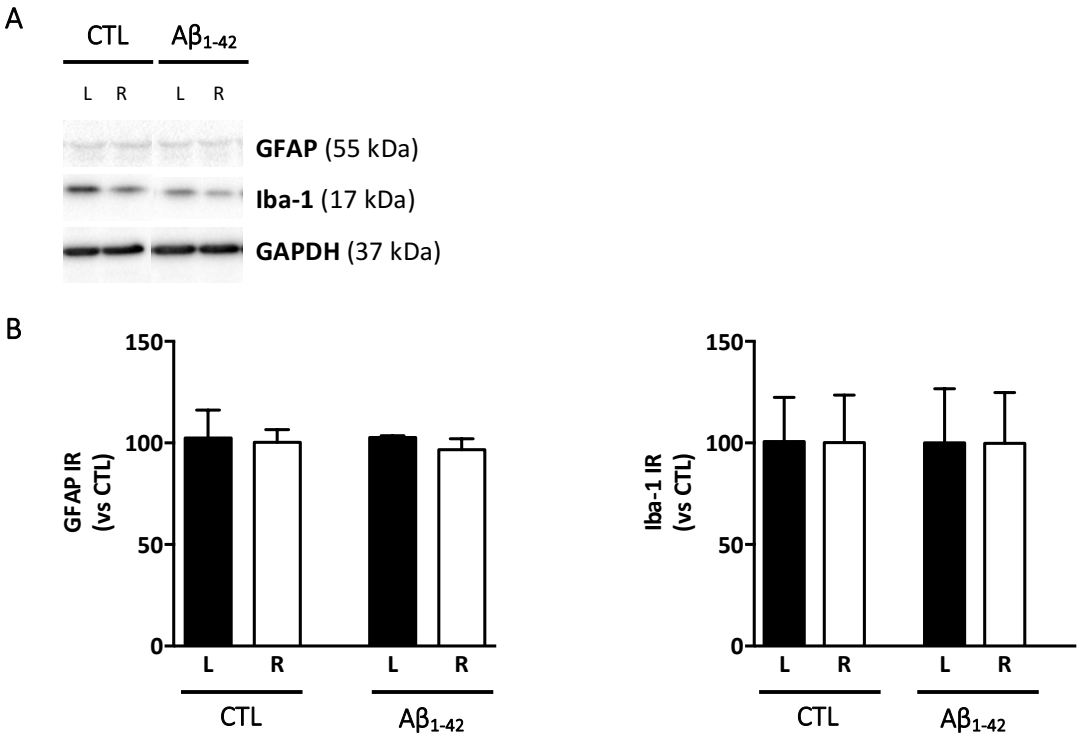


Figure 26. Western Blot analysis of GFAP and Iba-1 of Control (CTL) and $A\beta_{1-42}$ rats.

(A) Representative immunoblots of GFAP and Iba-1 of Left (L) and Right (R) hippocampus of CTL and $A\beta_{1-42}$ rats . GAPDH was used as loading control. **(B)** Densitometry analysis was performed with ImageJ software. No statistically significant differences were found between CTL and $A\beta_{1-42}$ rats, while comparing GFAP (astrocyte marker) and Iba-1 (microglia marker) immunoreactivity (IR) (n=3-4 animals per group; P>0.05, one-way ANOVA). Values are mean ± SEM.

GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

4.1.6. $A\beta_{1-42}$ did not induce morphological changes in astrocytes and microglia at the rat hippocampus

WB assay is a semi-quantitative technique and does not provide information about morphological changes of astrocytes and microglia. Therefore, an immunohistochemistry analysis of glia cell morphology was performed, in order to evaluate if any change in the morphology of these cells had occurred. Hoechst 33342 was used as the nuclear marker.

Astrogliosis, associated with changes in the morphology of astrocytes, may occur in AD in two opposite directions: when Aβ-plaques are absent, astrocytes become atrophic; when Aβ-plaques are present, astrocytes become hypertrophic surrounding the plaques.[169] As illustrated in Figure 27, no differences in the morphology of astrocytes (stained in red) were detected when samples from $A\beta_{1-42}$ rats were compared with those from CTL rats. As also showed in Figure 27, in both experimental groups,

microglia (stained in green) was in the ramified state, characterized by small cell bodies and numerous long branching processes, as typically happens under resting conditions.[170] This is in clear contrast with the known morphology of microglia under microgliosis, where retraction and thickness of microglia processes, as well as increase in the cell body, is expected to occur (amoeboid shape). [171–173]

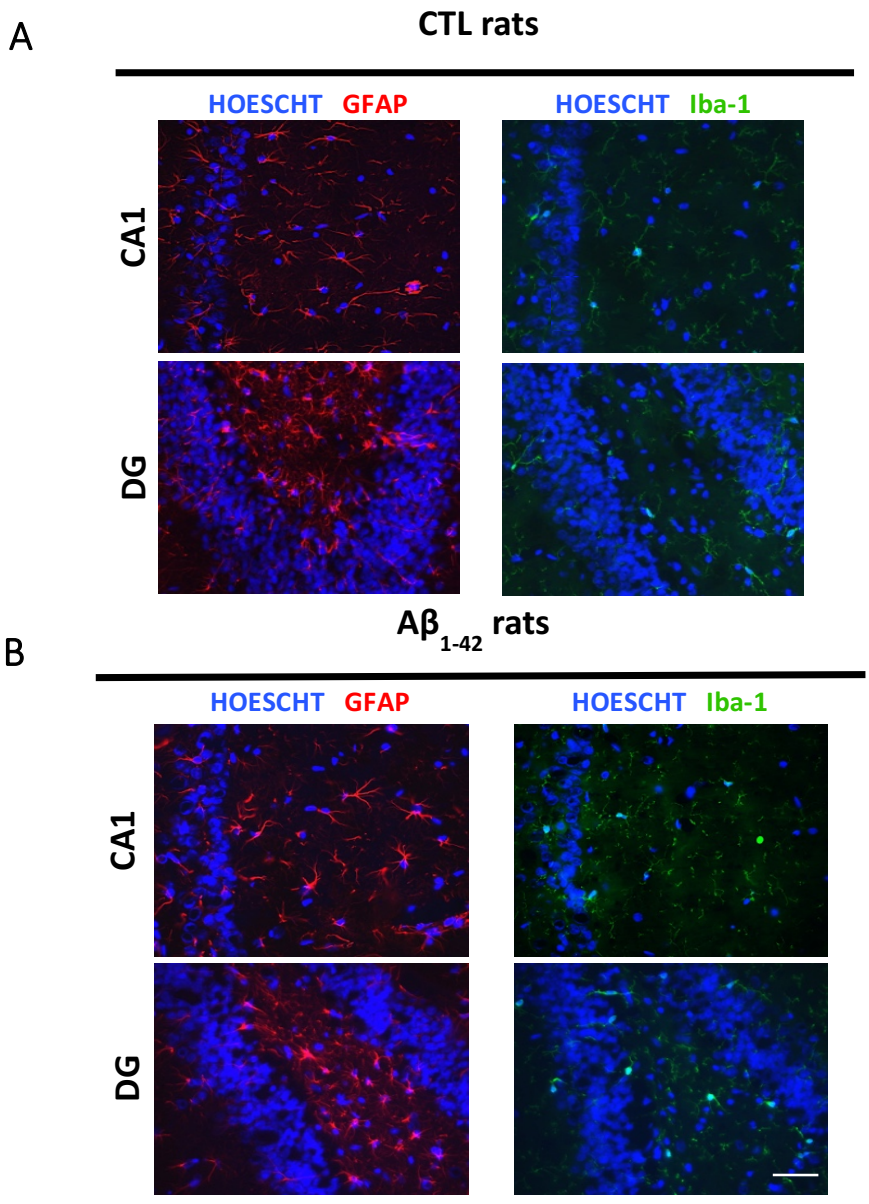


Figure 27. Detection of GFAP and Iba-1 in CA1 and DG hippocampal areas of Control (CTL) and $A\beta_{1-42}$ rats.

Representative images of Hoechst stained nucleus with GFAP stained astrocytes (red) or Iba-1 stained microglia (green) in **(A)** CTL rats and **(B)** $A\beta_{1-42}$ rats. There was no difference in GFAP and Iba-1 staining between the two conditions, suggesting absence of gliosis. Images were acquired on an inverted widefield fluorescence microscope (Zeiss Axiovert 200), with a 40x objective. Images representative of n=3 animals per group. Scale bar applies to all images, 50 µm.

GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; CA1, cornu ammonis *1; DG, dentate gyrus.*

To sum up, the animal model used in this work could mimic key hallmarks of AD: neuronal damage in the hippocampus associated with memory impairment. These changes occurred in the absence of marked neuroinflammation, since astrogliosis or microgliosis were not detected at the time of the analysis (21 days after i.c.v. surgery).

4.2. Impact of KTP-NH_2

After validating the animal model of AD, it was thus possible to test the effect of the prolonged treatment with KTP-NH_2. To do so, the same behavioural tests and molecular assays used to validate the animal model under our experimental conditions were performed. The results obtained are presented in the following sections.

4.2.1. KTP-NH_2 treatment mitigated the impairments in spatial working memory induced by $A\beta_{1-42}$ without affecting locomotor performance

In order to evaluate if the treatment with KTP-NH_2 could decrease the impairments in spatial working memory induced by $A\beta_{1-42}$, the animals were subjected to Y-Maze test.

Figure 28 shows the results of the performance of CTL and $A\beta_{1-42}$ rats, untreated and treated with KTP-NH_2, in the Y-Maze, where the percentage of spontaneous alternation was assessed. $A\beta_{1-42}$ rats had a lower percentage of spontaneous alternation compared with CTL rats ($A\beta_{1-42}$: 55 ± 3.6 *vs* CTL: 69 ± 2.0; n=6-7; P≤0.05, one-way ANOVA), indicating that the rats failed to successively alternate between the three arms of the maze, thus suggesting an impairment in spatial working memory. However, when the $A\beta_{1-42}$ rats were treated with KTP-NH_2 the percentage of spontaneous alternation in the Y-Maze was similar to CTL group ($A\beta_{1-42}$ KTP-NH_2: 67 ± 1.3; n=6; P>0.05, one-way ANOVA), which implies that this molecule reduced the deficits in spatial working memory induced by $A\beta_{1-42}$. Moreover, KTP-NH_2 alone had no measurable effects, since the percentage of spontaneous alternation of CTL KTP-NH_2 treated rats and of CTL rats was not statistically different (CTL KTP-NH_2: 68 ± 2.4; n=7, P>0.05, one-way ANOVA).

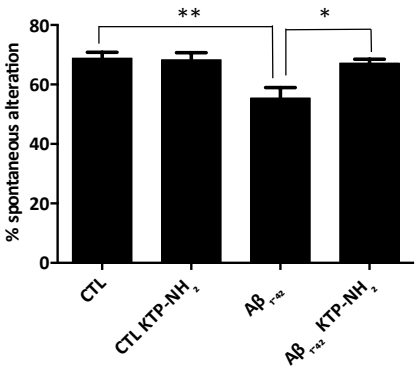


Figure 28. Performance in the Y-Maze test of Control (CTL) and $A\beta_{1-42}$ rats, treated with or without Amidated Kyotorphin (KTP-NH_2).

Spatial working memory of $A\beta_{1-42}$ rats untreated with KTP-NH_2 was impaired comparing with CTL rats and $A\beta_{1-42}$ KTP-NH_2 treated rats (n=6-7 animals per group; *P≤0.05, **P≤0.01, one way-ANOVA followed by Holm-Šídák's multiple comparisons test). Values are mean ± SEM.

Moreover, to determine if the locomotor activity was not affected by the treatment with KTP-NH_2, the total number of entries in the Y-Maze arms was analysed. No statistically significant differences were found between all animal groups in terms of total number of arm entries (n=6-7; P>0.05, one-way ANOVA) (Figure 29), indicating similarities of locomotion in all of the groups.

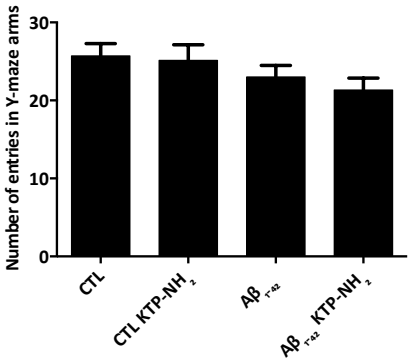


Figure 29. Number of entries in Y-Maze arms as a measure of locomotor performance of Control (CTL) and $A\beta_{1-42}$ rats, treated with or without Amidated Kyotorphin (KTP-NH_2).

Locomotor activity was similar among groups, since no statistically significant differences were found between all groups concerning the total number of arm entries (n=6-7 animals per group; NS P>0.05, one-way ANOVA). Values are mean ± SEM.

4.2.2. KTP-NH_2 treatment did not cause locomotor deficits and did not affect exploratory behaviour

To further confirm that the prolonged treatment with KTP-NH_2 had no effects in locomotor activity, the performance in the OF was evaluated. In addition, this test allowed to evaluate putative alterations in novel exploratory behaviour caused by the treatment with KTP-NH_2.

As shown in Figure 30, no statistically significant differences were found between all the experimental groups for the velocity parameter, time spent resting, total distance travelled and % of time spent in the periphery of the OF arena (n=6-7; P>0.05, one-way ANOVA).

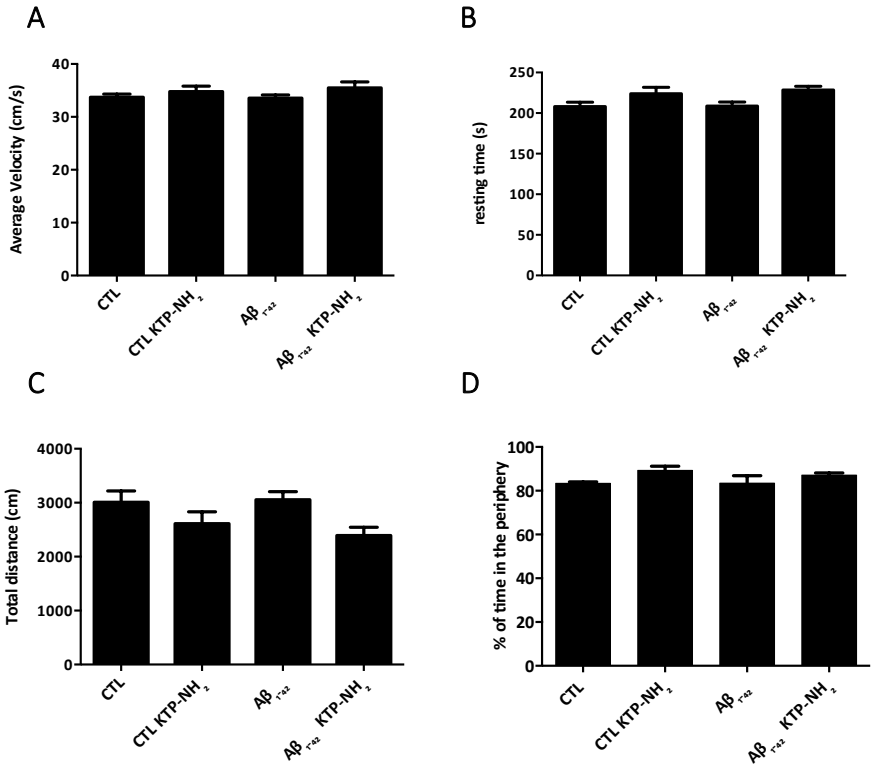


Figure 30. Locomotor performance and exploratory behaviour in the Open Field test of Control (CTL) and $A\beta_{1-42}$ rats, treated with or without Amidated Kyotorphin (KTP-NH_2).

Movement was video-recorded for a 5 min time period and data are shown as **(A)** average velocity, **(B)** time spent resting, **(C)** total distance travelled and **(D)** % of time spent in the periphery of the arena. No statistically significant differences between groups were found (n=6-7 animals per group; NS P>0.05, one-way ANOVA). Values are mean ± SEM.

4.2.3. KTP-NH_2 treatment attenuated episodic memory dysfunction induced by $A\beta_{1-42}$

To assess the consequences of chronic administration of KTP-NH_2 on episodic long-term memory, rats were evaluated in the NOR test.

As presented in Figure 31 (A), during the training phase (5 min), all the animals explored both objects similarly (F1 and F2), this being observed independently of which pair of objects was chosen as the familiar objects. Then, 24 h later, in the test phase (5 min), when CTL rats were exposure to the familiar object (F) and the novel one (N), they demonstrated a preference for explore during more time the novel object than the familiar one ($P \leq 0.05$; n=7; unpaired t-test). In contrast, $A\beta_{1-42}$ rats showed no preference for the novel object, since no statistically significant differences were found in the index of object preference between both objects ($P > 0.05$; n=6; unpaired t-test), indicating a loss of memory for the object to which they were exposed 24 h before. Interestingly, this memory impairment seems absent in $A\beta_{1-42}$ rats that had been treated with KTP-NH_2, since this group of animals have a clearly preference for explore more the novel object than the familiar one ($P \leq 0.05$, n=6; unpaired t-test), in the same way as CTL rats treated or not with KTP-NH_2 behaved.

Moreover, no statistically significant differences were found in the Object Recognition Index (Figure 31 (B)) between CTL rats treated with or without KTP-NH_2 and $A\beta_{1-42}$ rats treated with KTP-NH_2 (n=6-7; $P > 0.05$, one-way ANOVA), which demonstrates again that this treatment was beneficial in terms of amelioration of episodic long-term memory of $A\beta_{1-42}$ rats that received the drug.

Identical to what a higher object recognition index means, rats could discriminate between familiar and novel objects, preferring to explore more the novel one, as it can also be concluded by analysing the time spent exploring each object in the test phase (Figure 31 (C)). Therefore, preference for the novel object, demonstrated by an increase in the exploration time, indicates that a memory trace for the familiar object was properly encoded, consolidated and then retrieved to guide the behaviour during the test phase. In contrast, $A\beta_{1-42}$ rats not treated with KTP-NH_2 failed to discriminate the familiar object from the new one (F: 14 ± 3.3 *vs* N: 13 ± 1.9; n=6; $P > 0.05$, unpaired t-test), as can be concluded by the analysis of the exploration time for each object during 5 min (Figure 31 (C)).

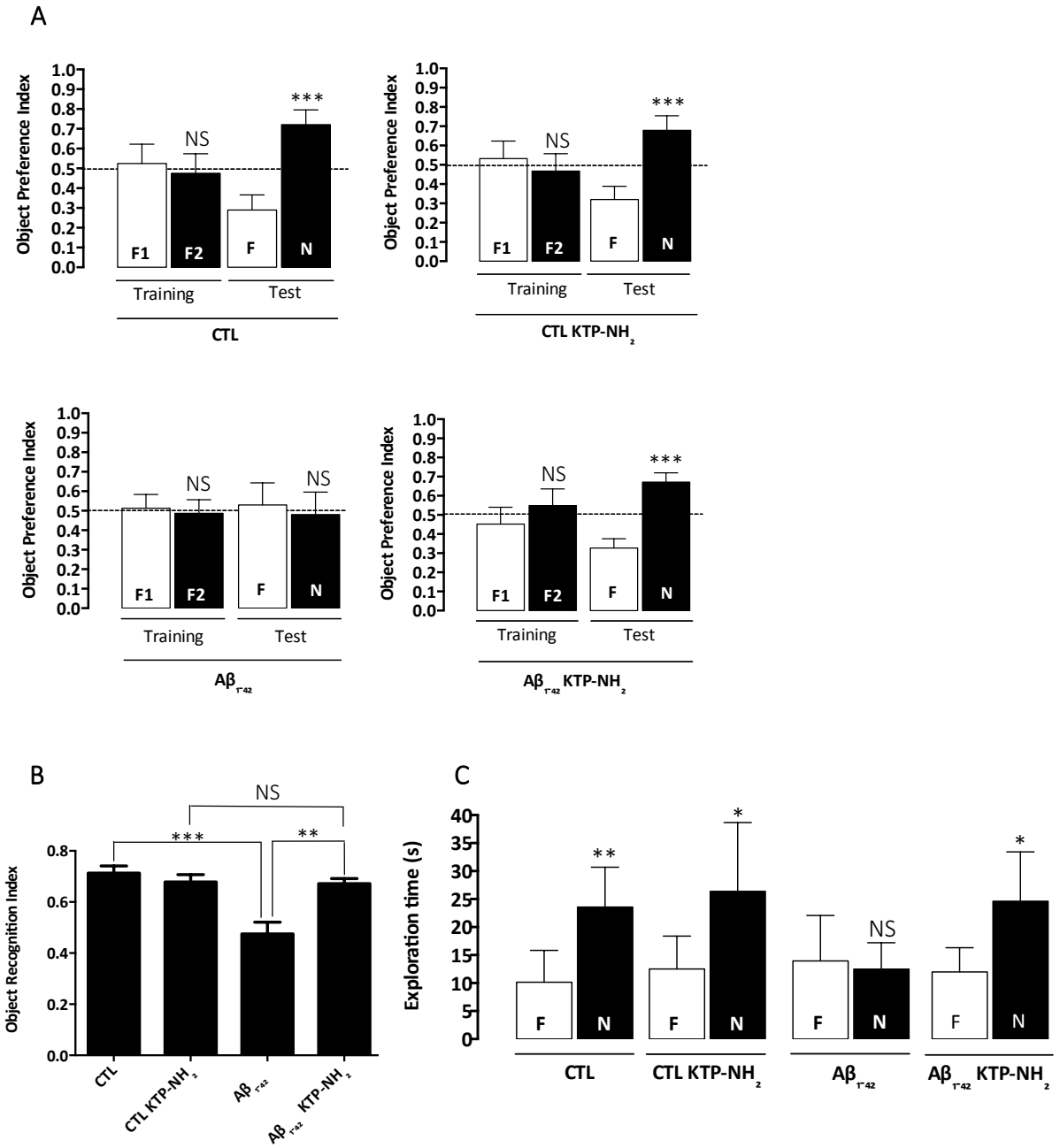


Figure 31. Performance in the Novel Object Recognition test of Control (CTL) and $A\beta_{1-42}$ rats, treated with or without Amidated Kyotorphin ($KTP\text{-}NH_2$).

(A) Object Preference Index is the ratio of time spent exploring any one of the two objects (F1 or F2) in training phase or the novel one (N) in test phase over the total time spent exploring both objects, i.e., F1 or F2/(F1 + F2) in the training phase and F or N/(F + N) in the test phase. Therefore, a preference index above 0.5 indicates novel object preference and below 0.5 indicates familiar object preference, wheraeas 0.5 represents no preference. Regarding the training phase, as expected, the animals did not show any preference between the two equal objects (F1 and F2). In the test phase, for $A\beta_{1-42}$ untreated rats, the data show that there was no preference between familiar (F) and novel (N) objects, but $A\beta_{1-42}$ treated rats had preference for the novel one, which means that $KTP\text{-}NH_2$ restored episodic long-term memory of the rats. **(B)** Object Recognition Index is calculated by the ratio between the time spent exploring a novel object over the total exploration time of both objects (novel + familiar). $A\beta_{1-42}$ did not induce memory impairment when the animals were treated with $KTP\text{-}NH_2$.

Figure 31. (Continuation) Performance in the Novel Object Recognition test of Control (CTL) and $A\beta_{1-42}$ rats, treated with or without Amidated Kyotorphin ($KTP\text{-}NH_2$).

(C) Exploration time spent by rats on the different (familiar (F) or new (N)) objects. $A\beta_{1-42}$ untreated rats showed signs of memory impairments since the time spent to explore each one of the two objects was similar, indicating that they did not discriminate between familiar and new objects.

(n=6-7 animals per group; NS P>0.05; *P≤0.05, **P≤0.01, ***P≤0.001, (A, C) unpaired t-test to compare within each group the time spent exploring each object, or (B) one-way ANOVA followed by Holm-Šídák's multiple comparisons test). Values are mean ± SEM.

4.2.4. $KTP\text{-}NH_2$ treatment rescued neurons from $A\beta_{1-42}$-induced damage

As mentioned previously, KTP has been pointed out as a putative neuroprotective molecule.[79,102–104] So, in order to assess if a prolonged treatment with a KTP derivative, $KTP\text{-}NH_2$, could avoid the neuronal damage , in CA1 and DG hippocampal areas, induced by i.c.v. injection of $A\beta_{1-42}$, the immunoreactivity of NeuN (neuronal marker) was evaluated.

As expected, $A\beta_{1-42}$ rats that did not receive $KTP\text{-}NH_2$ exhibited areas of neuron loss both in CA1 and DG, since zones deprived of both NeuN and Hoechst (nuclear marker) staining (indicated with arrows in Figure 32) could be clearly identified. Noteworthy, $KTP\text{-}NH_2$ treatment was able to reduce the neuronal injury caused by the administration of $A\beta_{1-42}$, since neuronal damage areas were less observed in $A\beta_{1-42}$ rats that received $KTP\text{-}NH_2$, in contrast with what occurred in $A\beta_{1-42}$ rats that did not receive $KTP\text{-}NH_2$ (Figure 32, (B)). No observable differences were found between CTL rats untreated or treated with $KTP\text{-}NH_2$ in both hippocampal areas (Figure 32, (A)), which indicates that this dipeptide *per se* does not affect neuronal viability.

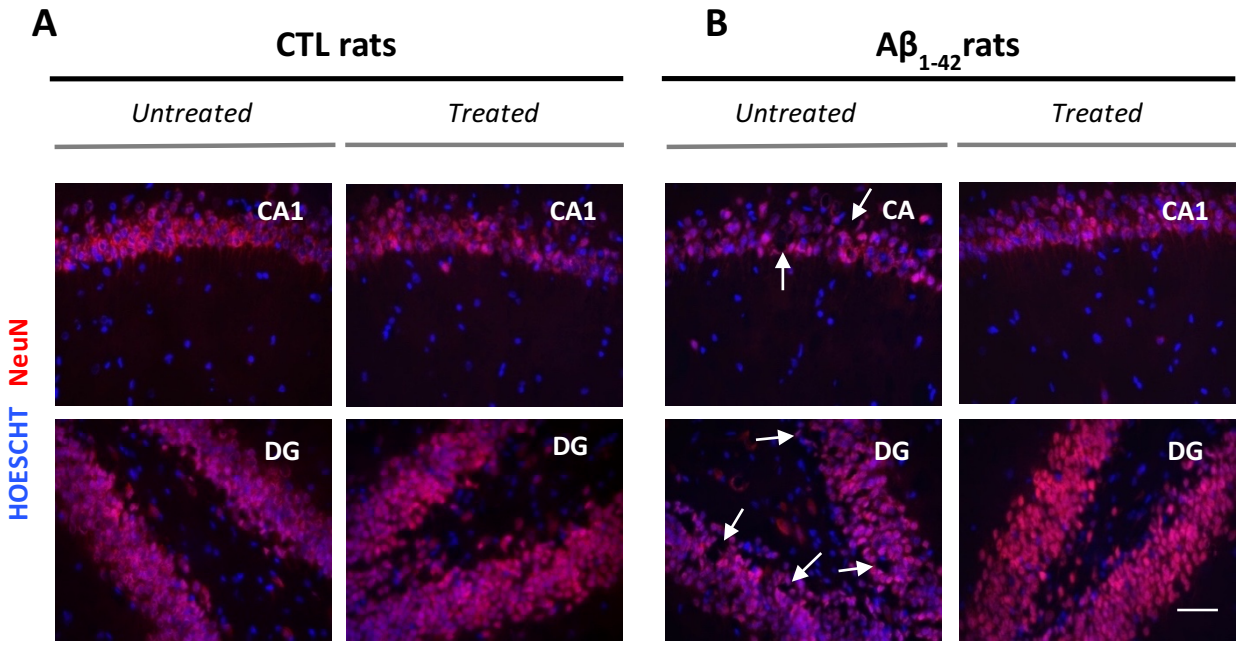


Figure 32. Detection of NeuN in CA1 and DG hippocampal areas of Control (CTL) and $A\beta_{1-42}$ rats, treated with or without Amidated Kyotorphin ($KTP\text{-}NH_2$).

Representative images of Hoechst stained nucleus (blue) with NeuN stained neurons (red) in **(A)** CTL rats and **(B)** $A\beta_{1-42}$ rats. In each condition, left and right panels represent untreated and treated animals with $KTP\text{-}NH_2$, respectively. The neuronal damage noticed in CA1 and DG areas of untreated $A\beta_{1-42}$ rats (indicated with arrows) decreased in treated $A\beta_{1-42}$ rats. Images were acquired on an inverted widefield fluorescence microscope (Zeiss Axiovert 200), with a 40x objective. Images representative of n=3 animals per group. Scale bar applies to all images, 50 µm.

NeuN, neuronal nuclear antigen; CA1, cornu ammonis *1; DG, dentate gyrus.*

Quantitative analysis of the fluorescence signal of NeuN (Figure 33) corroborates the significant decrease in NeuN content in $A\beta_{1-42}$ rats not treated with $KTP\text{-}NH_2$ compared with CTL rats, both in CA1 and DG hippocampal areas (**CA1 -** $A\beta_{1-42}$: 171 ± 17 *vs* CTL: 380 ± 20| **DG -** $A\beta_{1-42}$: 266 ± 10 *vs* CTL: 456 ± 34; n=3; P≤0.05, one-way ANOVA). The treatment of $A\beta_{1-42}$ rats with $KTP\text{-}NH_2$ avoided the neuron loss, since the NeuN fluorescence signal reverted to CTL values (**CA1:** 326 ± 19 | **DG:** 402 ± 30; n=3; P>0.05, one-way ANOVA, as compared with CTL). This analysis also confirmed that $KTP\text{-}NH_2$ alone was devoid of neurotoxic consequences (**CA1:** 369 ± 12 | **DG:** 440 ± 52; n=3; P>0.05, one-way ANOVA, as compared with CTL).

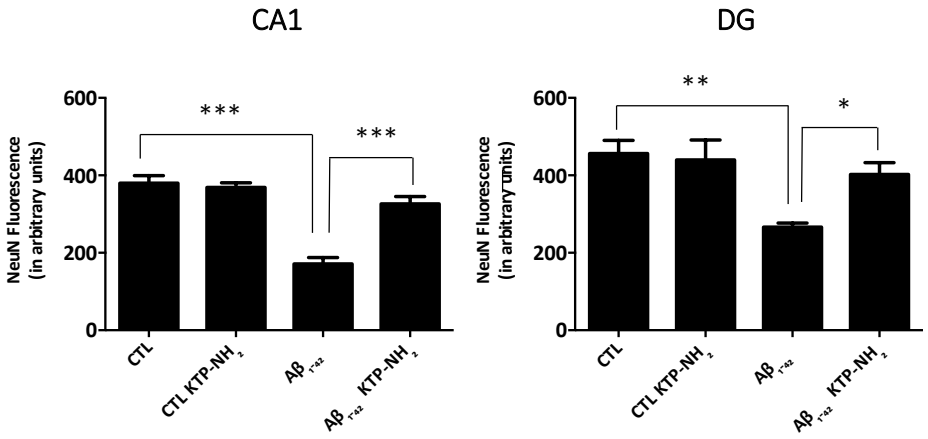


Figure 33. Quantification of the NeuN fluorescence signal in CA1 and DG hippocampal areas of Control (CTL) and $A\beta_{1-42}$ rats, treated with or without Amidated Kyotorphin ($KTP\text{-}NH_2$).

The quantification of NeuN signal in each hippocampal area is expressed in arbitrary units of fluorescence intensity. There was a significant decrease in NeuN signal in $A\beta_{1-42}$ rats compared with CTL rats, both in CA1 and DG areas, which reverted to control values when the animals were treated with $KTP\text{-}NH_2$, suggesting that this molecule decreased the neuron loss induced by $A\beta_{1-42}$ injection (n=3 animals per group; *P≤0.05; **P≤0.01; ***P≤ 0.001, one-way ANOVA). Values are mean ± SEM.

NeuN, neuronal nuclear antigen; CA1, cornu ammonis *1; DG, dentate gyrus.*

4.2.5. $KTP\text{-}NH_2$ treatment did not induce gliosis at the rat hippocampus

As mentioned previously, no signs of astrogliosis or microgliosis were detected in rats i.c.v. injected with $A\beta_{1-42}$. To evaluate the possibility that $KTP\text{-}NH_2$ could affect astrocytes and microglia proliferation a western blot assay was performed.

The western blot analysis of GFAP (marker of astrocytes) and Iba-1 (marker of microglia) is illustrated in Figure 34. GAPDH was used as loading control. Regardless the hippocampus analysed (left or right one), or if the animals were i.c.v. injected with $A\beta_{1-42}$ or vehicle, no differences in the expression of glial cells in response to $KTP\text{-}NH_2$ treatment (n=3-4; P>0.05, one-way ANOVA) were observed.

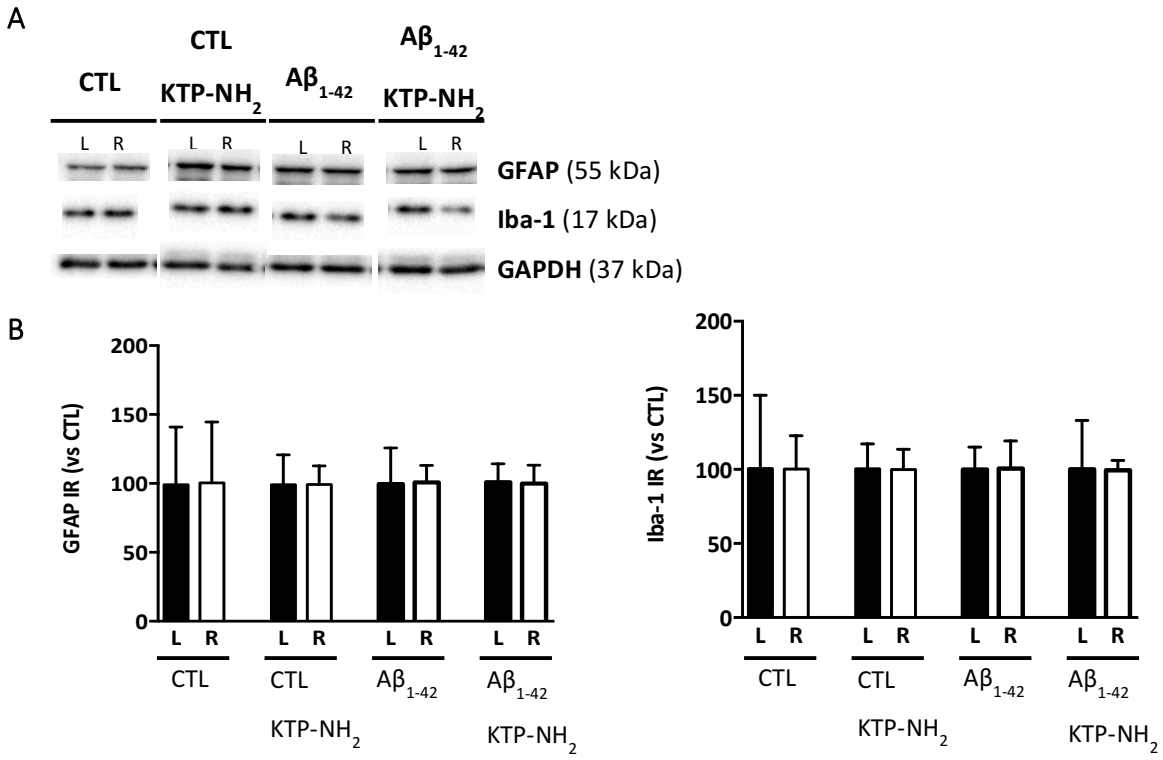


Figure 34. Western Blot analysis of GFAP and Iba-1 of Control (CTL) and $A\beta_{1-42}$ rats, treated with or without Amidated Kyotorphin ($KTP\text{-}NH_2$).

(A) Representative immunoblots of GFAP and Iba-1 of Left (L) and Right (R) hippocampus of CTL and $A\beta_{1-42}$ rats, untreated or treated with $KTP\text{-}NH_2$. GAPDH was used as loading control. **(B)** Densitometry analysis was performed with ImageJ software. No statistically significant differences were found regarding the GFAP (astrocyte marker) and Iba-1 (microglia marker) immunoreactivity (IR) between all conditions (n=3-4 animals per group; $P>0.05$, one-way ANOVA). Values are mean ± SEM.

GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

4.2.6. $KTP\text{-}NH_2$ treatment did not induce morphological changes in astrocytes and microglia at the rat hippocampus

Although no expression changes were detected in astrocytes and microglia, evaluated by the semi-quantitative WB technique, it was important to determine if the treatment with $KTP\text{-}NH_2$ had impact in the morphology of glial cells, which would be a sign of gliosis. To do so, the immunohistochemical staining for GFAP and Iba-1 was performed.

As exhibited in Figure 35, there was no evidence of astrogliosis (evaluated by GFAP immunoreactivity) or microgliosis (evaluated by Iba-1 immunoreactivity), neither in CTL rats or in $A\beta_{1-42}$ rats treated or untreated with the KTP derivative, since no morphologic differences in glial cells were noticeable, regardless the hippocampal area analysed (CA1 or DG).

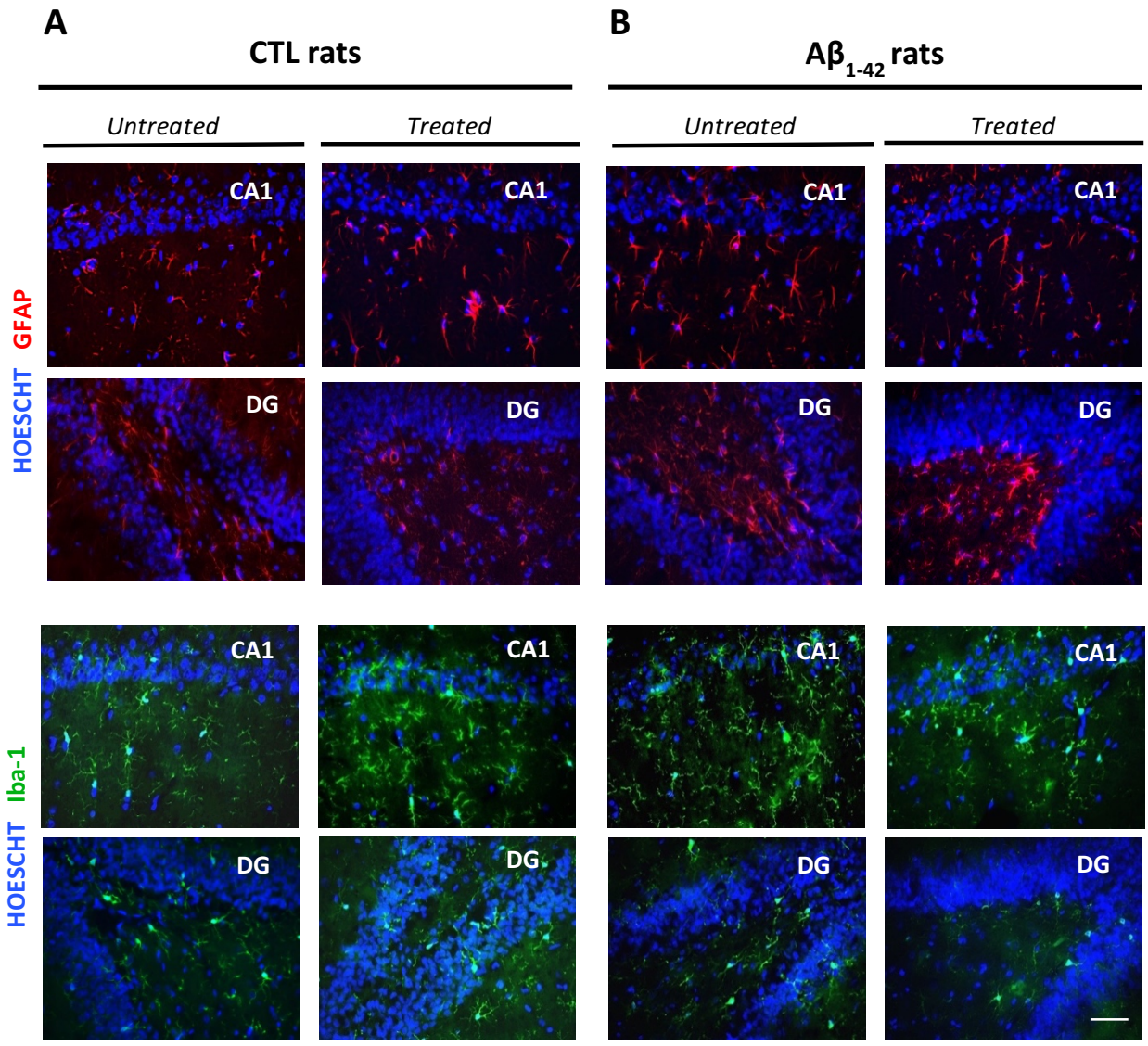


Figure 35. Detection of GFAP and Iba-1 in CA1 and DG hippocampal areas of Control (CTL) and $A\beta_{1-42}$ rats, treated with or without Amidated Kyotorphin ($KTP\text{-}NH_2$).

Representative images of Hoechst stained nucleus (blue) with GFAP stained astrocytes (red) and Iba-1 stained microglia (green), in **(A)** CTL rats and **(B)** $A\beta_{1-42}$ rats. In each condition, left and right panels represent untreated and treated animals with $KTP\text{-}NH_2$, respectively. There was no difference in GFAP and Iba-1 staining between conditions. Images were acquired on an inverted widefield fluorescence microscope (Zeiss Axiovert 200), with a 40x objective. Images representative of n=3 animals per group. Scale bar applies to all images, 50 µm.

GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; CA1, cornu ammonis *1; DG, dentate gyrus.*

In conclusion, the treatment with $KTP\text{-}NH_2$ decreased both the memory impairment and the neuron loss induced by i.c.v. injection of $A\beta_{1-42}$, without causing significant detectable changes in gliosis, both in CA1 and DG hippocampal areas at the time of analysis.

5. Discussion

The main findings of this work were:

(i) The i.c.v. injection of $A\beta_{1-42}$ induced impairments in spatial working memory and episodic long-term memory of the rats, without compromising their locomotor activity and without affecting the exploratory behaviour. Moreover, the administration of $A\beta_{1-42}$ caused neuronal damage at CA1 and DG hippocampal areas, without the occurrence of significant differences in gliosis.

(ii) The prolonged treatment with KTP-NH_2 improved memory impairments, without influencing locomotor activity and exploratory habits, as well as reduced neuronal injury induced by the administration of $A\beta_{1-42}$, without affecting gliosis. Moreover, KTP-NH_2 alone had no neurotoxic effects.

The results obtained while validating the animal model of AD were consistent with previous studies[117,123,165], using a similar animal model. Indeed, previous reports[117,123] showed that i.c.v. injection of $A\beta_{1-42}$ triggered a cascade of events leading to impairments in memory performance 15 days after surgery.

The first test applied to evaluate memory was the Y-Maze and the percentage of spontaneous alternation was assessed. The results obtained, besides being in concordance with a previous report[117], have a parallel in human studies that have evaluated spatial working memory in AD patients and found impairment of immediate visuospatial memory at early stages of the disease.[166,174] It was thus possible to recapitulate the deficits in spatial working memory in the animal model of AD. Nevertheless, it should be noticed that, in the type of Y-Maze test used, the presence of self-generated olfactory cues in the arm previously entered could indicate which of the two alternatives was not visited, without any significant reliance on memory.[126] Notwithstanding, this occurrence tend to be the exception rather than the rule, although some caution is necessary before taking any conclusions about the outcome of this test. Still, this test has the obvious advantage of avoiding excessive stressful handling of subjects as well as providing an additional useful measure of locomotor activity by counting the frequency of arm entries. Moreover, there is, in fact, a high incidence of studies in which the percentage of spontaneous alternation in the Y-Maze has been adopted as a measure of spatial working memory. The Y-Maze test based on spontaneous alternation behaviour continue to be a useful and popular test of responsiveness to novelty, but should not be performed as the only test to assess memory.[126]

Consequently, the NOR test was also performed, in which the episodic long-term memory was evaluated. Episodic memory loss is a defining feature of early-stage AD[175] and the NOR in rodents has

analogy in some ways to human declarative (episodic) memory[176], being increasingly considered as a useful tool for basic and preclinical research in the context of AD. Once again, animals i.c.v. injected with $A\beta_{1-42}$ showed deficits in the NOR test, which is also in concordance with a previous report[117].

Other test that was crucial to perform was the OF test. This test does not evaluate memory, but rather spontaneous locomotor activity and exploration of a novel environment. No significant differences were found regarding these two aspects, which is in agreement with a previous study[117]. In that study, they evaluated the locomotor activity and exploratory behaviour by analysing the number of crossings (frequency with which the rat crossed one of the grid lines with all four paws) and rearings (frequency with which the rat stood on its hind legs) in the OF arena, whereas in the present work the locomotor and exploratory behaviours was analysed using other parameters (average velocity, resting time, total distance travelled and % of time in the periphery of the OF arena). Nevertheless, this test reinforced the conclusion that the differences found in Y-Maze and NOR tests were not due to locomotor impairment. It is also important to mention that loss of motor function in AD patients is more evident in both moderate and severe stages of AD[177], and the animal model used in the present work better mimics a early stage of AD.

It was also observed that $A\beta_{1-42}$ rats, besides exhibiting memory impairments, displayed higher degrees of neuron loss at the hippocampus. Neuronal death was not observed in a previous report[117] that used a similar rat model of AD. One possible explanation for these dissimilar results is the fact that they assessed it 15 days after brain surgery, while in this work, the molecular studies were performed 21 days after the administration of $A\beta_{1-42}$. Additionally, it is important to mention that, in the present work, the neuronal damage was assessed by immunohistochemical staining for NeuN immunoreactivity and by the quantification of the immunofluorescence signal of NeuN, whereas in the previous study the neuronal morphology in hippocampal sections was evaluated by cresyl violet staining of Nissl bodies and degenerating neurons were assessed using Fluoro-Jade C, which fluorescently labels neurons independently of the mechanism of cell death.[117] Furthermore, they only evaluated neuronal injury at CA1 and CA3 hippocampal areas, while I evaluated, in addition to CA1 area, the DG area, which had a more pronounced neuronal injury than CA1.

Since $A\beta_{1-42}$ injected models commonly manifest considerable gliosis which, in some studies, has been linked to neuronal damage[168], we decided to evaluate if, in the experimental conditions used in the present work, $A\beta_{1-42}$ injection could also cause astrogliosis and microgliosis. Gliosis was evaluated by both immunohistochemistry technique and western blotting. In agreement with a previous report[117], no signs of gliosis evaluated by the immunohistochemistry detection of GFAP (a marker of astrocytes) and by the immunohistochemistry detection of Iba-1 (a marker of microglia) were detected,

independently of the hippocampus analysed , in spite of the different time points chosen to assess it (21 days after brain surgery in the present work *vs* 15 days in the previous study). Actually, it has been reported that the pick of the inflammatory response is one week after injection.[168] At day 3 following $A\beta_{1-42}$ injection, microglial and astrocytic cells are localized in the vicinity of the aggregates and exhibit characteristic properties of an activated phenotype.[168] So, I cannot exclude the possibility that neuroinflammation had occurred in some point of the neurodegeneration, but, if any, it has already remitted at the time of analysis. In this context, it is important refer that, neuroinflammation is not a hallmark of AD in humans.[169]

Absence of signs of astrogliosis or microgliosis at day 21 after i.c.v. injection was also confirmed by western blot. This technique allows to assess changes in the proliferation of the cells, independently of the morphological modifications. Note that, despite Iba-1 is widely employed as an marker for both ramified and activated microglia, Iba-1 staining can be used in western blot analysis for measuring microglial activation, especially when microglia is strongly activated and/or microglia number is substantially increased.[148] A word of caution has to be introduced in what concerns the loading control used in the present work: it has been reported[178] that GAPDH in AD can undergo many different oxidative modifications, which influence its structure and activity, potentially causing a decrease in the capacity of detecting any differences in western blot analysis. However, no consistent differences were found in the densitometry of GAPDH bands between sham-operated and $A\beta_{1-42}$-injected rats.

After validating the animal model, I tested the action of the drug of interest demonstrating, for the first time, that KTP-NH_2 has a neuroprotective role in an animal model of AD.

It is important to mention that the treatment with this molecule did not start after the appearance of memory impairments in rat models i.c.v. injected with $A\beta_{1-42}$ (2 weeks after surgery)[117,123], but instead 2 days after brain surgery, since I wanted to evaluate the potential of this KTP derivative in early stages of the disease, i.e. in cases where subjects are at high risk of developing AD. Indeed, Aβ deposition has been reported in humans well before cognitive signs of AD.[179] Therapies that have just been administered too late in the disease process, when the damage related to the proposed amyloid cascade is already irreversible, typically have not a significant impact in AD, being urgent to find new treatment approaches focused on a pre-symptomatic phase or on a prodromal phase (also known as Mild Cognitive Impairment – MCI) of the disease.[180] Therefore, the identification of the specific clinical features of early AD is increasingly relevant and will definitely lead to the development of more effective management strategies than those currently available.

Indeed, the early administration of the KTP derivative was proven to be extraordinarily effective in the amelioration of both spatial working memory and even more impressively of episodic long-term memory impairments induced by $A\beta_{1-42}$ i.c.v. injection.

Actually, the NOR test is more sensitive than the Y-Maze to detect alteration in animal behaviour and that is the reason why this test has being widely used to evaluate the influence of several drugs in episodic memory and recognition.[131] In addition, many indexes can be assessed in this test and, in the present work, the two indexes evaluated (Preference and Recognition index) demonstrated that the KTP-NH_2 treatment was beneficial in restoring the episodic long-term memory impairments induced by $A\beta_{1-42}$ administration.

In terms of locomotor activity, no differences were detected between rats treated with vehicle solution or KTP-NH_2, which is in agreement with a previous report[105], showing no signs of motor dysfunction in animals treated with KTP-NH_2. This is an important outcome since it allows to suggest that the differences in memory performance between $A\beta_{1-42}$ rats treated or not treated with KTP-NH_2 were not due to eventual differences in locomotor activity. In addition, it is also desirable that this drug does not induce *impairment of locomotor activity* as a secondary effect. Moreover, this study also indicates that KTP-NH_2 does not possess intrinsic anxiolytic properties and/or did not act on key areas responsible for this behaviour, since no statistically significant differences were found between the animal groups by analysing the % of time in the periphery of the OF arena.

Another interesting aspect of the present work was the confirmation of the neuroprotective effect of KTP derivative evaluated by immunohistochemistry and corroborated by quantification of the immunofluorescent signal of a neuronal marker. Therefore, one can strongly suggest that the improved memory abilities seen in the KTP-NH_2 -treated $A\beta_{1-42}$ rats was due to the neuroprotective effects of the drug. Actually, KTP-NH_2 decreased, in a significant manner, the neuronal loss (at CA1 and DG hippocampal areas) induced by i.c.v. injection of $A\beta_{1-42}$, without effecting gliosis, since neither signs of astrogliosis nor microgliosis were detected in the rats hippocampus using both immunohistochemistry and western blotting techniques.

One of the problems of AD therapy is usually the design of selective compounds without undesirable and potentially toxic side effects, making difficult to have ideal candidates to reach the stage of clinical testing. Data in this work indicated that KTP-NH_2 alone did not affect neuronal viability and did not act as a cognitive enhancer.

Thus, overall findings show that KTP-NH_2 may act in key brain areas as a neuroprotective molecule, without apparent neurotoxic effects, being potentially useful in the treatment of early phases of AD. Consequently, the obvious burning question is: what are the mechanisms by which KTP and its derivatives confer neuroprotection?

In fact, until now, the non-analgesic mechanisms of KTP action remain poorly investigated. However, as stated in 'Introduction', a growing body of evidence suggests that KTP may have

neuroprotective effects, namely by blocking voltage-dependent Ca^{2+} channels in Mauthner neurons of the medulla oblongata of goldfish, as well as in other neurons.[102]

Indeed, there is a strong evidence that intracellular Ca^{2+} dysregulation plays an important pathological role in AD and specifically that Aβ may induce increases in intracellular Ca^{2+} and lead to neuronal cell dysfunction and death.[181] Ca^{2+} is an intracellular second messenger in many cell types, and regulates many complex cellular processes, including cell activation, proliferation and apoptosis.[182] Maintenance of the precise intracellular Ca^{2+} homeostasis is fundamental to neuronal viability and functioning.[181] Mechanisms that perturb normal neuronal Ca^{2+} homeostasis include aberrant Ca^{2+} influx through plasma membrane channels, namely (i) the Voltage-dependent Ca^{2+} channels (VDCCs) that serve as the principal routes of Ca^{2+} entry into electrically excitable cells such as neurons and (ii) NMDA receptors.[183,184] Note that at presynaptic terminals, VDCCs (N and P/Q types) mediate the release of neurotransmitter upon arrival of action potentials.[185] L-type VDCCs (L-VDCCs) are found in electrically excitable neurons, become activated by strong membrane depolarization and serve as the principal routes of Ca^{2+} entry into neurons due to their slow deactivation rate.[182] A release of glutamate (the most important transmitter for normal brain function, involved in cognitive functions such as learning and memory), at central synapses, facilitates Ca^{2+} entry at postsynaptic sites through NMDA receptors and indirectly through L-type Ca^{2+} channels.[185] Excessive glutamate release leads to an imbalance of the postsynaptic Ca^{2+} load, triggering intracellular cascades that finally cause neuronal death.[185] This process, termed 'excitotoxicity', has been proposed to underlie the pathology of a variety of neurodegenerative disorders, including AD.[185–187] Excitotoxicity can be attenuated by blocking Ca^{2+} influx in both synaptic terminals, as well as postsynaptic sites.[185]

Thus, treatments that interrupt aberrant Ca^{2+} influx may be promising therapeutic strategies for AD. As mentioned in 'Introduction', Memantine, an antagonist of the NMDA receptors, improves cognition and reduces AD-like neuropathology in various animal models of AD and has beneficial effects in AD patients. On the other hand, in spite of the usefulness of L-VDCC blockers against AD pathology being still controversial, epidemiological studies have been suggesting that L-VDCC blockers can, in fact, prevent or slow the rate of progression of AD.[182,185,188,189]An example of a promisor molecule that acts as an L-VDCC blocker is Isradipine, that is approved for human use in the treatment of hypertension and is currently in use in clinical trials for Parkinson's disease. This drug demonstrates a neuroprotective effect at concentrations that are clinically relevant and achievable *in vitro* and *in vivo*, and it has been suggested that this particular calcium blocking agent may also have therapeutic value in the treatment of AD.[188,189]

Consequently, the KTP derivative used in the present work could act as voltage-dependent Ca^{2+} channel blocker, thus being useful in the AD treatment, since Ca^{2+} channel blockers may prevent some damage by buffering excessive Ca^{2+} influx through the plasma membrane. Yet, other cascades are also

likely to play an important role in AD and it is unlikely that Ca^{2+} channel blockers will attenuate those cascades. The ultimate treatment regimen may be a multidrug approach – perhaps combining therapeutics that already are used for AD with those decreasing Ca^{2+} entry by blocking voltage-dependent Ca^{2+} channels.

In addition, KTP-NH_2 could exhibit neuroprotective actions due to its anti-epileptic effect, which has also been reported.[98,99] In fact, there are studies that suggest that antiepileptic drugs may be effective at reversing memory loss in AD patients.[190–192]

Despite the evidences that the Aβ may trigger also neuronal hyperexcitability and seizures[193], these features were not analysed in the present work, being outside its scope. Nevertheless, the putative anti-epileptic action of KTP and its derivatives could confer in some way neuroprotection in the present experimental conditions.

In summary, independently of the mechanisms of action of KTP-NH_2, which could not be analysed in the time span of the present work, it became clear that this KTP derivative has the potential to be used as a neuroprotective molecule in early stages of AD.

6. Conclusion and Future Perspectives

In the present work, a kyotorphin derivative (KTP-NH_2), which can cross the BBB, was studied regarding its ability to mitigate memory dysfunction and neuronal damage caused by i.c.v. injection of $A\beta_{1-42}$ in male Wistar adult rats ($A\beta_{1-42}$ rats). The overall findings revealed that KTP-NH_2 was beneficial for reducing neuron injury at the hippocampus and for improving spatial working memory and episodic long-term memory abilities of $A\beta_{1-42}$ rats.

Therefore, this work suggests that KTP-NH_2 can be a drug candidate for the treatment of early phases of AD.

Indeed, the current understanding of AD pathogenesis indicates that early therapeutic intervention is likely to be needed.[194] This raises key challenges, such as how to identify patients who are likely to develop AD early enough in the course of the disease and how to monitor the effect of any intervention on disease progression.[194] It is worthwhile to note that Aβ deposition could be detected in the human brain even before any signs of disease.[179] In those cases it could be important to prevent symptoms appearance with a drug that proved to avoid memory impairment and neuronal loss caused by Aβ.

It is also worthy to mention that a single cure for AD is unlikely to be found. New information on pieces of the complex AD puzzle from preclinical research might mean that networks of interactions instead of single potential drug targets can be identified.[55] In fact, multi-target therapies can be designed in several ways.[195] The most conventional strategy is to prescribe several individual drugs. This approach is already used in AD, where acetylcholinesterase inhibitors can be given together with NMDA receptor antagonists for better symptomatic effects.[55]

Further studies are needed to unveil the pharmacodynamics aspects of KTP-NH_2, i.e. to identify its molecular targets. This would help to predict if this molecule better acts separately or if its neuroprotective action could be boosted if combined with other drugs already prescribed in AD and acting at different targets. Nevertheless, there are many examples of drugs that were developed long before their targets were known (e.g., aspirin and penicillin).[196] To understand the molecular targets of KTP-NH_2, a possibility would be to evaluate if it could modify the activity of NMDA receptors and/or VDCC by performing patch clamp recordings from hippocampal neurons. In addition, the influence of KTP-NH_2 upon intracellular free Ca^{2+} concentration could be assessed by using calcium imaging. Whether KTP derivatives affect other hallmarks of excitotoxicity, as enhanced glutamate release and decreased glutamate transport activity should also be evaluated.

Other studies can be performed in order to dissect the full potential of this molecule. It is necessary to study the pharmacokinetics of KTP to rationally define a therapeutic drug scheme. For

instance, it could be interesting to measure the plasmatic concentration of the KTP-NH_2 at different time points.

It is also important to test KTP derivatives in other animal models of AD and to use other methodologies to evaluate neuronal death and other types of memory tests too.

In addition, it is noteworthy to test other derivatives of KTP that are also able to cross the BBB. For instance, IbKTP-NH_2 (Amidated Kyotorphin linked to Ibuprofen, a nonsteroidal anti-inflammatory drug (NSAID)) can be interesting to test, despite some investigations about the therapeutic value of Ibuprofen in AD are contradictory, with some researchers suggesting a protective benefit for NSAID use in the context of AD and others showing no benefit.[197,198]

Since it has been reported that patients with AD, by failing to report pain, end up suffering from chronic pain and also that KTP has decreased levels in the CSF of AD patients, a dual neuroprotective analgesic action in a single drug would be of utmost importance.[79] Therefore, it could be also interesting to evaluate the nociception response of $A\beta_{1-42}$ rats not treated with KTP-NH_2 and compared with the ones that received the molecule. To do so, the Hot-plate test, which evaluates thermal pain reflexes due to footpad contact with a heated surface, could be performed.

AD has also emerged as a progressive disorder in which a decline in brain connectivity and disruption of synaptic plasticity are believed to occur in an early phase of the disease process, prior to extensive neuronal degeneration, and this correlates well with the cognitive decline observed in AD patients. Therefore, it is also important to evaluate if the animal model used in the present work can recapitulate the synaptotoxic effects induced by $A\beta_{1-42}$ (namely, long-term potentiation (LTP) inhibition in the CA1 region of the hippocampus, dendritic spine shrinkage and long-term depression (LTD) facilitation) and, consequently, if the treatment with KTP-NH_2 can decrease these effects.

In conclusion, the present work shows that a KTP derivative can have beneficial effects on major hallmarks of AD, thus highlighting a new possibility for AD treatment. However, much more has to be known on the mechanisms of action of this drug to maximally optimize the KTP-NH_2 therapeutic potential.

7. References

1'. Richards, M., Brayne, C. What do we mean by Alzheimer's disease? *BMJ*; 341:c4670 (2010).

1. Alzheimer, A., Stelzmann, R. A., Schnitzlein, H. N. & Murtagh, F. R. An English translation of Alzheimer's 1907 paper, 'Uber eine eigenartige Erkankung der Hirnrinde'. *Clin. Anat.* 8, 429–31 (1995).

2. WHO. *Dementia, a Public Health Priority.* (Geneva: World Health Organization., 2012).

3. Association, A. 2014 Alzheimer's disease facts and figures. *Alzheimer's Dement.* 10, e47–e92 (2014).

4. Citron, M. Alzheimer's disease: strategies for disease modification. *Nat. Rev. Drug Discov.* 9, 387–398 (2010).

5. Herrup, K. The case for rejecting the amyloid cascade hypothesis. *Nat Neurosci* 18, 794–799 (2015).

6. Sheng, M., Sabatini, B. L. & Su, T. C. Synapses and Alzheimer ' s Disease. (2015).

7. Benedikz, E., Kloskowska, E. & Winblad, B. The rat as an animal model of Alzheimer's disease. *J. Cell. Mol. Med.* 13, 1034–1042 (2009).

8. Corbett, A. *et al.* Drug repositioning for Alzheimer's disease. *Nat. Rev. Drug Discov.* 11, 833–846 (2012).

9. Bird, T. D. *Alzheimer Disease Overview*. *GeneReviews(®)* (1993). at <http://www.ncbi.nlm.nih.gov/pubmed/20301340>

10. *PLANO NACIONAL DE INTERVENÇÃO ALZHEIMER*. (2009).

11. Bagheri, M., Joghataei, M.-T., Mohseni, S. & Roghani, M. Genistein ameliorates learning and memory deficits in amyloid β(1–40) rat model of Alzheimer's disease. *Neurobiol. Learn. Mem.* 95, 270–276 (2011).

12. Findeis, M. A. The role of amyloid β peptide 42 in Alzheimer's disease. *Pharmacol. Ther.* 116, 266–286 (2007).

13. Menting, K. W. & Claassen, J. A. H. R. Î^{2}-secretase inhibitor; a promising novel therapeutic drug in Alzheimerâ€^{TM}s disease. *Front. Aging Neurosci.* 6, (2014).

14. Ballard, C. *et al.* Alzheimer's disease. *Lancet* 377, 1019–1031 (2011).

15. Hunter, S., Arendt, T. & Brayne, C. The Senescence Hypothesis of Disease Progression in Alzheimer Disease: an Integrated Matrix of Disease Pathways for FAD and SAD. *Mol. Neurobiol.* 48, 556–570 (2013).

16. Cruts, M. & Van Broeckhoven, C. Molecular genetics of Alzheimer's disease. *Ann. Med.* 30, 560–5 (1998).

17. Götz, J. & Ittner, L. M. Animal models of Alzheimer's disease and frontotemporal dementia. *Nat. Rev. Neurosci.* 9, 532–544 (2008).
18. Janota, C., Lemere, C. A. & Brito, M. A. Dissecting the Contribution of Vascular Alterations and Aging to Alzheimer's Disease. *Mol. Neurobiol.* (2015). doi:10.1007/s12035-015-9319-7
19. Rohn, T. Proteolytic Cleavage of Apolipoprotein E4 as the Keystone for the Heightened Risk Associated with Alzheimer's Disease. *Int. J. Mol. Sci.* 14, 14908–14922 (2013).
20. Blennow, K., de Leon, M. J. & Zetterberg, H. Alzheimer's disease. *Lancet (London, England)* 368, 387–403 (2006).
21. Yu, J.-T., Chang, R. C.-C. & Tan, L. Calcium dysregulation in Alzheimer's disease: from mechanisms to therapeutic opportunities. *Prog. Neurobiol.* 89, 240–55 (2009).
22. Selkoe, D. J. Alzheimer's Disease Is a Synaptic Failure. *Science (80-.).* 298, 789–791 (2002).
23. Selkoe, D. J. Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* 399, A23–A31 (1999).
24. Šalković-Petrišić, M. Amyloid cascade hypothesis: is it true for sporadic Alzheimer's disease. *Period. Biol.* 110, 17–25 (2008).
25. Selkoe, D. J. The molecular pathology of Alzheimer's disease. *Neuron* 6, 487–498 (1991).
26. Karran, E., Mercken, M. & De Strooper, B. The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nat. Rev. Drug Discov.* 10, 698–712 (2011).
27. Hardy, J. The Amyloid Hypothesis of Alzheimer's Disease: Progress and Problems on the Road to Therapeutics. *Science (80-.).* 297, 353–356 (2002).
28. Moreno-TreviÃ±o, M. G., Castillo-LÃ³pez, J. & Meester, I. Moving Away from Amyloid Beta to Move on in Alzheimer Research. *Front. Aging Neurosci.* 7, 1–4 (2015).
29. Musiek, E. S. & Holtzman, D. M. Three dimensions of the amyloid hypothesis : time , space and ' wingmen '. 18, (2015).
30. Lecanu, L. & Papadopoulos, V. Modeling Alzheimer ' s disease with non-transgenic rat models. *Alzheimer's Dement.* 5, 1–10 (2013).
31. Newman, M., Ebrahimie, E. & Lardelli, M. Using the zebrafish model for Alzheimer's disease research. *Front. Genet.* 5, (2014).
32. Gibbs, R. A. *et al.* Genome sequence of the Brown Norway rat yields insights into mammalian evolution. *Nature* 428, 493–521 (2004).
33. Jacob, H. J. & Kwitek, A. E. Rat genetics: attaching physiology and pharmacology to the genome. *Nat. Rev. Genet.* 3, 33–42 (2002).
34. Lin, J. H. Species similarities and differences in pharmacokinetics. *Drug Metab. Dispos.* 23, 1008–21 (1995).

35. Do Carmo, S. & Cuello, A. Modeling Alzheimer's disease in transgenic rats. *Mol. Neurodegener.* 8, 37 (2013).
36. Giacobini, E. Cholinesterase inhibitors: new roles and therapeutic alternatives. *Pharmacol. Res.* 50, 433–40 (2004).
37. Malin, D. H. *et al.* Hippocampal injections of amyloid beta-peptide 1-40 impair subsequent one-trial/day reward learning. *Neurobiol. Learn. Mem.* 76, 125–37 (2001).
38. Shi, X. *et al.* Rat hippocampal proteomic alterations following intrahippocampal injection of amyloid beta peptide (1-40). *Neurosci. Lett.* 500, 87–91 (2011).
39. Cetin, F. & Dincer, S. The effect of intrahippocampal beta amyloid (1-42) peptide injection on oxidant and antioxidant status in rat brain. *Ann. N. Y. Acad. Sci.* 1100, 510–517 (2007).
40. Ghasemi, R., Zarifkar, A., Rastegar, K., Maghsoudi, N. & Moosavi, M. Repeated intra-hippocampal injection of beta-amyloid 25–35 induces a reproducible impairment of learning and memory: considering caspase-3 and MAPKs activity. *Eur. J. Pharmacol.* 726, 33–40 (2014).
41. Miguel-Hidalgo, J. J. & Cacabelos, R. Beta-amyloid(1-40)-induced neurodegeneration in the rat hippocampal neurons of the CA1 subfield. *Acta Neuropathol.* 95, 455–65 (1998).
42. Hadlow, W. Criteria for development of animal models of diseases of the nervous system. *Am. J. Pathol.* 101.3 Supp, S213–S219 (1980).
43. Yau, S., Li, A. & So, K.-F. Involvement of Adult Hippocampal Neurogenesis in Learning and Forgetting. *Neural Plast.* 2015, 1–13 (2015).
44. *The Hippocampus Book*. (Oxford University Press, 2006). doi:10.1093/acprof:oso/9780195100273.001.0001
45. Mu, Y. & Gage, F. H. Adult hippocampal neurogenesis and its role in Alzheimer's disease. *Mol. Neurodegener.* 6, 85 (2011).
46. Li, Y., Mu, Y. & Gage, F. H. Development of neural circuits in the adult hippocampus. *Curr. Top. Dev. Biol.* 87, 149–74 (2009).
47. Bartsch, T. & Wulff, P. The hippocampus in aging and disease: From plasticity to vulnerability. *Neuroscience* 309, 1–16 (2015).
48. Tayeb, H. O., Yang, H. D., Price, B. H. & Tarazi, F. I. Pharmacotherapies for Alzheimer's disease: beyond cholinesterase inhibitors. *Pharmacol. Ther.* 134, 8–25 (2012).
49. Warburton, E. C. *et al.* Cholinergic neurotransmission is essential for perirhinal cortical plasticity and recognition memory. *Neuron* 38, 987–96 (2003).
50. Anand, R., Gill, K. D. & Mahdi, A. A. Therapeutics of Alzheimer's disease: Past, present and future. *Neuropharmacology* 76 Pt A, 27–50 (2014).
51. Dumas, J. A. & Newhouse, P. A. The cholinergic hypothesis of cognitive aging revisited again: cholinergic functional compensation. *Pharmacol. Biochem. Behav.* 99, 254–61 (2011).

52. Memantine | ALZFORUM. at <http://www.alzforum.org/therapeutics/memantine>
53. Esposito, Z. *et al.* Amyloid β, glutamate, excitotoxicity in Alzheimer's disease: are we on the right track? *CNS Neurosci. Ther.* 19, 549–55 (2013).
54. Mullane, K. & Williams, M. Alzheimer's therapeutics: continued clinical failures question the validity of the amyloid hypothesis-but what lies beyond? *Biochem. Pharmacol.* 85, 289–305 (2013).
55. Mangialasche, F., Solomon, A., Winblad, B., Mecocci, P. & Kivipelto, M. Alzheimer's disease: clinical trials and drug development. *Lancet. Neurol.* 9, 702–16 (2010).
56. Schneider, L. S. *et al.* Clinical trials and late-stage drug development for Alzheimer's disease: an appraisal from 1984 to 2014. *J. Intern. Med.* 275, 251–83 (2014).
57. Castellani, R. J. & Perry, G. Pathogenesis and disease-modifying therapy in Alzheimer's disease: the flat line of progress. *Arch. Med. Res.* 43, 694–8 (2012).
58. Hefti, F. *et al.* The case for soluble Aβ oligomers as a drug target in Alzheimer's disease. *Trends Pharmacol. Sci.* 34, 261–6 (2013).
59. Folch, J. *et al.* Masitinib for the treatment of mild to moderate Alzheimer's disease. *Expert Rev. Neurother.* 15, 587–96 (2015).
60. Iqbal, K. & Grundke-Iqbal, I. Alzheimer's disease, a multifactorial disorder seeking multitherapies. *Alzheimers. Dement.* 6, 420–4 (2010).
61. Cummings, J. L., Morstorf, T. & Zhong, K. Alzheimer's disease drug-development pipeline: few candidates, frequent failures. *Alzheimers. Res. Ther.* 6, 37 (2014).
62. Santos, S., Torcato, I. & Castanho, M. a. R. B. Biomedical applications of dipeptides and tripeptides. *Biopolymers* 98, 288–293 (2012).
63. Hancock, R. E. W. & Sahl, H.-G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* 24, 1551–1557 (2006).
64. Sun, L. Peptide-Based Drug Development. *Mod. Chem. Appl.* (2013). doi:10.4172/mca.1000e103
65. Uhlig, T. *et al.* The emergence of peptides in the pharmaceutical business: From exploration to exploitation. *EuPA Open Proteomics* 4, 58–69 (2014).
66. Craik, D. J., Fairlie, D. P., Liras, S. & Price, D. The future of peptide-based drugs. *Chem. Biol. Drug Des.* 81, 136–47 (2013).
67. Lax, R. The Future of Peptide Development in the Pharmaceutical Industry. *PharManufacturing Int. Pept. Rev.* 10-15, (2010).
68. Khafagy, E. S. & Morishita, M. Oral biodrug delivery using cell-penetrating peptide. *Advanced Drug Delivery Reviews* 64, 531–539 (2012).
69. Mahato, R. I., Narang, A. S., Thoma, L. & Miller, D. D. Emerging trends in oral delivery of peptide and protein drugs. *Crit. Rev. Ther. Drug Carrier Syst.* 20, 153–214 (2003).

70. Bellmann-Sickert, K. & Beck-Sickinger, A. G. Peptide drugs to target G protein-coupled receptors. *Trends in Pharmacological Sciences* 31, 434–441 (2010).
71. Kaspar, A. A. & Reichert, J. M. Future directions for peptide therapeutics development. *Drug Discovery Today* 18, 807–817 (2013).
72. Takagi, H., Shiomi, H., Ueda, H. & Amano, H. Morphine-like analgesia by a new dipeptide, L-Tyrosyl-L-Arginine (kyotorphin) and its analogue. *Eur. J. Pharmacol.* 55, 109–111 (1979).
73. Takagi, H., Shiomi, H., Ueda, H. & Amano, H. A novel analgesic dipeptide from bovine brain is a possible Met-enkephalin releaser. *Nature* 282, 410–412 (1979).
74. Serrano, I. D. *et al.* Correlation between membrane translocation and analgesic efficacy in kyotorphin derivatives. *Biopolymers* 104, 1–10 (2015).
75. Santos, S. Kyotorphin Derived Peptides in the Relationship between Analgesia and Alzheimer's Disease. (University of Lisbon, 2014).
76. Shiomi, H., Ueda, H. & Takagi, H. Isolation and identification of an analgesic opioid dipeptide kyotorphin (TYR-ARG) from bovine brain. *Neuropharmacology* 20, 633–638 (1981).
77. Shiomi, H. *et al.* Mechanism of kyotorphin-induced release of Met-enkephalin from guinea pig striatum and spinal cord. *Brain Res.* 221, 161–9 (1981).
78. Ueda, H., Shiomi, H. & Takagi, H. Regional distribution of a novel analgesic dipeptide kyotorphin (Tyr-Arg) in the rat brain and spinal cord. *Brain Res.* 198, 460–464 (1980).
79. Santos, S. M. *et al.* Neuropeptide kyotorphin (tyrosyl-arginine) has decreased levels in the cerebro-spinal fluid of Alzheimer's disease patients: Potential diagnostic and pharmacological implications. *Front. Aging Neurosci.* 5, (2013).
80. Nishimura, K. *et al.* Kyotorphin like substance in human cerebrospinal fluid of patients with persistent pain. *Masui.* 40, 1686–1690 (1991).
81. Ueda, H., Yoshihara, Y. & Takagi, H. A putative met-enkephalin releaser, kyotorphin enhances intracellular Ca2+ in the synaptosomes. *Biochem. Biophys. Res. Commun.* 137, 897–902 (1986).
82. Ueda, H., Fukushima, N., Yoshihara, Y. & Takagi, H. A Met-enkephalin releaser (kyotorphin)-induced release of plasma membrane-bound Ca2+ from rat brain synaptosomes. *Brain Res.* 419, 197–200 (1987).
83. Ueda, H. *et al.* How is kyotorphin (Tyr-Arg) generated in the brain? *Neuropeptides* 5, 525–8 (1985).
84. Fukui, K. *et al.* Isolation from bovine brain of a novel analgesic pentapeptide, neo-kyotorphin, containing The Tyr-Arg (kyotorphin) unit. *Neuropharmacology* 22, 191–196 (1983).
85. Ueda, H., Tamura, S., Satoh, M. & Takagi, H. Excess release of substance P from the spinal cord of mice during morphine withdrawal and involvement of the enhancement of presynaptic Ca2+ entry. *Brain Res.* 425, 101–5 (1987).

86. Lewis, R. V & Stern, A. S. Biosynthesis of the enkephalins and enkephalin-containing polypeptides. *Annu. Rev. Pharmacol. Toxicol.* 23, 353–72 (1983).
87. Rackham, A., Wood, P. L. & Hudgin, R. L. Kyotorphin (tyrosine-arginine): further evidence for indirect opiate receptor activation. *Life Sci.* 30, 1337–1342 (1982).
88. Stone, T. W. A comparison of the effects of morphine, enkephalin, kyotorphin and D-phenylalanine on rat central neurones. *Br. J. Pharmacol.* 79, 305–312 (1983).
89. Ribeiro, M. Improved Analgesic Kyotorphin Derivatives – correlating membrane interactions, brain targeting and pharmacological activities. (University of Lisbon, 2012).
90. Ueda, H. *et al.* The kyotorphin (tyrosine-arginine) receptor and a selective reconstitution with purified G(i), measured with GTPase and phospholipase C assays. *J. Biol. Chem.* 264, 3732–3741 (1989).
91. Machuqueiro, M. & Baptista, A. M. The pH-dependent conformational states of kyotorphin: a constant-pH molecular dynamics study. *Biophys. J.* 92, 1836–1845 (2007).
92. Arima, T., Kitamura, Y., Nishiya, T., Takagi, H. & Nomura, Y. Kyotorphin (L-tyrosyl-L-arginine) as a possible substrate for inducible nitric oxide synthase in rat glial cells. *Neurosci. Lett.* 212, 1–4 (1996).
93. Arima, T. *et al.* Effects of kyotorphin (L-tyrosyl-L-arginine) ON[3H]NG-nitro-L-arginine binding to neuronal nitric oxide synthase in rat brain. *Neurochem. Int.* 30, 605–611 (1997).
94. Shiomi, H., Ueda, H. & Takagi, H. Isolation and identification of an analgesic opioid dipeptide kyotorphin (Tyr-Arg) from bovine brain. *Neuropharmacology* 20, 633–638 (1981).
95. Ignat'ev, D. A., Vorob'ev, V. V & Ziganshin, R. K. Effects of a number of short peptides isolated from the brain of the hibernating ground squirrel on the EEG and behavior in rats. *Neurosci. Behav. Physiol.* 28, 158–166 (1998).
96. Sakurada, T. *et al.* Actions of intracerebroventricular administration of kyotorphin and an analog on thermoregulation in the mouse. *Peptides* 4, 859–863 (1983).
97. Bronnikov, G. *et al.* The effect of neuropeptides kyotorphin and neokyotorphin on proliferation of cultured brown preadipocytes. *FEBS Lett.* 407, 73–77 (1997).
98. Godlevsky, L. S., Shandra, A. A., Mikhaleva, I. I., Vastyanov, R. S. & Mazarati, A. M. Seizure-protecting effects of kyotorphin and related peptides in an animal model of epilepsy. *Brain Res. Bull.* 37, 223–226 (1995).
99. Bocheva, A. I. & Dzambazova-Maximova, E. B. Effects of kyotorphin and analogues on nociception and pentylenetetrazole seizures. *Folia Med. (Plovdiv).* 46, 40–44 (2004).
100. Summy-Long, J. Y. *et al.* Effects of central injection of kyotorphin and L-arginine on oxytocin and vasopressin release and blood pressure in conscious rats. *Brain Res. Bull.* 45, 395–403 (1998).

101. Dzambazova-Maximova, E. B. & Bocheva, A. I. The unique brain dipeptide kyotorphin – from discovery to nowadays. *J. Biomed. Clin.* (2010).

102. Santalova, I. M., Mavlyutov, T. A. & Moshkov, D. A. Morphofunctional changes in Mauthner neurons during exposure to the neuropeptide kyotorphin. *Neurosci. Behav. Physiol.* 34, 327–332 (2004).

103. Nazarenko, I. V, Zvrushchenko, M. S., Volkov, A. V, Kamenskiĭ, A. A. & Zaganshin, R. K. [Functional-morphologic evaluation of the effect of the regulatory peptide kyotorphin on the status of the CNS in the post-resuscitation period]. *Patol. Fiziol. i èksperimental'naia Ter.* 31–3 (1999). at <http://www.ncbi.nlm.nih.gov/pubmed/10379182>

104. Gorenkova, N. A., Nazarenko, I. V, Samorukova, I. V, Avrushchenko, M. S. & Volkov, A. V. [Therapy of postresuscitation behavioral disorders with mexidol and kyotorphin]. *Anesteziol. Reanimatol.* 63–6 (2002). at <http://www.ncbi.nlm.nih.gov/pubmed/12611163>

105. Sá Santos, S. *et al.* Amidated and Ibuprofen-Conjugated Kyotorphins Promote Neuronal Rescue and Memory Recovery in Cerebral Hypoperfusion Dementia Model. *Front. Aging Neurosci.* 8, (2016).

106. Ignat'ev, D. A., Vorob'ev, V. V. & Ziganshin, R. K. Effects of a number of short peptides isolated from the brain of the hibernating ground squirrel on the eeg and behavior in rats. *Neurosci. Behav. Physiol.* 28, 158–166 (1998).

107. Kolaeva, S. . *et al.* Effects of L-thyrosyl - L-arginine (kyotorphin) on the behavior of rats and goldfish. *Peptides* 21, 1331–1336 (2000).

108. Marinov, B. S. & Ziganshin, R. K. [Redox properties of peptides potentially regulating animal hibernation]. *Biofizika* 42, 147–53 (1997).

109. Chen, P., Bodor, N., Wu, W. M. & Prokai, L. Strategies to target kyotorphin analogues to the brain. *J. Med. Chem.* 41, 3773–3781 (1998).

110. Ribeiro, M. M. B. *et al.* Chemical conjugation of the neuropeptide kyotorphin and ibuprofen enhances brain targeting and analgesia. *Mol. Pharm.* 8, 1929–1940 (2011).

111. Santos, S. & Castanho, M. The Use of Visual Analog Scales to Compare Pain Between Patients With Alzheimer's Disease and Patients Without Any Known Neurodegenerative Disease and Their Caregivers. *Am. J. Alzheimers. Dis. Other Demen.* (2013). doi:10.1177/1533317513517046

112. Borsook, D. Neurological diseases and pain. *Brain* 135, 320–344 (2012).

113. Sabuncu, M. R. The Dynamics of Cortical and Hippocampal Atrophy in Alzheimer Disease. *Archives of Neurology* 68, 1040 (2011).

114. Seppälä, T. T. *et al.* CSF biomarkers for Alzheimer disease correlate with cortical brain biopsy findings. *Neurology* 78, 1568–1575 (2012).

115. Schraen-Maschke, S. *et al.* Tau as a biomarker of neurodegenerative diseases. *Biomark. Med.* 2, 363–384 (2008).

116. Ribeiro, M. M. B. *et al.* Inhibition of nociceptive responses after systemic administration of amidated kyotorphin. *Br. J. Pharmacol.* 163, 964–973 (2011).

117. Canas, P. M. *et al.* Adenosine A2A receptor blockade prevents synaptotoxicity and memory dysfunction caused by beta-amyloid peptides via p38 mitogen-activated protein kinase pathway. *J. Neurosci.* 29, 14741–14751 (2009).

118. Zhang, L. *et al.* Brain-Derived Neurotrophic Factor Ameliorates Learning Deficits in a Rat Model of Alzheimer's Disease Induced by Aβ1-42. *PLoS One* 10, e0122415 (2015).

119. EMLA cream: Indications, Side Effects, Warnings - Drugs.com. at <http://www.drugs.com/cdi/emla-cream.html>

120. Buprenorphine: Indications, Side Effects, Warnings - Drugs.com. at <http://www.drugs.com/cdi/buprenorphine.html>

121. Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates*. (Elsevier Academic Press, 2005).

122. Ribeiro, M. M. B. *et al.* Side-effects of analgesic kyotorphin derivatives: Advantages over clinical opioid drugs. *Amino Acids* 45, 171–178 (2013).

123. Cunha, G. M. A. *et al.* Adenosine A2A receptor blockade prevents memory dysfunction caused by β-amyloid peptides but not by scopolamine or MK-801. *Exp. Neurol.* 210, 776–781 (2008).

124. Y Maze Spontaneous Alternation Test. at <http://sbfnl.stanford.edu/cs/bm/lm/bml_ymaze.html>

125. Spellman, T. *et al.* Hippocampal–prefrontal input supports spatial encoding in working memory. *Nature* 522, 309–314 (2015).

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

127. Maurice, T., Lockhart, B. P. & Privat, A. Amnesia induced in mice by centrally administered beta-amyloid peptides involves cholinergic dysfunction. *Brain Res.* 706, 181–93 (1996).

128. Prut, L. & Belzung, C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur. J. Pharmacol.* 463, 3–33 (2003).

129. Bailey, Kathleen R.; Crawley, J. N. in *Methods of Behavior Analysis in Neuroscience. 2nd edition.* (2009). at <http://www.ncbi.nlm.nih.gov/books/NBK5221/>

130. Gould, T. D., Dao, D. T. & Kovacsics, C. E. in 1–20 (2009). doi:10.1007/978-1-60761-303-9_1

131. Antunes, M. & Biala, G. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cogn. Process.* 13, 93–110 (2012).

132. Taglialatela, G., Hogan, D., Zhang, W.-R. & Dineley, K. T. Intermediate- and long-term recognition memory deficits in Tg2576 mice are reversed with acute calcineurin inhibition. *Behav. Brain Res.* 200, 95–9 (2009).

133. Bevins, R. A. & Besheer, J. Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'. *Nat. Protoc.* 1, 1306–1311 (2006).

134. Coons, A. H., Creech, H. J. & Jones, R. N. Immunological Properties of an Antibody Containing a Fluorescent Group. *Exp. Biol. Med.* 47, 200–202 (1941).

135. Delisi, L. E. Handbook of Neurochemistry and Molecular Neurobiology. *Handb. Neurochem. Mol. Neurobiol. Schizophr.* 27, 107–241 (2009).

136. Ramos-Vara, J. A. Technical aspects of immunohistochemistry. *Vet. Pathol.* 42, 405–26 (2005).

137. Matos, L. L. de, Trufelli, D. C., de Matos, M. G. L. & da Silva Pinhal, M. A. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomark. Insights* 5, 9–20 (2010).

138. Odell, I. D. & Cook, D. Immunofluorescence Techniques. *J. Invest. Dermatol.* 133, e4 (2013).

139. Robinson, J. P., Bs, J. S. & Kumar, G. L. Immunofluorescence. *Immunohistochem. Stain. Methods Educ. Guid.* 160 (2013).

140. Key, M. Immunohistochemistry Staining Methods. *Immunohistochem. Stain. Methods* 57–60 (2009).

141. Fritschy, J.-M. & Hartig, W. Immunofluorescence. *Encycl. Life Sci.* 1–7 (1999). doi:10.1038/npg.els.0001174

142. Gage, G. J., Kipke, D. R. & Shain, W. Whole animal perfusion fixation for rodents. *J. Vis. Exp.* (2012). doi:10.3791/3564

143. Mullen, R. J., Buck, C. R. & Smith, A. M. NeuN, a neuronal specific nuclear protein in vertebrates. *Development* 116, 201–11 (1992).

144. Weyer, A. & Schilling, K. Developmental and cell type-specific expression of the neuronal marker NeuN in the murine cerebellum. *J. Neurosci. Res.* 73, 400–9 (2003).

145. Jacque, C. M. *et al.* Determination of glial fibrillary acidic protein (GFAP) in human brain tumors. *J. Neurol. Sci.* 35, 147–55 (1978).

146. Roessmann, U., Velasco, M. E., Sindely, S. D. & Gambetti, P. Glial fibrillary acidic protein (GFAP) in ependymal cells during development. An immunocytochemical study. *Brain Res.* 200, 13–21 (1980).

147. Sofroniew, M. V. & Vinters, H. V. Astrocytes: biology and pathology. *Acta Neuropathol.* 119, 7–35 (2010).

148. Hovens, I., Nyakas, C. & Schoemaker, R. A novel method for evaluating microglial activation using ionized calcium-binding adaptor protein-1 staining: cell body to cell size ratio. *Neuroimmunol. Neuroinflammation* 1, 82 (2014).
149. Yang, S. *et al.* Interlamellar CA1 network in the hippocampus. *Proc. Natl. Acad. Sci. U. S. A.* 111, 12919–24 (2014).
150. Ji, J. & Maren, S. Differential roles for hippocampal areas CA1 and CA3 in the contextual encoding and retrieval of extinguished fear. *Learn. Mem.* 15, 244–251 (2008).
151. Rolls, E. T. & Kesner, R. P. A computational theory of hippocampal function, and empirical tests of the theory. *Prog. Neurobiol.* 79, 1–48 (2006).
152. Padurariu, M., Ciobica, A., Mavroudis, I., Fotiou, D. & Baloyannis, S. Hippocampal neuronal loss in the CA1 and CA3 areas of Alzheimer's disease patients. *Psychiatr. Danub.* 24, 152–8 (2012).
153. Zarow, C. *et al.* Correlates of hippocampal neuron number in Alzheimer's disease and ischemic vascular dementia. *Ann. Neurol.* 57, 896–903 (2005).
154. Kerchner, G. A. *et al.* Hippocampal CA1 apical neuropil atrophy and memory performance in Alzheimer's disease. *Neuroimage* 63, 194–202 (2012).
155. Kesner, R. P. A behavioral analysis of dentate gyrus function. *Prog. Brain Res.* 163, 567–76 (2007).
156. Towbin, H., Staehelin, T. & Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* 76, 4350–4 (1979).
157. Burnette, W. N. 'Western Blotting': Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112, 195–203 (1981).
158. Yang, P.-C. & Mahmood, T. Western blot: Technique, theory, and trouble shooting. *N. Am. J. Med. Sci.* 4, 429 (2012).
159. Taylor, S. C. & Posch, A. The design of a quantitative western blot experiment. *Biomed Res. Int.* 2014, 8 (2014).
160. Kurien, B. T. & Scofield, R. H. Protein blotting: a review. *J. Immunol. Methods* 274, 1–15 (2003).
161. MacPhee, D. J. Methodological considerations for improving Western blot analysis. *J. Pharmacol. Toxicol. Methods* 61, 171–177 (2010).
162. KURIEN, B. & SCOFIELD, R. Western blotting. *Methods* 38, 283–293 (2006).
163. Ghosh, R., Gilda, J. E. & Gomes, A. V. The necessity of and strategies for improving confidence in the accuracy of western blots. *Expert Rev. Proteomics* 1–12 (2014). doi:10.1586/14789450.2014.939635
164. Eaton, S. L. *et al.* Total Protein Analysis as a Reliable Loading Control for Quantitative Fluorescent Western Blotting. *PLoS One* 8, e72457 (2013).

165. Zhang, L. *et al.* Brain-derived neurotrophic factor ameliorates learning deficits in a rat model of Alzheimer's disease induced by aβ1-42. *PLoS One* 10, e0122415 (2015).
166. Huntley, J. D. & Howard, R. J. Working memory in early Alzheimer's disease: a neuropsychological review. *Int. J. Geriatr. Psychiatry* 25, 121–32 (2010).
167. Cavallucci, V., D'Amelio, M. & Cecconi, F. Aβ Toxicity in Alzheimer's Disease. *Mol. Neurobiol.* 45, 366–378 (2012).
168. McLarnon, J. G. Correlated inflammatory responses and neurodegeneration in peptide-injected animal models of Alzheimer's disease. *Biomed Res. Int.* 2014, 923670 (2014).
169. Heppner, F. L., Ransohoff, R. M. & Becher, B. Immune attack: the role of inflammation in Alzheimer disease. *Nat Rev Neurosci* 16, 358–372 (2015).
170. Ransohoff, R. M. & Perry, V. H. Microglial physiology: unique stimuli, specialized responses. *Annu. Rev. Immunol.* 27, 119–45 (2009).
171. Kettenmann, H., Hanisch, U.-K., Noda, M. & Verkhratsky, A. Physiology of microglia. *Physiol. Rev.* 91, 461–553 (2011).
172. Eggen, B. J. L., Raj, D., Hanisch, U.-K. & Boddeke, H. W. G. M. Microglial phenotype and adaptation. *J. Neuroimmune Pharmacol.* 8, 807–23 (2013).
173. Kreutzberg, G. W. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* 19, 312–8 (1996).
174. Alescio-Lautier, B. *et al.* Visual and visuospatial short-term memory in mild cognitive impairment and Alzheimer disease: role of attention. *Neuropsychologia* 45, 1948–60 (2007).
175. Gold, C. A. & Budson, A. E. Memory loss in Alzheimer's disease: implications for development of therapeutics. *Expert Rev. Neurother.* 8, 1879–1891 (2008).
176. Rajagopal, L., Massey, B. W., Huang, M., Oyamada, Y. & Meltzer, H. Y. The novel object recognition test in rodents in relation to cognitive impairment in schizophrenia. *Curr. Pharm. Des.* 20, 5104–14 (2014).
177. Zidan, M. *et al.* Motor and functional changes in different stages of Alzheimer's disease. *Rev. Psiquiatr. Clínica* 39, 161–165 (2012).
178. Butterfield, D. A., Hardas, S. S. & Lange, M. L. B. Oxidatively modified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Alzheimer's disease: many pathways to neurodegeneration. *J. Alzheimers. Dis.* 20, 369–93 (2010).
179. Jack, C. R. *et al.* Serial PIB and MRI in normal, mild cognitive impairment and Alzheimer's disease: implications for sequence of pathological events in Alzheimer's disease. *Brain* 132, 1355–1365 (2009).
180. Corbett, A. *et al.* Drug repositioning for Alzheimer's disease. *Nat. Rev. Drug Discov.* 11, 833–46 (2012).

181. Bezprozvanny, I. & Mattson, M. P. Neuronal calcium mishandling and the pathogenesis of Alzheimer's disease. *Trends Neurosci.* 31, 454–63 (2008).

182. Yagami, T., Kohma, H. & Yamamoto, Y. L-Type Voltage-Dependent Calcium Channels As Therapeutic Targets for Neuro- degenerative Diseases. *Curr. Medicial Chem.* 19, 4816–4827 (2012).

183. Pollard, H. B., Rojas, E. & Arispe, N. A new hypothesis for the mechanism of amyloid toxicity, based on the calcium channel activity of amyloid beta protein (A beta P) in phospholipid bilayer membranes. *Ann. N. Y. Acad. Sci.* 695, 165–8 (1993).

184. Arispe, N., Rojas, E. & Pollard, H. B. Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum. *Proc. Natl. Acad. Sci. U. S. A.* 90, 567–71 (1993).

185. Nimmrich, V. & Eckert, A. Calcium channel blockers and dementia. *Br. J. Pharmacol.* 169, 1203–1210 (2013).

186. Harkany, T. *et al.* Mechanisms of beta-amyloid neurotoxicity: perspectives of pharmacotherapy. *Rev. Neurosci.* 11, 329–82 (2000).

187. Greenamyre, J. T. & Young, A. B. Excitatory amino acids and Alzheimer's disease. *Neurobiol. Aging* 10, 593–602 (1989).

188. Anekonda, T. S. & Quinn, J. F. Calcium channel blocking as a therapeutic strategy for Alzheimer's disease: The case for isradipine. *Biochim. Biophys. Acta - Mol. Basis Dis.* 1812, 1584–1590 (2011).

189. Anekonda, T. S. *et al.* L-type voltage-gated calcium channel blockade with isradipine as a therapeutic strategy for Alzheimer's disease. *Neurobiol. Dis.* 41, 62–70 (2011).

190. Nygaard, H. B. *et al.* Brivaracetam, but not ethosuximide, reverses memory impairments in an Alzheimer's disease mouse model. *Alzheimers. Res. Ther.* 7, 25 (2015).

191. Sanchez, P. E. *et al.* Levetiracetam suppresses neuronal network dysfunction and reverses synaptic and cognitive deficits in an Alzheimer's disease model. *Proc. Natl. Acad. Sci. U. S. A.* 109, E2895–903 (2012).

192. Shi, J.-Q. *et al.* Antiepileptics topiramate and levetiracetam alleviate behavioral deficits and reduce neuropathology in APPswe/PS1dE9 transgenic mice. *CNS Neurosci. Ther.* 19, 871–81 (2013).

193. Mendez, M. F., Catanzaro, P., Doss, R. C., ARguello, R. & Frey, W. H. Seizures in Alzheimer's disease: clinicopathologic study. *J. Geriatr. Psychiatry Neurol.* 7, 230–3 (1994).

194. Haas, M. *et al.* The European Medicines Agency's strategies to meet the challenges of Alzheimer disease. *Nat. Rev. Drug Discov.* 14, 221–2 (2015).

195. Hopkins, A. L. Network pharmacology: the next paradigm in drug discovery. *Nat. Chem. Biol.* 4, 682–690 (2008).

196. Lansbury, P. T. Back to the future: the 'old-fashioned' way to new medications for neurodegeneration. *Nat. Med.* 10 Suppl, S51–7 (2004).

197. Imbimbo, B. P., Solfrizzi, V. & Panza, F. Are NSAIDs useful to treat Alzheimer's disease or mild cognitive impairment? *Front. Aging Neurosci.* 2, (2010).

198. Breitner, J. C. *et al.* Extended results of the Alzheimer's disease anti-inflammatory prevention trial. *Alzheimers. Dement.* 7, 402–11 (2011).