International Ph.D. program in Neuroscience
XXXIV cycle

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TGF-β1 pathway as a new pharmacological target in depression and Alzheimer's disease

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List of Contents

List of Contents

LIST OF FIGURES

LIST OF ABBREVIATIONS

Acetylcholine (Ach)

Ach cholinesterase enzyme (AChE)

Ach cholinesterase inhibitors (AChEI)

Activin receptor-like kinases (ALKs)

Activities of daily living (ADL)/instrumental activities of daily living (IADL)

Alzheimer’s Disease (AD)

Alzheimer’s Disease Neuroimaging Initiative (ADNI)

Alzheimer's Disease Assessment Scale-Cognitive Behavior section (ADAS-Cog)

Amyloid precursor protein (APP)

Amyloid-beta (Aβ)

Amyloid-related imaging abnormalities (ARIA)

Apolipoprotein E (APOE)

Behavioral and Psychological Symptoms of Dementia" (BPSD)

Beta-site APP cleaving enzyme 1 (BACE1)

Blood-brain barrier (BBB)

Bone Morphogenetic Proteins (BMPs)

Brain Derived Neurotrophic Factor (BDNF)

Butyryl-cholinesterase (BuChE)

Central nervous system (CNS)

Cerebrospinal fluid (CSF)

Choline acetyltransferase (ChAT)

Chronic constriction injury (CCI)

Clinical Antipsychotic Trials of Intervention Effectiveness in Alzheimer's Disease (CATIE-AD)

Clinical Dementia Rating Scale (CDR)

Corticotropin release factor (CRF)

Cyclin-dependent kinase (cdk)

Delta opioid peptide receptor (DOPr)

Dementia Scale for Down syndrome (DSDS)

Dopamine (DA)

Down Syndrome (DS)

Early Onset Alzheimer Disease (EOAD)

Extracellular-regulated kinase (ERK)

Familial form of Alzheimer's disease (fAD)

Food Drug Administration (FDA)

Forced Swim Test (FST)

Functional MRI (fMRI)

Genome-wide Association studies (GWAS)

Geriatric Depression Scale (GDS)

Glial fibrillary acidic protein (GFAP)

Glial-derived neurotrophic factor (GDNF)

Glucocorticoid (GC)

Glutathione Peroxidase 1 (GPX1)

Growth and Differentiation Factors (GDFs)

Huntington’s disease (HD)

Hypothalamic-pituitary-adrenal (HPA)

Inducible nitric oxide synthase (iNOS)

Insulin-Like Growth Factor (IGF-1)

Intellectual disability (ID)

Interleukin (IL)

International Classification of Diseases (ICD)

Intracerebroventricular (i.c.v.)

Intrathecal (i.t.)

Latency-associated peptides (LAPs)

Late-onset sporadic Alzheimer Disease (sAD)

Long-term potentiation (LTP)

Matrix metalloproteinase 9 (MMP-9)

Microtubule-associated protein (MAP)

Mild cognitive impairment (MCI)

Mini-Mental State Examination (MMSE)

Montreal Cognitive Assessment (MoCA)

Mu opioid peptide receptor (MOPr)

Multiple sclerosis (MS)

National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS–ADRDA)

Nerve growth factor (NGF)

Neurofibrillary Tangles (Nfts)

Neurofilament Light Chain (Nf-L)

Neuropsychiatric Inventory Questionnaire (NPIQ)

Nerve Growth Factor (NGF)

Nitric Oxide (NO)

N-Methyl D-Aspartate (NMDA)

Noradrenaline (NA)

Novel Object Recognition Test (NOR)

Nuclear Factor Kappa-B (NF-Kb)

Parkinson’s Disease (PD)

Passive Avoidance Test (PAT)

Peripheral Blood Mononuclear Cells (PBMCS)

Phosphatidyl Inositol-3-Kinase (PI3K)

Phospho-Tau (P-Tau)

Positron Emission Tomography (PET)

Precursor for Nerve Growth Factor (ProNGF)

Prefrontal Cortex (PFC)

Presenilin-1 (PSEN1)

Presenilin-2 (PSEN2)

Reactive Nitrogen Species (RNS)

Reactive Oxygen Species (ROS)

Selective Serotonin Reuptake Inhibitors (SSRIs)

Serotonin (5-HT)

Single Nucleotide Polymorphisms (SNPs)

Smad Anchor For Receptor Activation (SARA)

Smad Ubiquitination Regulatory Factor 1 (Smurf1)

Small Latent Complexes (SLCs)

Spared Nerve Injury (SNI)

Structural Magnetic Resonance Imaging (MRI)

Test of Severe Impairment (TSI)

Transforming growth factor beta 1 (TGF-β1)

Tissue Inhibitor of Metalloproteases 1 (TIMP-1)

Tissue Plasminogen Activator (tPA)

Triggering Receptor Expressed on Myeloid Cells 2 (TREM2)

Tropomyosin Receptor Kinase B (TrkB)

Tumour Necrosis Factor-Alpha (TNF-α)

Type 2 Diabetes Mellitus (T2DM)

Vascular Endothelial Growth Factor (VEGF)

ABSTRACT

Different neurobiological and clinical links exist between depression and Alzheimer's disease (AD), among which neuroinflammation, oxidative stress and an impairment of the neurotrophin Transforming growth factor beta 1 (TGF-β1) exert a key role especially in the pathophysiology of cognitive deficits associated to these diseases. Second-generation antidepressants, in particular selective serotonin reuptake inhibitors (SSRIs), are currently studied for their neuroprotective activity in AD.

We identified in our non Tg-animal model of AD, obtained by i.c.v. injection of Aβ oligomers in mice, a selective impairment of hippocampal TGF-β1 signaling paralleling with a depressive-like phenotype and memory impairment. Interestingly, the chronic treatment with the SSRI fluoxetine and the new multimodal antidepressant vortioxetine was able to reverse this phenotype by completely rescuing TGF-β1 levels. In addition, in our animal model of amyloid-related depression we demonstrated that oxidative stress took place as a consequence of pro-oxidant enzymes activation induced by Aβ in the hippocampus, a brain area strongly affected both in depression and AD and one of the neurobiological links between these two diseases. Moreover, the antidepressant chronic treatment was able to prevent the pro-oxidant enzymes over-expression induced Aβ oligomers.

In order to identify drugs able to prevent Aβ-induced toxicity by rescue of TGF-β1, we adopted an experimental in vitro model of Aβ-induced neuroinflammation where BV-2 microglial cells were challenged with Aβ oligomers. We found that a natural dipeptide carnosine was able, in this experimental model, to decrease the secretion of pro-inflammatory cytokines and simultaneously increasing the synthesis and the release of TGF-β1. Interestingly, the ability of SB431542, a selective inhibitor of the type-1 TGF-β1 receptor, to completely prevent the effects of carnosine in mixed neuronal cultures treated with Aβ oligomers suggests that TGF-β1 release and activation of Smad-dependent signaling is essential in mediating the neuroprotective efficacy of carnosine against Aβ-induced neurodegeneration.

To study whether a deficit of TGF-β1 occurs also in a validated animal model of neuropathic pain, a central nervous system (CNS) disorder closely correlated in terms of comorbidity with depression, we used an established animal model of neuropathic pain obtained by chronic constriction injury (CCI) of sciatic nerve and we demonstrated a significant decrease of active TGF-β1 and of its type II

receptor (TGFβ-R2) levels in the spinal cord of rats as well as a selective deficit of TGF-β1 expression in microglial cells. The chronic treatment with LP2, a dual-target MOPr/DOPr agonist, was able to reduce CCI-induced mechanical allodynia by rescue of TGF-β1 and TGFβ-R2 levels suggesting that the rescue of TGF-β1 signalling by LP2 could be mediated by DOPr activation in spinal microglia.
Finally, we moved from rodent to humans, to validate the role of TGF-β1 signaling as a novel pharmacological target for the treatment of cognitive disorders, exploring the hypothesis that a deficit of TGF-β1 could represent a biomarker of cognitive decline in neuropsychiatric disorders characterized by an increased risk to develop AD such as Down Syndrome (DS) patients. We observed a significant decrease of TGF-β1 plasma levels in DS patients compared to age- and sex-matched healthy subjects and this deficit was correlated with global cognitive function evaluated by Test of Severe Impairment (TSI). The levels of TGF-β1 were significantly reduced also in young DS patients without AD suggesting that the deficit of TGF-β1 is an early and long-lasting event in the pathophysiology of cognitive decline in DS. In conclusion, overall the data presented in this PhD thesis suggest that TGF-β1 signaling pathway might represent a new pharmacological target for the treatment of cognitive deficits from depression to AD.

Chapter 1. INTRODUCTION

1.1 Alzheimer's Disease (AD): neurobiological and clinical phenotypes

1.1.1 AD epidemiology and risk factors

1.1.2 Neuropathology of AD: Amyloid plaques and neurofibrillary tangles

1.1.3 Pathogenetic hypotheses of AD

1.1.4 Diagnosis: the role of biological and neuropsychological markers in AD

1.1.5 Pharmacological treatment of AD

1.2 Neurobiological links between depression and AD

1.2.1 Stress and dysfunction of the hypothalamic-pituitary-adrenal axis

1.2.2 Neuroinflammation

1.2.3 The role of neurotrophin's signaling: focus on NGF, BDNF and TGF-β1

1.3 Neurobiological links between depression and chronic pain

1.4 TGF-β1 as a new pharmacological target in CNS disorders

1.4.1 TGF-β family and TGF-β1 signaling pathway

1.4.2 TGF-β1 pathway in the healthy brain, in aging and neurodegenerative diseases: focus on AD

1.5 Hypothesis and aims

Chapter 2. Fluoxetine and Vortioxetine Reverse Depressive-Like Phenotype and Memory Deficits Induced by $A\beta_{1-42}$ Oligomers in Mice: A Key Role of Transforming Growth Factor-β1.

Chapter 3. Antioxidant Activity of Fluoxetine and Vortioxetine in a Non-Transgenic Animal Model of Alzheimer's Disease.

Chapter 4. Carnosine Prevents Aβ-Induced Oxidative Stress and Inflammation in Microglial Cells: A Key Role of TGF-β1.

Chapter 5. The Multimodal MOPr/DOPr Agonist LP2 Reduces Allodynia in Chronic Constriction Injured Rats by Rescue of TGF-β1 Signalling.

Chapeter 6. Improving Cognition with Nutraceuticals Targeting TGF-β1 Signaling. Review.

Chapter 1.

INTRODUCTION

1.1 Alzheimer’s Disease (AD): neurobiological and clinical phenotypes

Alzheimer's disease (AD) is the most common type of dementia, representing about two-thirds of cases of dementia in people over 60 years old (Kumar, Sidhu et al. 2021). With the term of dementia is described a group of symptoms that impair cognitive functions, including memory, thinking, as well as behavioral and emotional functions, which characterize progressive neurodegenerative brain disorders (Roberts, Knopman et al. 2014). In addition to AD, other causes of dementia have been described including Lewy Bodies’ dementia, vascular dementia, frontotemporal lobar degeneration, and Parkinson’s disease (PD). Patients that suffering from dementia are unable to perform daily life activities with a slow and progressive cognitive and functional decline (Scott and Barrett 2007). AD is considered as an incurable form of dementia affecting about 33 million people worldwide and its incidence doubles every 5-10 years (Lanctôt, Amatniek et al. 2017). More than six millions of Americans are suffering from AD according to the Alzheimer's Association 2021. In the last year AD and other type of dementia have cost to the Nation $ 355 billion. By 2050, the number of people age 65 and older with AD dementia may grow to 12.7 million and the total costs of health care and long-

term care for individuals living with AD could rise to more than $ 1.1 trillion (Alzheimer's Association 2021, https://www.alz.org/alzheimer_s_dementia). In general, this disease influences intellectual ability, social skills, personality, and memory function and, over time, it leads to a reduction in quality of life and life expectancy of these patients. The current AD pharmacological treatments are only symptomatic, but do not interfere with the underlying pathogenic mechanisms of the disease.

In this context, the identification of biological markers could be useful for the diagnosis as well as should be a useful tool for the identification of early AD patients eligible for disease-modifying therapies.

1.1.1 *AD epidemiology and risk factors*

Alzheimer's disease (AD), the most common cause of dementia in the elderly, is a chronic neurodegenerative disease affecting approximately 44 million people worldwide that is predicted to more than triple by 2050 as the population ages (Lane, Hardy et al. 2018). AD is responsible for 50%–75% cause of dementia and its prevalence is considered approximately doubling in every 5 years in people over 65 years old. The incidence of AD increases every 5-10 years, with a prevalence of 10% in people over 65 and of 50% in people over 85 years old (Reitz, Brayne et al. 2011). Recent studies suggest that the incidence of dementia, particularly in men, might decline in western countries; it is not clear what mechanism would be responsible, but this could be given by the better management of vascular risk factors (Wu, Fratiglioni et al. 2016), (Lane, Hardy et al. 2018). The risk of dementia is higher in subjects with mild cognitive impairment (MCI) compared with cognitively normal people. The rates of AD incidence were similar in men and women at age from 70 to 79 years for MCI patients, but the rates were higher in women than men at age 80 to 89 years (Roberts, Knopman et al. 2014). For this reason, the MCI and mild dementia diagnosis is clinically relevant because in both, there is a heightened risk for further cognitive decline (Knopman and Petersen 2014). Aging is considered the primary AD risk factor, however the disease is the result of complex interactions between multiple factors including genetic, epigenetic and environmental factors that act in combination to induce brain deficits and increase the probability to AD development. Most of AD cases occur on an apparently sporadic basis, while mutations in three genes such as amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2) induce a rare (<0.5%) AD familial form (fAD) defined also as autosomal-dominant AD pathology or Early Onset Alzheimer Disease (EOAD) where the symptoms development occurs earlier than in sporadic AD form, typically between 30 and 50 years of age (Bateman, Aisen et al. 2011). In fAD the mutations within APP, PSEN1 and PSEN2 genes (the latter are proteases responsible for amyloid-beta (Aβ) production from

APP) affect a common pathogenic pathway in the synthesis and proteolysis of APP leading to an excessive Aβ production. Approximately 300 different pathogenic mutations in PSEN1 (located at 14q24.3), 13 mutations in PSEN2 (located at 1q31-q42) and almost 32 mutations or duplication in APP have been identified (Wu, Rosa-Neto et al. 2012), (Chávez-Gutiérrez and Szaruga 2020). Furthermore, PSEN1 mutations are responsible for about 75-80 % of genotyped families positive for a mutation, while APP and PSEN2 mutations are responsible for 20-15% and about 5%, respectively of AD familial cases (Wu, Rosa-Neto et al. 2012), (Campion, Dumanchin et al. 1999), (Janssen, Beck et al. 2003). In the late-onset sporadic AD (sAD) several genetic, epigenetic, environmental and lifestyle factors are responsible for its pathogenesis and progression (Karch and Goate 2015), (Pimenova, Raj et al. 2018). Apolipoprotein E (APOE), a protein involved in lipid metabolism encoded by APOE gene and located on chromosome 19, is expressed in many types of brain cells, primarily in astrocytes and results to be associated with age-dependent decline of learning and memory in humans, representing the most significant genetic risk factor for sAD development. APOE4 genotype is the major genetic marker associated with increased gray matter atrophy and lower Aβ-42 levels as well as higher tau/p-tau181 levels in cerebrospinal fluid (CSF) of AD patients (Spampinato, Rumboldt et al. 2011, Cruchaga, Kauwe et al. 2013, Chávez-Gutiérrez and Szaruga 2020). In addition, people with two allele copies for E4 are at a higher risk of AD development than those only one copy of this allele (Holtzman, Herz et al. 2012). In the sporadic AD form, ε4 heterozygotes have an odds ratio for AD of about 3 fold, rising to about 12 fold in homozygotes, compared to non-ε4 carriers (Verghese, Castellano et al. 2011). Conversely, the presence of ε2 allele reduces the risk of AD development. Genome-wide association studies (GWAS) have identified more than 20 genetic risk factors for sAD, involved in inflammatory, cholesterol metabolism and endosomal vesicle recycling pathways (Karch and Goate 2015). Of note, a rare R47H mutation of triggering receptor expressed on myeloid cells 2 (TREM2), a microglial surface receptor that triggers intracellular protein tyrosine phosphorylation, is associated with an increased risk of AD development (Wang, Cella et al. 2015). A number of acquired factors are risk factors for the development of AD including cerebrovascular diseases, diabetes, depression, hypertension, obesity and dyslipidemia (Mayeux and Stern 2012, Silva, Loures et al. 2019). Cerebrovascular diseases such as hemorrhagic infarcts, small and large ischemic cortical infarcts, vasculopathies, and changes in cerebral white matter represent the most commonly reported risk factors (Liu, Wong et al. 2015, Love and Miners 2016). A clear association between type 2 diabetes mellitus (T2DM) and the increased risk of AD development is suggested and several mechanisms are involved in this association including insulin resistance and insulin deficiency, impaired insulin receptor, hyperglycemia, cerebrovascular damage, and vascular inflammation (Li, Song et al. 2015). Multiple studies have emphasized the probable

links between these two pathologies, considering AD as a neuroendocrine disorder referred to as “diabetes type 3” or “brain diabetes” (Stanciu, Bild et al. 2020). In the early stages of AD, cerebral glucose utilization is reduced up to 45%, and blood flow about up to 18%. In the later stages, metabolic and physiological abnormalities worsen, resulting in 55–65% reductions in cerebral blood flow (de la Monte 2009). Insulin and insulin-like growth factors (IGFs) regulate neuronal survival, energy metabolism, and synaptic plasticity, which are required for learning and memory processes (de la Monte 2009). Moreover, insulin provide a support for the neurological functions by the stimulation of the enzyme expression that controls the acetylcholine (Ach) synthesis. Consequently, decreased insulin levels as well as low insulin receptor sensitivity could contribute to a reduction in Ach levels leading to a likely biochemical link between diabetes and AD (Pugazhenthi, Qin et al. 2017). Preclical studies demonstrated that insulin resistance or deficit promotes the action of β- and γ-secretases leading to brain Aβ accumulation and hyperphosphorylation of tau protein (Kimura 2016). Hyperglycaemia, an effect of peripheral insulin regulation impairment, can activate multiple biochemical pathways affecting negatively brain functions, such as glucose neurotoxicity, vascular injury, and accumulation of advanced glycation end products (Kellar and Craft 2020). Finally, it has been demonstrated that Beta-secretase 1, also known as beta-site APP cleaving enzyme 1 (BACE1) is increased in T2DM contributing to the cognitive impairment risk by both amyloidogenesis and insulin resistance (Bao, Liu et al. 2021). Among risk factors for AD development hypercholesterolemia acts as a risk factor compromising the integrity of blood-brain barrier (BBB) and increasing amyloid-beta (Aβ) accumulation, neurofibrillary tangles (NFT) formation, and promoting cognitive decline, neuroinflammation, and dysfunction of cholinergic neurons (Ricciarelli, Canepa et al. 2012, Xue-Shan, Juan et al. 2016). Among the modifiable risk factors for AD development, recent meta-analyses have underlined the relevance of stress (Baumgart, Snyder et al. 2015, Xu, Tan et al. 2015). It is known that in susceptible individuals prolonged stress increase the risk of physiological disorders as well as psychiatric and neurodegenerative disorders including AD. Stress by over-activation of hypothalamic-pituitary-adrenal (HPA) axis, which results into significant increase of plasma cortisol in humans and corticosterone in rodents, can enhance APP levels and tau phosphorylation leading to a synaptic dysfunction and neuronal death associated with AD (Caruso, Nicoletti et al. 2018). A lifetime history of major depression has been considered as a risk factor for later AD development and the presence of depressive symptoms can increase the conversion from MCI to AD (Caraci, Copani et al. 2010). Moreover, smoking increasing the generation of free radicals, promoting oxidative stress phenomena and the action of pro-inflammatory cytokines in the immune system has been proposed as modifiable risk factors for AD (Traber, van der Vliet et al. 2000, Silva, Loures et al. 2019). Smoking-related oxidative stress may directly promote the

amyloidogenic pathway involved in Aβ oligomers production and extracellular fibrillar Aβ aggregation (Durazzo, Mattsson et al. 2014).

The higher incidence of early-onset AD in people with 21 trisomy (Down Syndrome, DS) is due to the location of APP gene on chromosome 21 and consequently the APP overexpression results in an increase and deposition of cerebral Aβ peptide (Caraci, Iulita et al. 2017). Rovelet-Lecrux et al. reported that APP gene duplication is sufficient to cause early-onset AD in the absence of missense mutations (Rovelet-Lecrux, Hannequin et al. 2006). The main neuropathological hallmarks of AD can be detected in virtually all people with DS over the age of 40 (Zigman, Devenny et al. 2008) although not all DS patients develop AD symptoms during adult age (Iulita, Caraci et al. 2016). In summary, aging alone is not sufficient to cause AD development supporting the multifactorial basis of the disease.

Epidemiological evidence suggest that life-style changes such as education, physical exercise and diet are able to lower the risk of AD acting as protective factors. Several studies indicate that individuals with a higher level of schooling and education have a lower risk of developing dementia (Scarmeas, Levy et al. 2001). In this context, it has been proposed "the cognitive reserve hypothesis" suggesting that at a particular level of AD pathology, highly educated individuals are less likely to manifest clinical symptoms of dementia compared to less-educated individuals (Roe, Xiong et al. 2007).

Physical activity is able to prevent AD and exert a neuroprotective activity by increasing neurotrophic factors such as BDNF (Brain Derived Neurotrophic Factor), IGF-1 (Insulin-Like Growth Factor), VEGF (Vascular Endothelial Growth Factor), stimulating neurogenesis and synaptic plasticity (Vecchio, Meng et al. 2018). Aerobic exercise increases BDNF production and it may be a useful non-pharmacological strategy to improve the treatment of cognitive and affective symptoms in depression (Guerrera, Furneri et al. 2020). For example, a meta-analysis conducted by Hamer and Chida showed that physical activity practice reduces AD risk by 45% (Hamer and Chida 2009). Furthermore, some dietary components are essential for neurocognition protection such as fish oil, vitamins E and C, fruits and vegetables, vitamins B6, B12 and folate (Smith and Blumenthal 2010). Supplementation of omega-3 fatty acids emerged as a possibility for prevention and management of AD (Fraga, Carvalho et al. 2017).

In conclusion, together with advanced age and APOE-4 genotype, female sex is a major risk factor for late-onset AD development (Scheyer, Rahman et al. 2018). It has been demonstrated that estrogens, especially estradiol, are able to prevent mitochondrial dysfunction in nerve cells and neuroinflammatory phenomena (Zárate, Stevnsner et al. 2017). Evidence supported a protective role of estrogens in reducing AD risk (by approximately 40%), emphasising the positive role of the

exogenous hormone exerting a neuroprotective effect in preventing AD incidence (Xu, Tan et al. 2015).

1.1.2 Neuropathology of AD: Amyloid plaques and neurofibrillary tangles

AD is a neurological disorder characterized by hippocampal and cortical atrophy, loss of synapses and neurodegeneration. Alois Alzheimer described this pathology in 1906 by an autopsy of a 56-year-old woman with severe dementia noting the presence of senile plaques outside neurons and neurofibrillary tangles within neurons (Norfray and Provenzale 2004). At cellular level, AD is characterized by progressive cortical neurons decline, especially pyramidal neurons, which mediate cognitive functions (Mann 1996). Neuronal degeneration begins in the medial temporal lobe, specifically at the level of entorhinal cortex and hippocampus, leading to memory deficits as observed in the early clinical manifestations of AD. As the disease progresses, the neurodegeneration affects the frontal cortex and the neocortex causing damage to limbic structures including hippocampus and the fibers that connect it to cerebral cortex, amygdala, and thalamus (Bozoki, Korolev et al. 2012). At the macroscopic level, there is brain atrophy, specially observed in the frontotemporal cortex. The cognitive impairment in AD patients is closely associated with the progressive degeneration of the limbic system, neocortical regions and the basal forebrain. This neurodegenerative process is characterized by early damage to the synapses with retrograde degeneration of the axons and eventual atrophy of the dendritic tree and the cell body of neurons. Indeed, the loss of synapses in the neocortex and limbic system is the best correlate of the cognitive impairment in AD patients (Serrano-Pozo, Frosch et al. 2011).

At the microscopic level, the hallmarks of the disease are amyloid plaques, NFTs, and extensive neuronal loss (**Figure 1**, (Holtzman, Morris et al. 2011)).

Amyloid plaques are accumulations of Aβ peptide in the extracellular space of the brain. Aβ peptide, a 38-43 amino acid peptide derived from a much larger protein known as the APP, within plaques is present in insoluble aggregated forms including fibrils and oligomers (Koffie, Meyer-Luehmann et al. 2009). The APP is cleaved by two pathways: in the non-amyloidogenic pathway the full-length APP is cleaved by α- and γ-secretases, while in the amyloidogenic pathway the cleavage by the β- and γ-secretases produces several species of Aβ fragments. Neurotoxic forms of Aβ generated by cleavage of APP initially by BACE1, the major β-secretase in the brain, produce the C99 fragment and soluble APPβ, and then C99 is cleaved by γ-secretase to produce Aβ (Sadigh-Eteghad, Sabermarouf et al. 2015). Aβ42 is more abundant than Aβ40 within plaques due to its higher rate of fibrillization and insolubility. Aβ peptide can undergo aggregation through a step-by-step process, starting with soluble monomers and evolving to the formation of oligomers, protofibrils, and mature

fibrils, with the oligomeric structures representing the more toxic species of Aβ causing neuronal dysfunction and death in AD brain (Brorsson, Kumita et al. 2010, Klein 2013). When Aβ is deposited and aggregated in a non-fibrillar conformation, it is detected via immunohistochemical techniques as "diffuse" plaques while when it is aggregated in deposits in the extracellular space in fibrillar forms with a β-sheet conformation can be recognized by electron microscopy and light microscopy with stains such as Congo red and Thioflavin-S (Holtzman, Morris et al. 2011). In AD brain, the neuritic plaques are surrounded by degenerating axons and dendrites as well as in areas surrounding plaques there is also "gliosis" with hypertrophy and alteration of the morphology and proliferation of astrocytes and microglia. Of note, microglia, a subtype of brain glial cells constituting up to 10% of all cells in the healthy human cortex, are in intimate contact with neurons and are involved in many basic physiological processes (Kraft and Harry 2011). It has been shown that activated microglia (by the release of different cytotoxic molecules, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), and pro-inflammatory cytokines) is able to contribute to the neurodegenerative disorders development and progression including AD (Caruso, Fresta et al. 2019). When Aβ aggregates are found in blood vessel walls independently of deposition in the neuropil (space between neuronal and glial cell bodies that is comprised of dendrites, axons, synapses, glial cell processes, and microvasculature) that occurs in many cases of AD, these deposits are called cerebrovascular plaques or cerebral amyloid angiopathy (Smith and Greenberg 2009, Holtzman, Morris et al. 2011). Another neuropathological hallmark of AD is the accumulation of intracellular NFTs, composed by paired helical filaments containing a hyperphosphorylated form of tau protein. Tau is a microtubule-associated protein (MAP) that normally is located to the axon, where physiologically facilitates the axonal transport by binding and stabilizing the microtubules. In AD brain, tau is translocated to the giving rise to NFTs formation (Serrano-Pozo, Frosch et al. 2011). In fact, under normal physiologically conditions, tau undergoes phosphorylation and dephosphorylation processes, thus forming insoluble aggregates. An imbalance in this dynamic equilibrium results in increased levels of abnormally hyperphoshorylated tau (P-tau 181, P-tau 199, P-tau 231, P-tau 396, P-tau 404), which in turn sequesters normal tau and other MAPs (MAP1 and MAP2) (Blennow, Zetterberg et al. 2007).

Tau pathology typically begins in the entorhinal cortex and hippocampus before the propagation to the associative isocortex. Primary sensory, motor and visual areas tend to be relatively spared (Serrano-Pozo, Frosch et al. 2011). Inside the neurons, the NFTs accelerate neuronal death by impeding intraneuronal transport. Many neurons develop intracellular aggregates of tau in NFTs and there is an increase in the amount of total tau and phosphorylated forms of tau (p-tau) into CSF of AD patients. There is strong evidence that tau pathology contributes to clinical progression of AD;

this is likely partly through pathways downstream of Aβ but there is also likely tau-related brain damage in AD that is independent of Aβ (Small and Duff 2008).

Both tau and amyloid protein assemble into insoluble aggregates of NFTs and senile plaques, respectively, promoting neuronal degeneration and are deposited along the course of cortical memory pathways dominated by pyramidal cells causing neuronal destruction along these pathways (Norfray and Provenzale 2004).

It has been suggested that many years before the appearance of clinical signs of AD, the deposition of both NFTs and Aβ occurs within the neocortex, hippocampus, and other cognition-related subcortical structures. Increasing evidence showed that Aβ can be internalized or produced inside of the cells. This provides the opportunity for Aβ to facilitate NFTs formation. On the other hand, the disruption in tau formation can influence the production of Aβ and amyloid plaques deposition (Sadigh-Eteghad, Sabermarouf et al. 2015). In fact, senile plaques formation may precede NFTs, and vice-versa. For instance, in AD late-onset, NFTs are deposited before senile plaques, whereas in DS patients amyloid is deposited before NFTs formation.

The development of senile plaques and NFTs in the striatum and hippocampus causes dopamine (DA) and serotonin (5-HT) receptor loss that can be detected by using metabolic imaging techniques such as positron emission tomography (PET). Likewise, their formation in the basal forebrain and entorhinal areas causes early loss of cholinergic neurons and nicotinic cholinergic receptors (Newhouse, Potter et al. 2001). These last, in the normal brain are related to acquisition and retention of verbal and non-verbal informations, suggesting the basis for the “cholinergic hypothesis” in the onset of AD cognitive symptoms. In addition, the decrease in the number of nicotinic cholinergic receptors in AD provided the rationale for cholinesterase therapy in treating AD-related memory deficits (Norfray and Provenzale 2004).

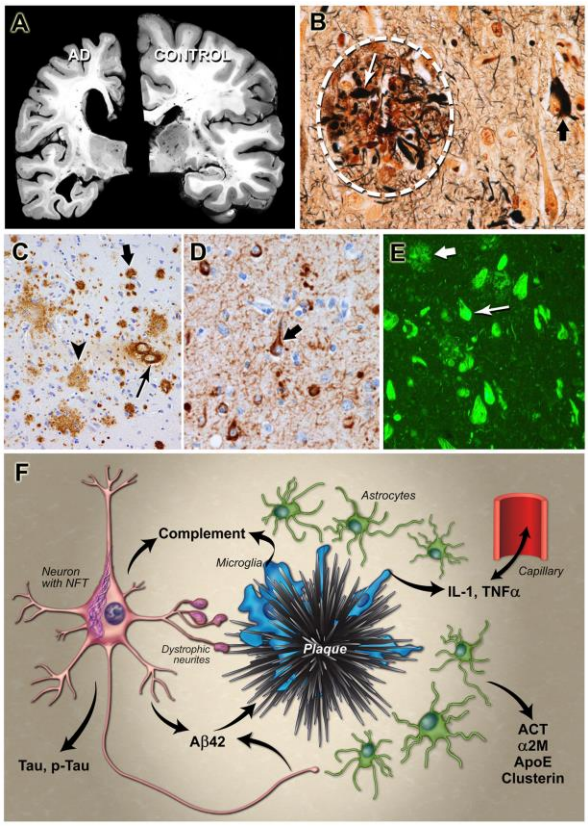

Figure 1. AD neuropathology (Adapted from Holtzman DM et al., 2011).

A. Severe brain atrophy in AD patient compared to normal subjects. **B.** Amyloid plaques, with dystrophic neurites and neurofibrillary tangles. **C.** Anti-Aβ antibody immunohistochemical staining of an AD brain. **D.** Hyperphosphorylated tau accumulation in neuronal cell bodies. **E.** The presence of Aβ in a β-pleated sheet structure in amyloid plaques and tau in a β-pleated sheet structure in neurofibrillary tangles by Thioflavin-S stain. **F.** AD neuropathology.

1.1.3 Pathogenetic hypotheses of AD

Extensive research has laid the basis of our current understanding of AD etiology and pathogenesis. During the past decades, many hypotheses have been postulated in AD pathogenesis. Of note, the cholinergic synapses are ubiquitous present in the human CNS. Their high density in the thalamus, striatum, limbic system, and neocortex suggest that cholinergic transmission is essential to memory, learning, attention and other higher brain functions (Hampel, Mesulam et al. 2018).

The systematic biochemical investigation of AD brains' patients began in the late 1960s providing the basis for the development of rational therapeutic interventions. Support for this perspective is given by reports of substantial neocortical deficits in the choline acetyltransferase (ChAT) enzyme responsible for the synthesis of ACh, a major neurotransmitter in the brain with activity throughout the cortex, basal ganglia, and basal forebrain (Davies and Maloney 1976, Francis, Palmer et al. 1999). Subsequent discoveries of reduced choline uptake, ACh release and loss of cholinergic neurons from the nucleus basalis of Meynert (a distinct population of basal forebrain neurons) confirmed a

substantial pre-synaptic cholinergic deficit in the cortex of patients with AD (Whitehouse, Price et al. 1982, Rylett, Ball et al. 1983, Francis, Palmer et al. 1999). These studies, together with the emergent role of ACh in learning and memory processes, led to the "cholinergic hypothesis of Alzheimer's disease" (Drachman and Leavitt 1974, Francis, Palmer et al. 1999). According to this hypothesis, the progressive loss of limbic and neocortical cholinergic innervation and the degeneration of cholinergic neurons in the basal forebrain contributed significantly to cognitive impairment in AD.

Perry et al. (1978) correlated the reduced ChAT activity with the increase in the number of neuritic plaques in the post-mortem brains of AD patients (Perry, Tomlinson et al. 1978). Neurofibrillary degeneration in the basal forebrain is believed to be the primary cause for the dysfunction and death of forebrain cholinergic neurons, giving rise to a widespread presynaptic cholinergic denervation (Hampel, Mesulam et al. 2018). Even nowadays cholinesterase inhibitors increasing Ach availability at synapses in the brain, represent one of the few drug therapies providing clinically useful in the treatment of AD dementia, thus validating the cholinergic system as an important therapeutic target in the disease (Hampel, Mesulam et al. 2018).

The major pathogenic events leading to AD development as proposed by the "amyloid hypothesis" suggesting that accumulation of pathological forms of Aβ produced by sequential cleavage of APP by β- and γ-secretase enzymes in the brain is the primary pathological process, driven through an imbalance between Aβ production and its clearance, leading to the aggregation of $A\beta_{1-42}$ monomers in oligomeric form suggesting that soluble forms of Aβ are the proximate effectors of synapse loss and neuronal injury (Hardy and Selkoe 2002, Lane, Hardy et al. 2018). Three secretases (α, β, and γ) are involved in the APP cleavage by two pathways. In the non-amyloidogenic pathway, APP is cleaved by α-secretase enzyme to produce the neuroprotective soluble APPα fragment, while in the amyloidogenic pathway, APP is sequentially cleaved by β, and then γ-secretases leading to Aβ formation and its subsequently accumulation in the senile plaques (Hardy and Selkoe 2002). The formation of NFTs and subsequent neuronal dysfunction and neurodegeneration, probably mediated by inflammation, are thought to be downstream processes. Aβ oligomers may directly cause synaptic and neuritic damage and induce tau hyperphosphorylation, in addition to the activation of damaging inflammatory cascades. According to the amyloid hypothesis, the accumulation of Aβ plaques acts as a pathological trigger for a cascade of events that includes neuritic injury, NFTs formation, neuronal dysfunction and cell death in AD brain (**Figure 2**; (Barage and Sonawane 2015). The Aβ deposition and diffused plaque formation lead to local microglial activation, cytokine release, reactive astrocytosis and a multi-protein inflammatory response (Eikelenboom, Zhan et al. 1994, Barage and Sonawane 2015). Also, the different biochemical and structural changes in surrounding axons,

dendrites and neuronal cell bodies that are characterized by synapse and neuron loss as well as the cerebral atrophy in AD brain are in accordance with this hypothesis (Terry, Masliah et al. 1991).

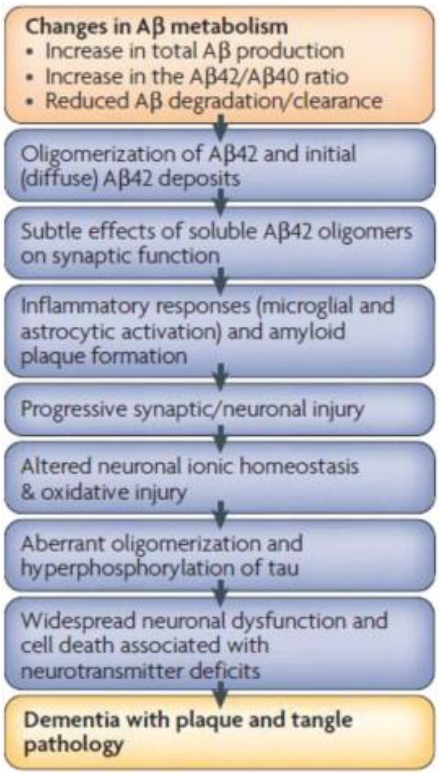

Figure 2. The amyloid hypothesis. Aβ oligomers may directly injure the synapses and neurites of brain neurons, and actives microglia and astrocytes **(Adapted from Barage and Sonawane 2015)**.

The strongest support for the Aβ cascade hypothesis originates from genetic studies suggested that neurodegenerative processes in AD are the consequences of an imbalance between Aβ peptide production and clearance. Autosomal dominant mutations in three different genes (APP, PSEN1 and PSEN2) related to familial form of AD have been linked to abnormal Aβ production or aggregation (Selkoe 2001). Specific point mutations in the APP gene shift the processing of APP towards the Aβ pathway by reducing the affinity to α-secretase, or increasing turnover by β-secretase (Almkvist, Basun et al. 1997). The fact that such simple point mutations greatly facilitate AD development support the concept that Aβ is the main cause of AD pathology (Li and Hölscher 2007) ApoE4 carriers, typical of late-onset AD, is an allele found to markedly increase AD risk and decrease Aβ brain clearance leading to excess Aβ aggregation and typical downstream AD neuropathology. Aβ42 oligomers in late-onset AD brains decrease synapse density, inhibit long-term potentiation (LTP), and enhance long-term synaptic depression (Selkoe and Hardy 2016).

Nonetheless, Aβ-peptide accumulation does not correlate well with the extent of neuronal loss and cognitive dysfunction observed in AD brain. In fact, the demonstration of direct Aβ-peptide

neurotoxicity has been difficult in most animal model highlighting the existence of key intermediate between amyloidosis and neurodegeneration (Nelson, Alafuzoff et al. 2012).

While the genetic evidence strongly support the pivotal role of Aβ aggregation in the induction of AD cascade pathogenetic, it seems clear that Aβ is necessary but not sufficient to mediate the neurodegenerative process and that there are other downstream factors that play a key role such as neuroinflammation and tau accumulation. In this context, the tau hypothesis is based on the observations that tau oligomers are neurotoxic, and that anomalous tau hyperphosphorylation constitute a common final pathway for the different altered molecular signals that affect brain neurons (Maccioni, Farías et al. 2010). Evidence indicated that severity of dementia's type are correlated with the increasing accumulation of NFTs in the brain, and the hyperphosphorylated tau species in the CSF of AD patients is highly correlated with the extent of cognitive impairment and a decrease in tau filaments by target-directed drugs alleviate cognitive impairment in AD patients (Maccioni, Muñoz et al. 2001, Maccioni, Lavados et al. 2006). Beyond the classical formulation of the tau hypotheses, several studies support the role of neuro-immunomodulation changes in the pathogenesis of AD reinforcing the idea that damage signals activate microglia, which in turn overproduce pro-inflammatory cytokines triggering deleterious signal cascades in neuronal cells with deregulation of protein kinases and phosphatases controlling thus tau phosphorylations and the consequent neurofibrillary degeneration (Fernández, Rojo et al. 2008). As a result of neuronal death, tau oligomeric species are released into the extracellular environment, thus contributing to microglial activation and providing positive feedback on the deleterious cycle that lead to progressive degeneration of neurons in AD brains (Morales, Farías et al. 2010).

GWAS have been identified a number of genes from innate immune pathways that act as significant risk factors for AD. Among them, microglial genes such as TREM2, CR1, and CD33, have been identified. Proteomic and transcriptomic analyses have described microglial proteins such as TREM2 and Transmembrane Immune Signaling Adaptor TYRO-BP to be upregulated in the late onset AD brains. These studies also showed an altered immune network connectivity in AD brains suggesting that the altered immune networks are causally associated with disease pathogenesis (Zhang, Gaiteri et al. 2013).

Thus, there is strong evidence that suggests neuroinflammation as a key player in AD pathogenesis. Stimuli from the microenvironment may induce microglia cells to switch to a classically activated inflammatory phenotype M1, or, on the contrary to an alternatively activated M2 phenotype characterized by the secretion of different types of cytokines (Businaro, Corsi et al. 2018). Microglia are a subtype of brain glial cells constituting up to 10% of all cells in the healthy human cortex. Microglia, the resting macrophage population in the brain and spinal cord, is in intimate contact with

neurons and is involved in many basic physiological processes. Microglial cells, often found near Aβ plaques in AD patients, are able to produce different neurotrophic and anti-inflammatory factors essential for cell growth and protection; but they can also release different cytotoxic molecules, such as ROS, RNS, and pro-inflammatory cytokines such as Interleukin (IL)-1β, IL-6, and Tumour Necrosis Factor-alpha (TNF-α) (Kraft and Harry 2011) playing a central role in the inflammatory processes in AD brain. Neuroinflammation is considered as a complex set of brain processes that may begin with microglial activation and may produce both beneficial and harmful effects. Sustained activation of microglia and following release of inflammatory mediators is an important process of chronic neuroinflammation (Kraft and Harry 2011, Cai, Hussain et al. 2014). Upon an inflammatory stimulus such as Aβ, the microglia of the CNS is activated and secrete pro-inflammatory cytokines and chemokines to facilitate cellular recruitment to the inflammation site. The excessive production of Aβ, inflammatory stimuli and the lack of an efficient control of microglial and astrocytic cells for the clearance of Aβ peptide drives the neurotoxic process triggering a further pro-inflammatory response (**Figure 3**, (Minter, Taylor et al. 2016)).

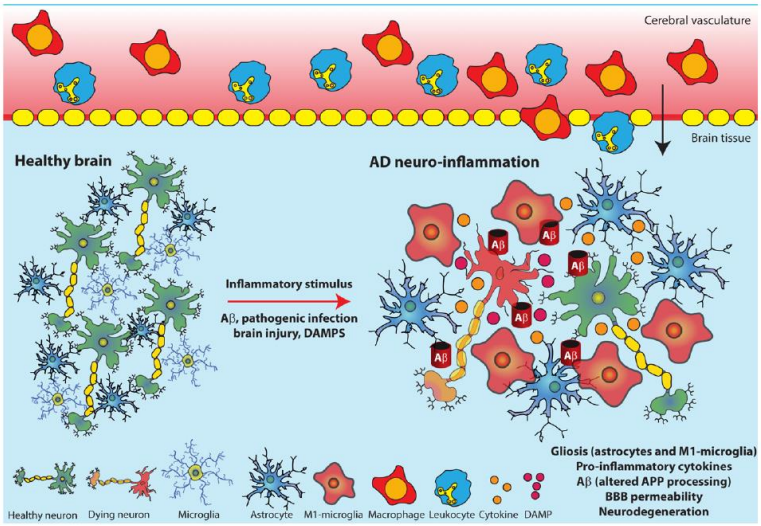

Figure 3. Microglial activation and neuroinflammation Aβ-induced neurotoxicity (Adapted from Minter, Taylor et al. 2016).

In healthy brain individuals the microglia inflammatory response is protective and crucial for pathogen removal, involving pro-inflammatory initiation and phagocytic clearance. In AD patients this process is chronically dysregulated leading to a pro-inflammatory environment detrimental to neuronal integrity. Inflammatory cytokines-mediated neurotoxicity induced by Aβ has been considered as a main actor in AD pathogenesis. The mechanisms and pathways of neuroinflammation triggered by Aβ have been demonstrated by in vivo and in vitro studies, where activated glial cells lead to a release of pro-inflammatory cytokines, inflammatory mediators such as Cox-2, inducible

nitric oxide synthase (iNOS) and nitric oxide (NO), free radicals, and chemokine (Ayasolla, Khan et al. 2004, Garção, Oliveira et al. 2006). In addition, microglial activation induced by Aβ is mediated not only by the release of pro-inflammatory cytokines microglia-induced but also by the production of hydrogen peroxide from NADPH oxidase (Jekabsone, Mander et al. 2006).

Oxidative stress phenomena Aβ-induced is considered as both a cause and a consequence of neuroinflammation and microglial activation in AD pathogenesis acting as a striking mediator among them (Zhang, Chen et al. 2009). Neurotoxic Aβ-induced oxidative stress leads to neuroinflammation and/or microglial activation through not only the oxidative reaction mechanism of free radicals but also by the involvement of NF-κB, ROS, NO and the activation of NADPH oxidase and apoptosis (Liew, Huang et al. 2010, Cai, Hussain et al. 2014). Evidence suggested a vicious circle between the induction of oxidative stress and Aβ aggregation/toxicity (Caruso, Spampinato et al. 2019). Markers of oxidative stress have been found also in plasma and erythrocytes other than at brain level of MCI and AD patients (Minati, Edginton et al. 2009) as well as in AD brain animal models before plaques deposition. These informations suggested that oxidative damage appears in the early stage of AD, and therefore the disease progression might be connected to the redox imbalance (Cheignon, Tomas et al. 2018).

A sustained inflammatory environment in the brain triggers oxidative stress and inhibits synaptic transmission, causing synaptic dysfunction. Neuroinflammation and oxidative stress also result in altered mitochondria and impaired energy metabolism. Each of them stimulates other pathological features, resulting in the progressive cognitive decline observed in AD (Pimplikar 2014).

1.1.4 Diagnosis: role of biological and neuropsychological markers in AD

The presence of senile plaques and NFTs begin to build up in the brain many years before the development of clinical symptoms. MCI is considered a transitional stage between normal aging and AD that is diagnosed on the basis of early mild memory impairment, absence of deficits in cognitive domains other than memory, and progressive decline in cognitive functions leading to the development of dementia (Bozoki, Giordani et al. 2001, Morris, Storandt et al. 2001). The diagnosis of MCI and mild dementia is important because in both, there is a heightened risk for further cognitive decline development (Knopman and Petersen 2014). One of the most common signs of AD, especially in the early stage, is forgetting recently learned information. Among the main cognitive symptoms of AD there are amnesia, aphasia, apraxia, and agnosia. Over the course of the disease, these symptoms become more pronounced with the progress of disease severity. AD patients often find it hard to complete daily tasks. For some people, having vision problems is a sign of AD and this may lead to difficulty with balance or trouble reading (https://www.alz.org/alzheimers-dementia/10_signs).

In the 1996, the International Psychogeriatric Association defined the term “Behavioral and Psychological Symptoms of Dementia” (BPSD). These non-cognitive symptoms including agitation, apathy, depression, hallucinations and delusions that occur at some stage in most patients. BPSDs can both precede cognitive symptoms, especially apathy and depression, but also occur with them and worsen during the illness. In AD patients, depressive symptoms are associated with poor quality of life whereas mood disorders and psychotic symptoms predict changes in the poor quality of life 2 years later (Prado-Jean, Couratier et al. 2010). Depression affects up to 43% of patients with dementia and it predicts an increased number of neuropsychiatric symptoms, particularly agitation, anxiety, and irritability (Prado-Jean, Couratier et al. 2010). Moreover, increased number of BPSDs it has been negatively correlated with survival rates of patients over a 3-year period and the presence of psychosis in AD it has been associated with increased mortality rate and speeding up of cognitive decline (Cerejeira, Lagarto et al. 2012).

The clinical criteria for the diagnosis of AD has been the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS–ADRDA) combining clinical and neuropathological patterns and assigning diagnoses of “possible”, “probable” and “definite AD” (McKhann, Drachman et al. 1984). The evaluation of cognitive deficits by neuropsychological tools including screening tests such as Mini-Mental State Examination (MMSE) and Alzheimer's Disease Assessment Scale-Cognitive Behavior section (ADAS-Cog), as well as specific tools for the assessment of neuropsychiatric symptoms, such as the Neuropsychiatric Inventory Questionnaire (NPIQ) combined with instruments evaluating normal activities of daily living /instrumental activities of daily living (IADL) (McKhann, Drachman et al.) (Caraci, Castellano et al. 2014). The Clinical Dementia Rating Scale (CDR) is used by the physician to assess patients’ performance on everyday activities in five different cognitive domains (Schneider, Olin et al. 1997). Cognitive deficits can be assessed in MCI patients by the use of different psychometric tools such as Montreal Cognitive Assessment (MoCA) combined with other neuropsychological instruments for the evaluation of executive function, as well as attention, orientation, memory, language and visuo-constructional functions (Larner 2012). The combination of cognitive screening tests (i.e. MMSE and MoCA) with specific neuropsychological tools represents a new strategy to identify the subgroup of amnestic MCI patients at higher risk to develop AD-dementia. Neuropsychiatric symptoms, such as depression and apathy, can be evaluated in MCI patients by validated and reliable psychometric tools, such as NPIQ and Geriatric Depression Scale (GDS) (Rosenberg, Mielke et al. 2013). The development of depressive symptoms in these patients, independently from an history of depression, should be monitored in longitudinal studies, because depressive symptoms can be prodromal AD symptoms and can also increase the risk of conversion

from MCI into AD (Caraci, Castellano et al. 2014). In this context, biological markers could be fundamental to predict the disease progression or the rate of cognitive decline in MCI patients at higher risk to develop AD. To date neuroimaging biomarkers, genetic biomarkers (i.e Apo E4 allele) and CSF-derived biomarkers are available to detect AD dementia development. Neuroimaging techniques can detect Aβ accumulation and deposition as well as NFTs (Positron emission tomography (PET) by using a variety of specific ligands), whereas others techniques, such as fluorodeoxyglucose PET [(FDG)-PET], structural and functional magnetic resonance imaging (MRI), can detect synaptic dysfunction and neuronal injury in an early phase of AD pathogenesis. The ligand [18^F] florbetapir PET may help to identify individuals at increased risk for progressive cognitive decline (Doraiswamy, Sperling et al. 2012). FDG-PET uses [18^F] FDG as a ligand to measure brain glucose metabolism and cerebral blood flow in AD brain. 18^F-(FDG) PET hypometabolism, a marker of synaptic dysfunction, in the parieto-temporal association areas, posterior cingulate and precuneus, regions which have a critical role in episodic memory function, is supportive for an AD diagnosis (Kato, Inui et al. 2016).

Structural Magnetic Resonance Imaging (MRI) is the imaging technique, which allows the identification of hippocampal selective atrophy in mild AD patients and, most importantly, already in MCI subjects. In particular, a specific pattern of neurodegeneration, including medial temporal lobe, posterior cingulate and orbitofrontal cortex, has been found to predict the progression to AD in MCI patients recruited in the Alzheimer's Disease Neuroimaging Initiative (ADNI) project (Misra, Fan et al. 2009). Structural imaging, using computed tomography or MRI, is recommended for all patients investigated for cognitive impairment in order to exclude structural abnormalities and other neurodegenerative diseases (Lane, Hardy et al. 2018).

Functional MRI (fMRI) is an important imaging biomarker that it has been used in the last years to evaluate abnormal cognitive task-related changes in the basal brain activity during resting state in AD patients as well as in MCI patients (an hyperactivation in the medial temporal lobe predicts subsequent cognitive decline in MCI patients) (Miller, Fenstermacher et al. 2008).

The typical CSF pattern in AD is low Aβ42 and elevated levels of both tau and phospho-tau (p-tau) considering these as the most informative biomarkers of AD and this pattern has a role in predicting MCI subject will develop AD (Olsson, Lautner et al. 2016). In addition, combining baseline CSF p-tau181 levels and medial temporal lobe atrophy evaluated by MRI imaging significantly increases the overall prediction accuracy in the diagnosis of AD from 74% to 84%. Moreover, an inverse correlation has been found between low CSF $A\beta_{1-42}$ concentrations and brain Aβ deposition in AD patients as assessed by PiB-PET-Amyloid imaging.

There are international scientific efforts to identify and validate innovative blood-based biomarkers able to reflect pathophysiological mechanisms associated with different neurodegenerative diseases, including AD. Recently, plasma neurofilament light chain (Nf-L) protein, a scaffolding cytoskeleton protein released upon neuronal damage, appeared as a promising blood-based biomarker for the prediction of neurodegenerative phenomena and AD development. Recent published data have identified potential blood-based biomarkers (eg, neuronally derived exosome levels of p-tau, $A\beta_{1–42}$, neurogranin) that predict the risk of AD development and progression, and discriminate between disorder and cognitively normal older adults (Zvěřová 2018). According to this scenario, decreased plasma $A\beta_{1-42}$ levels paralleled to an increased precursor for nerve growth factor (proNGF) concentrations combined with inflammatory biomarkers predict the worsening of AD pathophysiology and the subsequent cognitive decline in DS patients (Hampel, Vergallo et al. 2019). More recently, it has been demonstrated that increased plasma BACE1 activity may predict the progression from MCI into AD acting as a non-invasive blood-based biomarker (Shen, Wang et al. 2018).

1.1.5 Pharmacological treatment of AD

Current approved drug treatments for AD provide only symptomatic relief but without modifying the course of the disease and not interfere with the neuropathological mechanisms underlying the illness. The use of Ach cholinesterase inhibitors (AChEI) as a treatment for AD comes from the cholinergic hypothesis according to the deterioration in cholinergic neuron function causes cognitive and behavioral impairments of AD. These compounds reversibly bind to the hydrophobic region of the Ach cholinesterase enzyme (AChE) in order to allosterically modulate its catalytic activity increasing Ach availability into the synaptic cleft to enhance the cholinergic neurotransmission. The first drug to get Food Drug Administration (FDA) approval in the treatment of AD was tacrine and later on it was withdrawn due to its hepatotoxicity (Abeysinghe, Deshapriya et al. 2020). Currently available FDA-approved AChEI for AD treatment are donepezil (1996), approved to treat all stages of AD, rivastigmine (2000), and galantamine (2001) approved for mild-to-moderate AD. These drugs are prescribed to treat symptoms related to memory, thinking, language, judgment and other thought processes. Memantine, a voltage-gated and uncompetitive N-methyl d-aspartate (NMDA) receptor antagonist with moderate affinity, is the drug of choice in severe AD and a combination of donepezil and extended release memantine is used in moderate to severe AD form (Abeysinghe, Deshapriya et al. 2020).

Donepezil is a piperidine derivative that reversibly inhibits AChE with a binding to plasma proteins in a proportion of 96%, and is metabolised by isoenzyme 2D6 and 3A4 of cytochrome P450. Starting

and minimal effective dose is 5 mg once daily while the maximal recommended dose is 10 mg/die. The most common side-effects reported following the treatment with donepezil are nausea, vomiting, diarrhoea, muscle cramps, dizziness, fatigue, and anorexia, that are manifested in dose-dependent manner (Massoud and Gauthier 2010)

Rivastigmine is a carbamate derivative that reversibly inhibits both acetyl- and butyryl- (BuChE) cholinesterase enzymes. This latter is an enzyme widely distributed in the CNS that plays a role in cholinergic function and neurodegeneration. Rivastigmine binds to plasma proteins in a proportion of 40%, and it is hydrolysed by esterases and is excreted in the urine. It is not metabolized by cytochrome P450 isoenzymes thus minimizing pharmacokinetic interactions. Starting effective dose is 1.5 mg twice a day with the minimal effective dose of 3 mg twice daily. Rivastigmine dose may be gradually titrated up to a maximum of 6 mg twice daily. A transdermal patch is available on most markets since 2008 providing a drug delivery over 24 hours and similar efficacy to the highest recommended dose of oral rivastigmine with improved tolerability (Winblad, Grossberg et al. 2007, Kurz, Farlow et al. 2009). Rivastigmine (6 to 12 mg daily orally or 9.5 mg daily transdermally) has been shown to be beneficial in mild to moderate AD in comparison with placebo. The better outcomes were observed in improving cognition and daily activities but were small and of uncertain clinical importance (Birks and Grimley Evans 2015).

Galantamine is a tertiary alkaloid agent that reversibly inhibits AChE (Robinson and Plosker 2006) and binds allosterically to nicotinic receptors enhancing the intrinsic action of ACh on these receptors and increasing the cholinergic function. It is rapidly absorbed after oral administration and it binds to plasma proteins in a proportion of 18%. Galantamine is metabolized by isoenzyme CYP2D6 and 3A4. Starting dose of galantamine extended-release formulation is 8 mg/die with the minimal effective dose of 16 mg once daily and maximal dose of 24 mg/die. Galantamine's side effects are nausea, vomiting, loss of appetite and increased frequency of bowel movements (Massoud and Gauthier 2010). A dose of 16-24 mg/die has been shown to improve cognitive functions in AD patients significantly better than donepezil (Razay and Wilcock 2008) and patients who received galantamine 24 mg/die for 6 months showed an improvement in cognition and less incidence of disease worsening when compared with placebo group (Orgogozo, Small et al. 2004).

AChEIs are able to improve cognitive, daily and global functions, and some behavioral symptoms of AD patients, compared with placebo group (Massoud and Gauthier 2010).

Glutamate is the main excitatory neurotransmitter in CNS that binds with NMDA receptors and is implicated in learning and memory processes. Excessive glutamate binding to NMDA causes an influx of ion Ca2+ leading to excitotoxicity and consequently synaptic functions loss and neuronal death and results to be involved in the neurodegeneration of AD (Liu, Chang et al. 2019). Memantine

acting as an NMDA receptor antagonist provides a symptomatic relief as well as is able to reduce neuronal damage occurring in AD and to restore the Ca^{2+} imbalance in cells Aβ-induced playing thus a neuroprotective effect (Abeysinghe, Deshapriya et al. 2020). Memantine is well absorbed after oral administration with starting dose of 5 mg/die (in one or two doses). Minimal and maximal therapeutic doses are 10 mg and 20 mg daily, respectively. It can cause side effects, including headache, constipation, confusion and dizziness. Memantine at the dose of 20 mg dose is effective in improving daily activities deficits and cognitive impairment compared with placebo (Wilcock 2003). A combination therapy with AChEIs and memantine is considered as the best treatment choice for the treatment of moderate to severe AD (Deardorff and Grossberg 2016).

Several types of drugs have been used to BPSDs treatment including typical and atypical antipsychotics, antidepressants, anticonvulsants and mood stabilizers.

Atypical antipsychotics have shown to be effective in the treatment of neuropsychiatric symptoms of dementia, such as aggression, psychosis, and agitation and despite serious side effects, including extrapyramidal symptoms, are still widely used off-label. Olanzapine, quetiapine, and risperidone's efficacy and safety in the treatment of dementia were examined in the Clinical Antipsychotic Trials of Intervention Effectiveness in Alzheimer's Disease (CATIE-AD) study (Ismail, Dagerman et al. 2007). The safety and efficacy of aripiprazole and ziprasidone in treating AD as well as in Lewy Bodies' dementia is also demonstrated. Currently, more data is needed to determine whether different atypical antipsychotics vary with regard to their effectiveness, or their risk of mortality or cerebrovascular side-effects. Today, new drugs such as pimavanserin and brexiprazole are undergoing evaluation to treat psychosis, agitation and aggression in AD (Magierski, Sobow et al. 2020).

Second-generation antidepressants, in particular selective serotonin reuptake inhibitors (SSRIs), can be an effective and well-tolerated alternative to atypical antipsychotics for BPSDs treatment. Citalopram and sertraline could improve agitation and psychosis in subjects with dementia showing a similar efficacy, but better tolerability and safety, compared to haloperidol and risperidone treatment (Cerejeira, Lagarto et al. 2012). It is known that depression acts as a risk factor for AD but on the other hand it has been proved that depression is a typical presentation of the initial phases of dementia or MCI. As with all BPSDs, the management of clinical depression should start with the optimization of dementia treatment (Magierski, Sobow et al. 2020). SSRIs represent a good option for mood disorders in people with cognitive impairment and dementia.

In the last years, the drug discovery process has focused on the development of disease modifying drugs able to counteract the progression of AD considering drugs able to reduce Aβ production and/or aggregation, to promote Aβ clearance and drugs that targeting tau phosphorylation (Salomone, Caraci

et al. 2012). Active and passive immunization targeting Aβ peptide in AD preclinical models showed clearance of aggregated Aβ deposits and improved memory and learning. In passive immunization, exogenous antibodies are administered able to bind with Aβ fibrils and thus prevent its aggregation (Abeysinghe, Deshapriya et al. 2020). Among them solanezumab, a human IgG that binds with Aβ increasing its clearance, is now on a large scale phase III trial in mild AD cases. It reduces the inflammatory process Aβ-induced playing a neuroprotective role. On June 7, 2021, the FDA approved aducanumab, the first new drug for AD in the last two decades, that should be initiated in patients with MCI or mild dementia stage of the disease for the treatment of memory and cognitive deficits (https://www.alz.org/alzheimers-dementia/treatments/medications-for-memory).

Aducanumab is a monoclonal human IgG with a high affinity towards Aβ that can reduce both soluble and insoluble Aβ levels and reduce neurodegeneration. The most common side effects include amyloid-related imaging abnormalities (ARIA), headache and fall. Another potentially serious side effect is an allergic reaction.

Moreover, BAN2401 a human IgG which selectively binds to Aβ protofibrils and reduces amyloid aggregation is undergoing clinical trials for AD (Abeysinghe, Deshapriya et al. 2020).

Some drugs which have not received the FDA approval yet and are currently undergoing phases III and IV of clinical trials are ALZT-OP1 that is a combination of cromolyn and ibuprofen, these latter are two FDA approved drugs acting as neuroprotective agents by reducing the Aβ levels and inflammation (Abeysinghe, Deshapriya et al. 2020), and TRx0237, a tau protein aggregation inhibitor, that is able to inhibit the NFTs formation and reduces neuronal death.

Drug development research targeting the molecular mechanisms underlying the pathogenetic process of the disease may present us the first approved disease modifying therapies for AD in the near future.

1.2 Neurobiological links between depression and AD

During the last years, several studies have been demonstrated the neurobiological and clinical links between depression and AD. Starting from epidemiological studies, depression is considered as a risk factor for AD development. On the other hand, it is useful to have some understanding whether a history of depression acts simply as an independent risk factor for AD development or whether depressive disorders correspond to a prodromal phase of AD increasing the conversion from MCI to AD (Modrego and Ferrández 2004, Ownby, Crocco et al. 2006). Different studies showed that depressive symptoms determine an additive risk effect to the progression to dementia in MCI subjects and in particular patients with amnestic MCI present an increased risk to develop AD when their clinical condition is associated with relevant depressive symptoms (Mourao, Mansur et al. 2016, Moon, Kim et al. 2017). The first study that showed a central role of depression as a risk factor for

the conversion from MCI into AD was conducted by Modrego and Fernandez in 2004, where in a 3-years prospective cohort study found that a high percentage of depressed MCI patients developed dementia (85%) in comparison with non-depressed MCI patients (32%) (relative risk, 2.6) (Modrego and Ferrández 2004). Moreover, depression in MCI individuals may be associated with underlying neuropathological changes in AD-affected regions (atrophy in frontal, parietal, and temporal cortex) and with an increased cognitive decline (Lee, Lu et al. 2012). Depressed-AD patients showed a greater and faster cognitive impairment compared with non-depressed patients and senile plaques and NFTs are more marked in the hippocampus of AD patients with comorbid depression (Rapp, Schnaider-Beeri et al. 2008). Patients with late-onset depression showed regional hippocampal abnormalities and present an increased risk to develop cognitive impairment over time than patients with early-onset depression (Ballmaier, Narr et al. 2008).

The levels of Aβ in the CSF of depressed and AD patients are similar and, it has been highlighted that low CSF $A\beta_{(1-42)}/A\beta_{(1-40)}$ ratio reflects a higher risk of AD or cognitive dysfunction, particularly in depressed patients (Graff-Radford, Crook et al. 2007, Direk, Schrijvers et al. 2013, Nyarko, Quartey et al. 2019).

In this context, the identification of common neurobiological markers in depression and AD represents an essential step for an early diagnosis and treatment of preclinical AD or MCI subjects at higher risk to AD development (Caraci, Spampinato et al. 2018).

Preclinical studies demonstrated that the central soluble $A\beta_{1-42}$ injection induces a depressive like-behavior in rats, with altered HPA axis activation, reduced 5HT cortical levels and neurotrophin amount suggesting that Aβ oligomers administration may be associated with the development of "amyloid-related depression", a clinical phenotype characterized by a low response to "monoaminergic antidepressants drugs" (Morgese, Schiavone et al. 2017, Schiavone, Tucci et al. 2017).

Of note, the depressive-like phenotype Aβ-induced could be reverted by fluoxetine and ketamine, which are able to increase the noradrenaline (NA) release, a neurotransmitter with a neuroprotective activity against Aβ-induced neurotoxicity (Liu, Ye et al. 2015).

Moreover, intracerebroventricular (i.c.v.) injection of $A\beta_{1-42}$ oligomers in mice is able to induce cognitive deficits and depressive-like symptoms, and induce neuroinflammatory phenomena that plays a central role in the pathophysiology of both depression and AD (Ledo, Azevedo et al. 2013, Caraci, Spampinato et al. 2018). Neuroinflammation can also contribute to the pathogenesis of both diseases by impairment of neurotrophins signaling including Transforming Growth Factor-beta 1 (TGF-β1).

1.2.1 Stress and dysfunction of the hypothalamic-pituitary-adrenal axis

It is known that stress is able to increase the levels of Aβ peptide and on the other hand, a dysregulation of HPA axis occurs early in AD and is associated with an increase in the cortisol levels. In addition, high levels of Aβ have been associated with an increase in the glucocorticoid (GC) amount and corticotropin release factor (CRF) levels (Morgese, Schiavone et al. 2017). In animal AD models has been demonstrated that acute or chronic stress is able to increase Aβ interstitial level and that chronic isolation stress exacerbates Aβ plaque deposition associated with increased GC and type 1 CRF receptor expression (Kang, Cirrito et al. 2007, Dong, Yuede et al. 2008).

Several studies demonstrated that an increase in CRF release was associated with the speed up of neuropathology and cognitive decline in AD animal models (Dong, Murphy et al. 2012) and an increase in GC levels has been associated with cognitive impairment in humans (Lupien, Fiocco et al. 2005). GC hormones quickly cross the BBB and bind to glucocorticoid and mineralcorticoid receptors with low- and high-affinity, respectively, exerting a negative feedback on HPA axis in the hippocampus and regulating normal cellular metabolic activity finally influencing many CNS functions, such as learning and memory (de Kloet, Joëls et al. 2005, Caraci, Copani et al. 2010).

Recently, it has been demonstrated that $A\beta_{1-42}$ oligomers i.c.v. is able to induce a depressive-like behavior in rats and this phenotype is associated to altered HPA axis activation (Morgese, Tucci et al. 2017) as observed in major depressive disorder with an impairment of the inhibitory feedback of circulating glucocorticoids on the secretion of HPA hormones. Thus, an impairment of the negative feedback of GC on the HPA axis activity results in increased cortisol levels assuming that high basal cortisol levels are associated with more rapid cognitive decline in AD patients.

Stress and increased GC levels, by driving APP processing, enhance Aβ production as well as an increased vulnerability to stress is associated with a depressive and anxiety-like phenotype and corresponded to increased plasma Aβ levels. Furthermore, the duration and the type of stress have a crucial role for cognitive impairment onset (Morgese, Schiavone et al. 2017).

An excess in GC levels may have a key role in the pathophysiology of both diseases by multiple mechanisms. In particular, GC increased levels by the activation of GC receptors can reduce neurogenesis in the hippocampal dentate gyrus, lead to the retractation of hippocampal apical dendrites, and cause hippocampal neurons death. GC can trigger apoptotic death in hippocampal neurons by the activation of their receptors and increasing neuronal susceptibility to different neurotoxins including Aβ oligomers.

Finally, it has been suggested that alterations in APP processing and dysregulation in Aβ production and/or clearance could occur in response to an increase in stress hormones release. In addition, the

reduction in 5-HT and neurotrophin levels, the trigger neuroinflammation and microglial activation have also been involved (Morgese, Schiavone et al. 2017).

1.2.2 Neuroinflammation

Neuroinflammation is considered as the innate immune response of the nervous system to injury, infection or neurodegenerative disease including AD and it is characterized by the activation of resident glial cells (microglia and astrocytes), release of cytokines and chemokines, activation and infiltration of leukocytes (Campos, Antunes et al. 2020). Neuroinflammation is a complex response that is able to involve a host of cellular and molecular changes, the recruitment of peripheral immune cells, the induction of some intracellular signaling pathways as well as the pro-inflammatory mediators release in the brain contributing to the development of neuronal dysfunction and death in AD brain. Thus, one of the major mechanisms of AD neuropathology has been accredited to the chronic neuroinflammation induced by cytokines released from activated microglia and astrocytes (Zhang and Jiang 2015).

An increase in pro-inflammatory IL-6 cytokine is observed in animal model of AD with consequently more permeable of BBB during peripherally evoked inflammation (Takeda, Sato et al. 2013). In AD brain, IL-6 stimulates and promotes the recruitment of microglia and astrocytes to release pro-inflammatory cytokines and it also promotes the phosphorylation of tau in neurons (Quintanilla, Orellana et al. 2004). Moreover, an increase in TNF-α serum levels was found in AD patients and this pleiotropic cytokine is able to induce Aβ production by increasing the BACE 1 expression and the γ-secretase activity. Aβ is able to induce the expression of pro-inflammatory mediators such as COX-2 and iNOS. In turn, pro-inflammatory cytokines such as TNF-α and IL1-β enhance the production of APP and the process of APP proteolytic cleavage increasing the production of Aβ peptide (Zhang and Jiang 2015). An increase in IL1-β CSF levels and in IL-6 plasma levels have been found in AD patients contributing to reach the signal for triggering neuronal death in AD brain.

It is known that intrahippocampal Aβ injection is able to increase pro-inflammatory cytokines levels such as IL1-β release and leading to microglial activation (this latter is considered an early event in AD pathogenesis) (Sanz, Chiozzi et al. 2009). Aβ oligomers are able to induce in non-transgenic animal model of AD, obtained by i.c.v. injection of $A\beta_{1-42}$ oligomers, a neuroinflammatory phenomenon, which play a central role in the pathophysiology of major depression and AD. Aβ oligomers induce a depressive-like behavior paralleled to memory deficits, hippocampal recruitment of microglia and astrocytes and the activation of brain inflammatory pathways with an increase in IL1-β and TNF-α levels in Aβ-injected mice (Ledo, Azevedo et al. 2013). In addition, an increase of pro-inflammatory cytokines (IL-1β, TNF-a) and a decrease of anti-inflammatory cytokines (IL-10,

IL-4, and TGF-β1) have been observed in hippocampus and cortex of animal models of depression. Depressed patients showed higher levels of pro-inflammatory cytokines (IL-1, IL-6, IL-8, IL-12, IFN-γ and TNF-α) as well as increased acute phase proteins, chemokines and cellular adhesion molecules. On the other hand, reduced levels of anti-inflammatory cytokines including TGF-β1 have been found in depressed patients at plasma level. Of note, several studies have found a positive correlation between depressive symptoms' severity and the increase in the inflammatory status that is able to alter 5-HT metabolism and reduce both synaptic plasticity and hippocampal neurogenesis in depressed patients (Maes, Yirmyia et al. 2009).

These data suggest that chronic inflammation is a common pathophysiological link between depression and AD and might explain why an increased pro-inflammatory status could increase the vulnerability to Aβ toxicity and induce hippocampal atrophy favoring the onset of cognitive decline and the progression from depression to AD.

1.2.3 The role of neurotrophin's signaling: focus on NGF, BDNF and TGF-β1

The "Neurotrophic Factor Hypothesis" for the neurodegenerative diseases including AD was postulated in 1981 suggesting that the loss of activity of neurotrophic factors such as nerve growth factor (NGF) and BDNF may exacerbate the effects of Aβ and explains how the pathology precipitates more readily (Allen, Watson et al. 2011). Neurotrophins are essential for the maintenance of neuronal homeostasis and modulation of synaptic plasticity as well as are involved in learning, memory and neuronal health (Fumagalli, Molteni et al. 2008). In normal brain, **NGF** is produced as a 36kDa precursor protein (proNGF) which is cleaved to the mature form, by furin intracellularly or plasmin extracellularly. NGF binds with high affinity to tyrosine kinase receptor TrkA, facilitated by the co-receptor p75NTR, and it is retrogradely transported to the cell bodies of cholinergic neurons in the basal forebrain. The remaining NGF is degraded in the extracellular space by matrix metalloproteinase 9 (MMP-9), which is also released from neurons in dependent manner activity, together with its endogenous inhibitor TIMP-1 (tissue inhibitor of metalloproteases 1). This leads to Ach release and activation of M1 muscarinic receptors whit consequently increased α-secretase activity, which is counter to Aβ production. In AD brain proNGF is not processed properly to NGF. The increased level of proNGF will lead to increased binding at p75 and probably sortilin, leading to a greater likelihood of cell death. Due to an increase in MMP-9 activity, NGF is degraded more quickly. Thus less Ach will be released, less communication with other neurons, less activation of M1 receptors and an increase in β-secretase activity occur, leading to an increase in Aβ formation (Allen, Watson et al. 2011). Cuello and colleagues demonstrated that the conversion of proNGF to mature NGF is reduced in AD, while the activity of MMP-9, the metallo-protease responsible for its

degradation, is enhanced suggesting that this is likely an initiating factor in the degeneration of the cholinergic neurons (Cuello, Bruno et al. 2010, Iulita, Caraci et al. 2016). The failure in proNGF maturation and the increased MMP-9 activity should lead to a reduction in mature NGF-mediated trophic support of basal forebrain cholinergic neurons. This is of particular significance given that NGF dysmetabolism occurs in a context of reduced expression of the high affinity mature NGF receptor, TrkA, relative to the high affinity proNGF receptor, p75, which is aggravated by the fact that TrkA expression itself depends on mature NGF signalling. Comparable to AD patients, DS subjects show reduced cortical ChAT activity and nucleus basalis neurons atrophy. In line with these evidence, Cuello et al., hypothesized that NGF dysmetabolism should be present in DS individuals, given that these patients present increased proNGF levels in frontal, temporal and parietal cortex accompanied by reductions in plasminogen and tissue plasminogen activator (tPA) as well as increased neuroserpin expression (involved in NGF maturation and degradation in the extracellular space) (Iulita, Caraci et al. 2016). The reduced conversion levels of proNGF to mature NGF in these brain areas found in AD or DS-AD patients with consequently elevated MMP-9 activity determines increased degradation of mature NGF leading to synaptopathy and degeneration of cholinergic neurons with memory and executive function deficits in these patients. Given that proNGF may have cellular effects independent of mature NGF, it is possible that proNGF accumulation in AD and DS brains further exacerbates the effect of the trophic disconnect on basal forebrain cholinergic neurons (Caraci, Iulita et al. 2017).

BDNF is highly expressed and widely distributed throughout the CNS especially in the hippocampus and cerebral cortex and is important in the survival and function of hippocampal, cortical, cholinergic and dopaminergic neurons (Allen, Watson et al. 2011). In the hippocampus, BDNF is a vital component of synaptic plasticity and memory formation. LTP is a prerequisite for memory formation and is maintained by the action of BDNF at tropomyosin receptor kinase B (TrkB), which results in structural changes at the synapses level. TrkB receptors are also present on some cholinergic neurons and help to support cholinergic functions (Phillips, Hains et al. 1990, Allen, Watson et al. 2011).

In AD brain pro-BDNF is down-regulated (perhaps directly due to Aβ), thus BDNF levels are reduced leading to a reduction in LTP and synapses formation (Allen, Watson et al. 2011). In addition, the levels of BDNF and its receptor TrkB are reduced in AD brain (Murer, Yan et al. 2001) and pro-inflammatory cytokines, such as IL-1β, render neurons more vulnerable to degeneration by interfering with BDNF-induced neuroprotection (Tong, Balazs et al. 2008).

Reduced levels of BDNF have been connected to dendritic atrophy, neuronal apoptosis, and inhibition of neurogenesis in depression (Nowacka and Obuchowicz 2013). A significant decrease in BDNF levels have been found in animal models of depression stress-induced (Krishnan and Nestler 2008)

as well as in depressed patients (Karege, Vaudan et al. 2005, Caraci, Copani et al. 2010). Stress reduces BDNF-mediated signaling in the hippocampus and prefrontal cortex (PFC), whereas chronic treatment with antidepressants increases BDNF-mediated signaling (Duman and Monteggia 2006, Caraci, Copani et al. 2010). BDNF is also involved in the pathogenesis of treatment-resistant depression, because an association was been found between a genotype of BDNF (val66met polymorphism) that significantly impairs the intracellular trafficking and activity-dependent release of BDNF (Egan, Kojima et al. 2003) and an increased risk to develop treatment-resistant depression (Tryselius, Samakovlis et al. 1992, Caraci, Copani et al. 2010).

It has been recently demonstrated that Aβ induced a reduction of cortical 5-HT levels as well as a reduction of BDNF and NGF signaling levels related a depressive-like behavior (Schiavone, Tucci et al. 2017).

Interestingly, genetic variations of BDNF play a central role in AD-related depression. Borroni et al., demonstrated that the presence of the functional single-nucleotide polymorphism (G196A; Val 66 → Met 66), which impairs BDNF signaling, significantly increases the risk to develop depression in AD patients (Borroni, Archetti et al. 2009, Caraci, Copani et al. 2010). The same polymorphism inducing abnormal accumulation of Aβ plays a key role also in AD pathogenesis, where Met carrier is correlated with lower BDNF concentrations, hippocampal atrophy, and impaired cognitive ability in AD patients (Song, Yu et al. 2015). According to this scenario, the presence of a Met-BDNF allele is associated with a higher risk of disease-progression in MCI patients (Forlenza, Diniz et al. 2010) and a significant decline in episodic memory and hippocampal volume in prodromal AD patients (Lim, Villemagne et al. 2014). This evidence suggest that a deficit of BDNF release might be a common pathophysiological event both in depression and AD (Caraci, Spampinato et al. 2018).

BDNF can also interact in the CNS with other neurotrophins such as **TGF-β1** that is an anti-inflammatory cytokine which regulates the balance between T helper-1 and T helper-2 cytokines, but it can also act as a neurotrophic factor in the CNS protecting neurons against a several number of neurotoxic insults, including excitotoxicity, hypoxia, ischemia, and most importantly Aβ (Vivien and Ali 2006, Caraci, Copani et al. 2010). It also exert a key role in recognition memory formation promoting the transition from early to late LTP (Caraci, Gulisano et al. 2015).

TGF-β1 also affect neuronal survival through other mechanisms because it acts synergistically with other neurotrophins and is required for a full neuroprotective activity of NGF, BDNF and glial-derived neurotrophic factor (GDNF) (Caraci, Battaglia et al. 2011).

It is known that depression acts as a risk factor for AD development (Ownby, Crocco et al. 2006) and it has been demonstrated that patients with MCI and depression present an increased risk of AD development compared to those without depression (Modrego and Ferrández 2004). Different

neurobiological links have been found between these two pathologies including a deficit of TGF-β1 signaling (**Figure 4**, (Caraci, Spampinato et al. 2018)).

Plasma TGF-β1 levels are reduced in depressed patients and are correlate with depression severity. Moreover, a deficit of TGF-β1 significantly contribute to treatment resistance in depression (Myint, Leonard et al. 2005, Musil, Schwarz et al. 2011). Interestingly, a study performed in bonnet macaque showed that stressful condition, a link between depression and AD, induces a reduction of TGF-β1 levels in CSF (Coplan, Gopinath et al. 2017).

A deficit of TGF-β1 is associated to Aβ pathology and NFTs formation and the levels of its receptor TGF-βRII are reduced in human AD brain and correlated with pathological hallmarks of the disease (Tesseur, Zou et al. 2006). AD patients also showed a reduction in the plasma levels of both active and inactive TGF-β1 forms (Juraskova, Andrys et al. 2010) as well as a reduction in its secretion from circulating peripheral blood mononuclear cells has been observed (Luppi, Fioravanti et al. 2009).

A TGF-β1 deficit in AD brain may contribute to enhance both chronic inflammation and neuronal vulnerability to Aβ oligomers, thus accelerating AD progression (Caraci, Copani et al. 2010). Finally, it has been proposed that a deficit of this neurotrophic factor increases the vulnerability to Aβ toxicity and hippocampal atrophy, thus promoting the onset of cognitive deficit and finally the progression from depression to AD.

During the following sections, we will focus on the role of TGF-β1 in physiological and pathological conditions focusing our attention on the key role played by this neurotrophin as a potential biomarker for neurodegenerative diseases including AD.

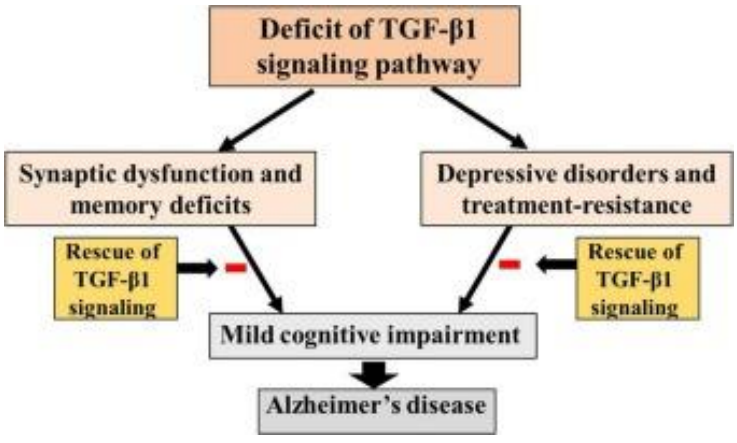

Figure 4. TGF-β1 deficit: a common link between depression and AD (Adapted by (Caraci, Copani et al. 2010).

1.3 Neurobiological links between depression and chronic pain

Depression is a neuropsychiatric disorder which often occurs in comorbidity with chronic pain. Several studies suggested that both diseases are highly-connected and may co-aggravate physical and

psychological symptoms. It has been estimated that 85% of people suffering of chronic pain experience and severe depression supporting the hypothesis that both conditions coexist and exacerbate one another. 5-HT as well as NA systems play an important role in this comorbidity (Sánchez-Salcedo, Cabrera et al. 2021) along with dopamine (DA) system seems to be involved in the pathophysiology of chronic pain, in fact DA system in the limbic midbrain area is reduced in patients with this pathological condition. In particular, D2 receptors, involved in the occurrence and development of depression, have been observed to be reduced in animal models of chronic neuropathic pain (Sheng, Liu et al. 2017). Pain severity is strongly associated with depression in the elderly and depressed women patients are an increased risk to develop chronic pain. Depressed patients are six times more likely to suffer from neuropathic pain suggesting that neuroinflammation could represent a common pathogenic factor between these two diseases (Zis, Daskalaki et al. 2017). In chronic pain, neuroinflammation often results from peripheral damage and excessive neuronal activity of primary sensory neurons (Ji, Xu et al. 2014). Cytokine sensitization of nociceptors following peripheral injury can occur via several pathways in the spinal cord. In response to nerve damage, the central cytokine cascade is stimulated with the activation of spinal microglia in pain-related areas and the following cytokines release. The expression of genes encoding pro-inflammatory cytokines are increased in PFC and hippocampus, limbic system's areas involved in emotions, memory and learning processes, resulting responsible for pain processing and depression (Campos, Antunes et al. 2020). The elevated pro-inflammatory cytokines levels have been found in depressed patients and some of these (IL-6 and TNF-α) has been observed in the sciatic nerve of neuropathic pain animal models corresponding to the development of mechanical allodynia (Cui, Holmin et al. 2000). A decrease in IL-10 anti-inflammatory cytokine and an increase in TNF-α levels in the PFC were found in a preclinical co-model of pain and depression induced by spinal nerve ligation and olfactory bulbectomy model suggesting that a condition of persistent neuroinflammation plays an important role in the induction and maintenance of depression-pain syndrome (Campos, Antunes et al. 2020).

At the same time, a decrease of anti-inflammatory cytokines (IL-10, IL-4, and TGF-β1) have been observed in hippocampus and cortex of animal models of depression while an increase in IL-10 levels showed to have beneficial effects for nociceptive pain. In an animal model of both neuropathic pain and depression, it has been observed microglial and astrocyte activation as well as an increase in IL-1β in the amygdala, a key brain region for the modulation of emotion and pain (Burke, Geoghegan et al. 2013). In addition, a condition of persistent pain may cause a reduction of hippocampal volume and an impairment of LTP and neurogenic process as observed in depressed patients. An increase in cortisol levels induces hippocampal dendritic atrophy, suggesting that depression can induce neural

plasticity changes in areas also involved in controlling the nociceptive system (PFC, hippocampus) and may induce central pain sensitivity (Campos, Antunes et al. 2020). In this regard, fluoxetine, a selective inhibitor of 5-HT uptake used in the treatment of depression, is able to decrease microglial activation and pro-inflammatory cytokines levels in the hippocampus as well as increases IL-10 levels in a rat model of depression and chronic pain comorbidity (Hu, Dong et al. 2017). Moreover, this classical antidepressant decreases TNF-α hippocampal levels in animal models of neuropathic pain and it is able to reduce plasma pro-inflammatory cytokines levels, increase anti-inflammatory IL-10 cytokine, decrease myelin degeneration and leukocyte infiltration in peripheral nerves and decreases astrocyte activation in the spinal cord (Murad and Ayuob 2015, Cai, He et al. 2019, Campos, Antunes et al. 2020).

Moreover, in both pathological conditions, inflammation contributes to glutamate-induced excitotoxicity increasing the neurotransmitter's release in the synaptic cleft. A hyperfunction of the glutamatergic system as well as a hypofunction of the GABAergic system (this latter is the major inhibitory neurotransmitters in the CNS) have been reported in depression. It has been demonstrated that a loss of effective inhibitory neurotransmission in the spinal dorsal horn is involved in neuropathic pain development (Benson, Mifflin et al. 2015). Moreover, an increase in the level of GluA1 subunits of AMPA-type glutamate receptors in the nucleus accumbens (a key component of the brain reward system that is affected in both chronic pain and depression) was observed in spared nerve injury (SNI) animal model contributing to the development of depressive-like behavior associated with chronic pain (Goffer, Xu et al. 2013).

Changes in neurotrophic factors, especially for BDNF levels with following alterations in synaptic plasticity, play a key role in the comorbidity between chronic pain and depression. At the brain level, BDNF plays an opposed role on pain sensitivity, in fact BDNF promotes the nociceptive facilitation while on the other hand, this factor seems to indirectly stimulate descending nociceptive inhibition by contributing to the DA availability. In inflammatory pain conditions, the release of BDNF is regulated by the NGF-upregulation when nociceptors are activated acting as a central modulator of pain; furthermore in peripheral inflammation, an increase in BDNF levels in neurons of dorsal root ganglion is correlated to enhanced release in the spinal dorsal horn (Pezet and McMahon 2006, Nijs, Meeus et al. 2015, Cappoli, Tabolacci et al. 2020). Clinical studies showed increased plasma BDNF levels in patients with fibromyalgia and depressive symptoms. However, pre-clinical studies suggested that the regulation of BDNF in chronic pain and depression is regulated in a region-dependent manner. It has been demonstrated an increase in BDNF level in the spinal cord of neuropathic pain animal models compared to control animals consistent with chronic inflammation (Humo, Lu et al. 2019). In addition, rats subjected to spared nerved ligation with depressive-like

phenotype showed reduced levels of BDNF in the medial prefrontal cortex (mPFC), hippocampus and spinal cord suggesting that reduced BDNF signaling in the mPFC might contribute to neuropathic pain-induced anhedonia (Fang, Yang et al. 2020) together with a down-regulation in BDNF cortical levels was observed in chronic constriction injury (CCI) animal models.

Of note, the BDNF Val66 SNP seems to be closely related to the pain and depression comorbidity, in fact BDNF met carriers more often develop chronic pain (Generaal, Milaneschi et al. 2016) as well as the presence of BDNF Val66Met functional polymorphism increases the risk to develop depression in AD patients (Borroni, Archetti et al. 2009).

As mentioned above the well-known neurotropic factor TGF-β1 is involved in the pathophysiology of AD and a deficit of this anti-inflammatory cytokine can contribute to the development of cognitive decline in both depression and AD. In experimental animal models of chronic neuropathic pain, the expression of TGF-β and its type 1 receptor is down-regulated in red nucleus contralateral to the nerve injury side (Wang, Yu et al. 2015). Moreover, preclinical studies carried out in animal models of neuropathic pain showed that TGF-β1 has an antinociceptive effect by the attenuation of spinal neuroinflammation suppressing neuroimmune responses of neurons and glia as well as by the promotion of endogenous opioids' expression at the spinal cord level. Peripheral nerve injury leads to a down-regulation of endogenous TGF-β1 in the lumbar spinal dorsal horn grey matter. TGF-β1 through its pleiotropic effects is able to inhibit peripheral nerve injury-induced spinal microgliosis, astrocytic activation and the up-regulation of pro-inflammatory cytokines such as TNF-α. Furthermore, TGF-β1 is able to decrease the spinal extracellular excitatory amino acids by inhibition of nerve injury-induced down-regulation of glutamate transporters, thus exerting an antinociceptive effect (Echeverry, Shi et al. 2009, Lantero, Tramullas et al. 2012, Chen, Huang et al. 2013, Lees, Fivelman et al. 2015). In addition, a crosstalk between TGF-β1 signaling and opioid receptors lead to an improvement of endogenous as well as exogenous opioid analgesia in experimental models of neuropathic pain. TGF-β1 therefore exercises a protective effect against the chronic neuropathic pain development by the inhibition of the neuroinflammatory phenomena and the promotion of endogenous opioids' expression within the spinal cord (Lantero, Tramullas et al. 2012, Caraci, Merlo et al. 2019).

1.4 TGF-β1 as a new pharmacological target in CNS disorders

In mammals, three members compose the TGF-β family, which are TGF-β1, 2 and 3, whith the main isoform expressed in the immune system represented by TGF-β1. This neurotropic factor exerts pleiotropic effects on adaptive immunity, especially in the regulation of effector and regulatory CD4 positive T-cell responses. Moreover, TGF-β1 is an anti-inflammatory cytokine that is able to mediate

the development and function of immunosuppressive regulatory T cells (Bettelli, Carrier et al. 2006). TGF-β1 is a member of TGF-β superfamily, which includes several groups of highly conserved multifunctional cell-cell signaling proteins fundamental for the control of cell growth, differentiation, and embryogenesis, as well as immune suppression and neuroprotection (ten Dijke and Hill 2004). It is known that TGF-β1 controls key events in normal development and physiology, and the deregulation of its signaling has been implicated in the pathogenesis of diseases such as connective tissue disorders, fibrosis, and cancer (Morikawa, Derynck et al. 2016).

1.4.1 TGF-β family and TGF-β1 signaling pathway

It is clear that mammalian genomes encode 33 TGF-β-related polypeptides. TGF-β family comprises more than 30 structurally related proteins including three TGF-β isoforms, Activins, Nodals, Bone Morphogenetic Proteins (BMPs), and Growth and Differentiation Factors (GDFs). Members of this family, present in both vertebrates and invertebrates, are ubiquitously expressed in different tissues with several functions during the earliest stages of development and in the adult organism (Weiss and Attisano 2013). Multiple membrane-bound proteins are able to modulate the ligands' activity of the TGF-β superfamily, often in a context-dependent manner and with varying strength and ligand specificity.

All members of TGF-β family are encoded by much larger precursor polypeptides composed by three segments: an amino-terminal signal peptide that is removed during protein translocation into the lumen of the rough endoplasmic reticulum, a large precursor segment or prosegment (vary in length from 150 to 450 residues), and the C-terminal monomer polypeptide that is fundamental for the production of active and fully mature TGF-β family protein (composed by 112 amino acid residues in the case of TGF-β) (Morikawa, Derynck et al. 2016). Mature TGF-β superfamily ligands are released by cleavage from a large precursor and in the case of the three TGF-β isoforms is represented by latency-associated peptides (LAPs). Following secretion, the LAPs remain non-covalently associated with the mature TGF-β dimer and maintain the TGF-βs latent, that is, unable to bind and activate the receptors, in complexes known as "small latent complexes" (SLCs) (Morikawa, Derynck et al. 2016) that control ligand activity by sequestering TGF-β in the extracellular matrix or by mediating interactions with integrin receptors to release the mature ligand (Harrison, Al-Musawi et al. 2011).

Members of TGF-β family exert their cellular function by two structurally related transmembrane proteins, i.e. Type I (also called activin receptor-like kinases (ALKs), in humans seven Type I receptors have been identified) and Type II receptors (five types in humans) transmembrane proteins with a cytoplasmic serine/threonine kinase domain (Weiss and Attisano 2013). Ligands of TGF-β

family trasmit signals by forming a heterotetrameric complex between Type I and Type II receptor at the plasma membrane level with the following interaction of the intracellular domains with each other permitting the Type II receptor to phosphorylate the Type I receptor (Wrana, Attisano et al. 1992). Following the transphosphorylation process, the Type I receptor kinase is activated and in turn phosphorylates cytoplasmic effectors in order to trigger intracellular signaling cascades such as the SMAD-signaling pathway. This cascade of events results in the extracellular signal transduction across the plasma membrane into the cytosol and subsequently to the nucleus regulating thus the gene transcription (Hata and Chen 2016). Among TGF-β superfamily, the isoform of TGF-β, BMPs, and activins proteins act as dimers by binding heterotetrameric cell surface complexes. TGF-βs and activins bind primarily to the type II receptors and promote recruitment of type I receptors, whereas BMPs often bind primarily to type I receptors but mostly require the combination of both type of receptors for higher affinity binding (Budi, Duan et al. 2017).

Upon release/dissociation of TGF-β from LAP, the active form of TGF-β1 is able to activate different cellular responses by binding to, and activating specific cell surface receptors where downstream intracellular effectors, known as Smad proteins, are able to mediate the intracellular signaling transduction (Morikawa, Derynck et al. 2016). Following the assembly of type I and type II receptors into complexes, the TGF-β type II receptor phosphorylates the TGF-β type I receptor on specific serine and threonine residues in the juxtamembrane region or GS domain with consequent activation. Of note, although the ligand is able to bind efficiently to complexes, accessory receptors such as homodimeric betaglycan promotes the binding of TGF-β to type II receptor. The activation of ALK5 results in the propagation of the signal downstream by directly phosphorylation of receptor-regulated Smads (R-Smads) that is facilitated by the scaffolding protein called Smad anchor for receptor activation (SARA). R-Smad2 and R-Smad3 then are combined with a co-Smad (Smad4) forming heterotrimeric or dimeric complexes that, in combination with transcription factors, accumulate in the nucleus regulating gene transcription directly activating or repressing target gene expression. Inhibitory Smads (I-Smads: Smad6 and Smad7) form a distinct subclass of Smads that act in an opposing manner to R-Smads and antagonize signaling, in fact complexes of Smad7 and Smad ubiquitination regulatory factor 1 (Smurf1) or Smurf2 by binding to activated type I receptor lead to a receptor's ubiquitination and degradation, and termination of signaling. The canonical or SMAD-dependent pathway is able to modulate the gene expression both transcriptionally and post-transcriptionally level in order to propagate the physiological and pathological activities of TGF-β (ten Dijke and Hill 2004).

In the non-canonical pathway, the activated receptor complex transmits a signal by other factors, such as the extracellular-regulated kinase (ERK) pathways, the nuclear factor kappa-B (NF-kB) pathway

and the phosphatidyl inositol-3-kinase (PI3K)/Akt pathway that are involved in several physiological processes such as cell-cycle inhibition, suppression of immune response, and neuroprotection. These non-Smad transducers can mediate signaling responses alone or in combination with Smads, also converging onto Smads to control Smad activities participating synergically in diverse events (**Figure 5**).

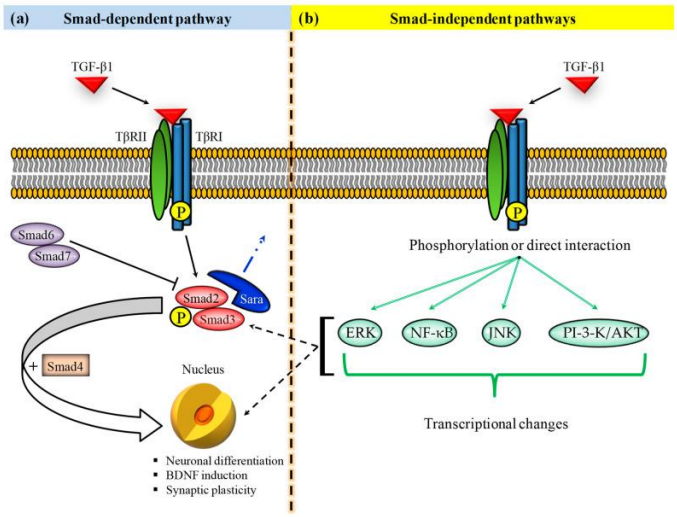

Figure 5. TGF-β1 pathway regulates various cellular processes (Adapted from (Grasso, Caruso et al. 2021)).

1.4.2 TGF-β1 in the healthy brain, in aging and neurodegenerative diseases: focus on AD

At the brain level, TGF-β1 is widely considered as an injury-related cytokine, particularly associated with astrocyte scar formation in response to brain injury. Several evidence showed that TGF-β1 is able to regulate cell survival and differentiation, brain homeostasis, angiogenesis, and it is involved in memory formation, and neuronal plasticity. In fact, this factor is involved in cognitive processes in healthy brain. TGF-β1 and its receptors are present in progenitor zones of the developing cerebral cortex, at spinal cord level, and mesencephalon as well as are widely distributed in different adult brain regions (Diniz, Matias et al. 2019). TGF-β1 has a pivotal role in neuronal homeostasis function and synaptogenesis; it is involved in the regulation of neurite outgrowth and synapse formation. In vitro and in vivo studies showed that TGF-β1 is able to induce sprouting and elongation of hippocampal axons and it promotes the axonal re-elongation from brain injuries as well as during the development of neocortex, TGF-β1 signaling is necessary in order to trigger axon formation and neuronal migration (Abe, Chu et al. 1996, Yi, Barnes et al. 2010).

It is known that members of TGF-β family have an antiproliferative effect by inducing G1/S phase arrest in different cell types, thus they might control the proliferation of neuroepithelial stem cells with the consequent enhanced neurogenesis. TGF-β1 promotes neuronal differentiation from

hippocampal and cortical progenitors in a Smad-dependent way and promotes the exit of cell cycle by induction of the cyclin-dependent kinase (cdk) inhibitor 1 (p21) and downregulation of Cdk activators (Vogel, Ahrens et al. 2010). This support the hypothesis that TGF-β1 has an inhibitory effect of cell-cycle preventing Aβ-challenged cortical neurons from entering the S-phase exerting its neuroprotective effect against Aβ toxicity (Caraci, Battaglia et al. 2008). Moreover, it is postulated that TGF-β1 protects neurons by its anti-apoptotic effects through the inactivation of Bad, activation of the Erk/MAP kinase pathway and increasing the production of the antiapoptotic protein Bcl-2 (Zhu, Yang et al. 2002).

In addition, TGF-β1 signaling by Smad2 and/or Smad3 is also essential for maintaining quiescent microglia after injury and it is able to inhibit free radical production and to induce apoptosis of stem/progenitor cells. The lack of TGF-β1 expression in neonatal knocked out mice increased neuronal degeneration, reduced synaptogenic markers expression (synaptophysin, laminin) and induced microgliosis (Brionne, Tesseur et al. 2003). Cortical astrocytes are the main source of TGF-β1 in CNS but this cell type could act also as a target of this cytokine in the healthy brain and pathological states: on the hand TGF-β1 acts as strong inducer of astrocytic differentiation from neural progenitor and on the other hand, cortical neurons activate the glial fibrillary acidic protein (GFAP) gene promoter by inducing TGF-β1 secretion by astrocytes. Thus, astrocytes modulated endothelial functions by activating the pathway, which contributed to vascular integrity and establishment of the BBB. TGF-β1 plays a key role in the regulation of endothelial differentiation, vascular network formation and maintenance of brain blood vessel integrity (Pepper 1997, Diniz, Matias et al. 2019).

It is known that the exposure to prenatal inflammatory stimuli influences adult brain functions; in vivo studies demonstrated an impairment of adult neurogenesis as well as memory deficits due to a down-regulation of hippocampal TGF-β1 levels confirming the role of this cytokine in brain developmental events and neural plasticity (Graciarena, Depino et al. 2010). In addition, the block of the endogenous TGF-β1 signaling pathway by the specific type I TGF-β1 receptor inhibitor (SB431542) has been shown to impair hippocampal LTP formation and memory functions demonstrating that this neurotrophic factor is essential for the transition from early to LTP potentiation (Caraci, Gulisano et al. 2015). Moreover, TGF-β1 can enhance synaptic plasticity by promoting the expression of BDNF and its receptor. It has been demonstrated an increase of TGF-β1 level in the PFC during learning process suggesting an involvement of this neurotropic factor in memory trace consolidation (Arkhipov, Pershina et al. 2019). Of note, intranasal delivery of TGF-β1 improved cognitive functions and reduces hippocampal damage in pilocarpine-treated rats (animal models of status epilepticus) by its neuroprotective and anti-apoptotic effects (Li, Li et al. 2013).

Upon aging, the communication between astrocytes and microglia, that in normal conditions regulate the brain homeostasis, is disrupted and not control the onset of neuroinflammation. Behind this event, the impairment of TGF-β1 secretion by astrocytes leads to an uncontrolled activation of microglial cells in aged animals. Different studies showed that aging and chronic inflammation decrease the canonical TGF-β1/Smad pathway, lead to a microglia's cytotoxic activation and microglia-mediated neurodegeneration (Estrada, Oliveira-Cruz et al. 2018). On the other hand increased hippocampal levels in adult mice of TGF-β1-Smad3 pathway on microglia associated with aging appears to impair the beneficial effect of TGF-β1 (Tichauer, Flores et al. 2014) and in postmortem human brain tissue with aging the TGF-β1 hippocampal levels was found increased (Werry, Enjeti et al. 2010). An increase in TGF-β1 expression is observed with age suggesting a protective role for this neurotrophic factor in longevity, in particular in the process known as "inflammaging" counteracting the pro-inflammatory status observed during aging, thus preventing the development of age-related disorders such as cancer and AD (Salvioli, Capri et al. 2009).

An impairment of TGF-β1 signaling has been implicated in several neurodegenerative diseases, such as Huntington's disease (HD), motorneuron disease and AD indicating that the dysregulation of this pathway might be involved in the pathogenesis of different neurodegenerative disorders.

Asymptomatic HD patients showed a reduction of TGF-β1 levels in the peripheral blood suggesting that TGF-β1 could act as a potential biomarker of disease development during the asymptomatic phase (Battaglia, Cannella et al. 2011). Moreover reduced TGF-β1 levels were also observed in animal models of HD in both plasma and cortical neurons accelerating the neurodegerative process (Martínez-Canabal 2015). A deficit of TGF-β family signaling has been reported in animal models of hereditary spastic paraplegia and the TGF-β-Smad2/3 pathway rescues motor functions in animal models of amyotrophic lateral sclerosis (Katsuno, Adachi et al. 2011). It has been shown a direct correlation between the stages of amyotrophic lateral sclerosis and TGF-β1 levels in both plasma and CSF of patients, although its role in this neurodegenerative disease remains elusive. The down-regulation of TGF-β1 could exacerbate multiple sclerosis (MS) symptoms preventing microglia to destroy myelin but on the other hand TGF-β1 and its receptors are expressed in CNS inflammatory lesions of these patients (Martínez-Canabal 2015). Recently it has been demonstrated that TGF-β1 administration promoted remyelination and restored neurological functions in a MS animal model (Hamaguchi, Muramatsu et al. 2019).

TGF-β1 is elevated in DA striatal regions as well as in CSF of PD patients compared with control individuals but in PD animal model the co-infusion of TGF-β1 and GDNF into the ventral tegmental area exerted neurotrophic effects (Gonzalez-Aparicio, Flores et al. 2010). It exerts a neuroprotective

activity in PD by inhibition of microglial inflammatory response via Smad3 signaling and has a protective role against the synaptic loss induced by α-synuclein oligomers (Chen, Liu et al. 2017).

The production and release of TGF-β1 increase significantly in response to CNS lesions, with astrocytes and microglia being the major sources of this factor in the injured brain, for example, transgenic mice that overexpress TGF-β1 develop AD-like vascular alterations (Gaertner, Wyss-Coray et al. 2005). The levels of TGF-β1 were found increased in CSF of AD patients compared to non-demented subjects and brain TGF-β1 mRNA levels are increased in AD brains and result correlate positively with the extent of cerebrovascular amyloid deposition or cerebral amyloid angiopathy suggesting the hypothesis that increased TGF-β1 acts as a neuroprotective factor and its decreased levels lead to a neurodegeneration (Wyss-Coray 2006).

Therefore, Aβ oligomers induce a down-regulation of TGF-β1 in culture astrocytes with a decrease in synaptogenic potential of this cell type (Tapella, Cerruti et al. 2018), Aβ oligomers induce in fact astrocytic cellular atrophy, reduced TGF-β1 levels and synaptogenic astrocyte ability suggesting that increased astroglial secretion of TGF-β1 may provide a useful strategy for the treatment of AD early stages (Diniz, Tortelli et al. 2017). Moreover, Aβ oligomers induce the release of proinflammatory cytokines from activate microglia contributing to a neuronal death in AD brain. Interestingly, several studies demonstrated that TGF-β1 is able to reduce microglia activation and promote Aβ clearance by the microglia (Magnus, Chan et al. 2002).

An early dysfunction of TGF-β1 signaling may trigger the aggregation and toxicity of Aβ oligomers in neurons and in this regard, Tesseur et al. reported that a decrease in TGF-β1 signaling is associated with enhanced deposition of Aβ peptide and neuronal degeneration in AD animal model suggesting that a reduction in neuronal TGF-β1 signaling increases age-dependent neurodegeneration (Tesseur, Zou et al. 2006). An alteration of TGF-β1 pathway increases the neurodegeneration vulnerability, in fact knockout TGF-β1 mice showed neuronal damage, reduced synaptic density and synaptogenesis paralleled to microglial activation (Brionne, Tesseur et al. 2003). A deficit of TGF-β1 leads to a disinhibition of GSK-3β and promotes tau hyperphosphorylation; therefore, TGF-β1 exerts its neuroprotective effect against Aβ-toxicity by the prevention of Aβ-induced tau hyperphosphorylation and by activating its type I receptor and PI-3-K/AKT pathway that plays an established role in mechanisms of neuroprotection (Caraci, Battaglia et al. 2008).

Moreover, a reduction in TGF-β1 plasma levels was found in AD patients as well as a reduction of its secretion from circulating peripheral blood mononuclear cells (PBMCs). A deficit in neuronal TGF-β1 type II receptor was observed in the early stage of AD suggesting that a deficit of TGF-β1/type II receptor signaling might exert a pathogenetic role in AD, depriving cortical neurons of trophic support, and finally promoting Aβ-induced neurodegeneration. It has been demonstrated that

the inhibition of endogenous TGF-β1 signaling by using SB431542 amplified Aβ-induced neurotoxicity in the rat hippocampus (Caraci, Battaglia et al. 2008) and TGF-β1 protects in vitro against $A\beta_{1-42}$-induced hippocampal neuronal inflammation and apoptosis by its type I receptor (Fang, Sun et al. 2018). The i.c.v. injection of TGF-β1 in AD model rats ameliorated cognitive deficits, neuronal loss and apoptosis phenomena induced by Aβ emphasizing once again the neuroprotective activity of this neurotropic factor (Shen, Chen et al. 2014).

We recently found a deficit of hippocampal TGF-β1 signaling in Aβ-injected mice paralleled to memory deficits and depressive-like behavior; in our "amyloid-related depression" animal model, these events are reversed by the chronic treatment with SSRIs that are currently studied for their neuroprotective activity in AD. In particular, fluoxetine is neuroprotective against Aβ-induced neurodegeneration via a paracrine signalling mediated by TGF-β1, which does not result from a simplistic SERT blockade (Caraci, Tascedda et al. 2016).

Finally, TGF-β1 protein levels are predominantly under genetic control, and the TGF-β1 gene, located on chromosome 19q13.1–3, contains several single nucleotide polymorphisms (SNPs) upstream and in the transcript region which may affect protein levels (Grainger, Heathcote et al. 1999). SNPs at codons +10 (T(C) and +25 (G/C), that reduce TGF-β1 expression, have been associated with an increased conversion from MCI into AD (Arosio, Bergamaschini et al. 2007). The authors also showed that the CC genotype of TGF-β1 gene was associated with reduced serum level of TGF-β1 and an increased conversion from MCI into AD. The human +10 CC genotype of TGF-β1 gene, increases the risk to develop Late-Onset AD (LOAD) independently from APOE4 status and it is also associated with depressive symptoms in AD with an increased risk of 5-fold independently of a history of depression (Caraci, Bosco et al. 2012).

It is known that depression acts as a risk factor for AD development and the presence of depressive symptoms significantly increases the conversion from MCI into AD. As observed with the +10 (T/C) and +25 (G/C) polymorphism, other genetic polymorphisms in neurotrophic factors increase the risk of depression in AD patients such as BDNF Val66Met functional polymorphism where BDNF A/A carriers had a threefold-time risk for depression in AD (Borroni, Archetti et al. 2009). The presence of Met-BDNF allele, particularly in association with APOE-E4, may predict a worse cognitive impairment in MCI patients (Forlenza, Diniz et al. 2010).

It might be interesting to examine whether the +10 (T/C) and +25 (G/C) functional polymorphisms of TGF-β1 might act simultaneously with the BDNF Val66Met polymorphism in increasing the risk of conversion from MCI into AD as well as in the increase to develop depressive disorders in MCI patients at higher risk to develop AD.

1.5 Hypothesis and aims

Based on the reviewed data, the present thesis has focused on the validation of TGF-β1 signaling as a new pharmacological target for the treatment of CNS disorders focused on AD and has also investigated drugs able to prevent Aβ-induced toxicity by the rescue of TGF-β1 pathway.

The following aspects were investigated:

1. To examine whether i.c.v. injection of $A\beta_{1-42}$ oligomers induces the onset and/or the development of depressive-like behavior and memory deficits in young male C57BL/6 mice by impairing the TGF-β1 pathway
2. To determine whether fluoxetine and/or vortioxetine are able to prevent these events by the rescue of TGF-β1 pathway
3. To analyze the effect of Aβ oligomers on pro- and anti-oxidant system in our non-transgenic animal model of AD and the effect of second generation antidepressants on these pathways
4. To examine the neuroprotective effects of TGF-β1 by using in vitro experimental models of Aβ-induced neuroinflammation and to identify drugs able to prevent Aβ-induced toxicity by TGF-β1 rescue
5. To study whether a deficit of TGF-β1 occurs in a validated animal model of neuropathic pain and whether mu or delta opioid peptide receptors agonists can positively modulate TGF-β1 pathway.

Chapter 2.

Fluoxetine and Vortioxetine reverse depressive-like phenotype and memory deficits induced by $A\beta_{1-42}$ oligomers in mice: a key role of Transforming Growth Factor-β1

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Abstract

Depression is a risk factor for the development of Alzheimer's disease (AD), and the presence of depressive symptoms significantly increases the conversion of Mild Cognitive Impairment (MCI) into AD. A long-term treatment with antidepressants reduces the risk to develop AD and different second-generation antidepressants such as selective serotonin reuptake inhibitors (SSRIs) are currently studied for their neuroprotective properties in AD.

In the present work, the SSRI fluoxetine and the new multimodal antidepressant vortioxetine were tested for their ability to prevent memory deficits and depressive-like phenotype induced by intracerebroventricular injection of amyloid-β (1-42) ($A\beta_{1-42}$) oligomers in 2-month-old C57BL/6 mice. Starting from 7 days before Aβ injection, fluoxetine (10 mg/Kg) and vortioxetine (5 and 10 mg/Kg) were intraperitoneally injected daily, for 24 days. Chronic treatment with fluoxetine and vortioxetine (both at the dose of 10 mg/Kg) was able to rescue the loss of memory assessed 14 days after Aβ injection by the passive avoidance task and the object recognition test. Both antidepressants reversed the increase in immobility time detected 19 days after Aβ injection by forced swim test. Vortioxetine exerted significant antidepressant effects also at the dose of 5 mg/Kg. A significant deficit of Transforming Growth Factor-β1 (TGF-β1), paralleling memory deficits and depressive-like phenotype, was found in the hippocampus of Aβ-injected mice in combination with a significant reduction of the synaptic proteins synaptophysin and PSD-95. Fluoxetine and vortioxetine completely rescued hippocampal TGF-β1 levels in Aβ-injected mice as well as synaptophysin and PSD-95 levels. This is the first evidence that a chronic treatment with fluoxetine or vortioxetine can prevent both cognitive deficits and depressive-like phenotype in a non-transgenic animal model of AD with a key contribute of TGF-β1.

Keywords: Alzheimer's disease, amyloid-β, vortioxetine, antidepressants, fluoxetine, memory, TGF-β1, depression

* *Published in Front. Pharmacol. 2019 Jun 21;10:693.*

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by memory loss, cognitive decline, and neuropsychiatric symptoms, such as depression and psychotic signs, which strongly interfere with normal daily activities (Lanctôt et al., 2017). Different neurobiological and clinical links have been found between depression and AD (Caraci et al., 2018). Depression is a risk factor for the development of AD, and the presence of depressive symptoms significantly increases the conversion of mild cognitive impairment (MCI) into AD (Modrego and Ferrández, 2004). Common pathophysiological events have been identified in depression and AD, including activation of the hypothalamic–pituitary–adrenal (Deshpande, Irani et al.) axis with increased glucocorticoid levels, neuroinflammation with an aberrant tumor necrosis factor-α (TNF-α) signaling, and an impairment of transforming growth factor-β1 (TGF-β1) signaling (Caraci et al., 2018). Intracerebroventricular (i.c.v.) injection of oligomers of amyloid-β (1-42) (Aβ1-42), the most toxic form of amyloid aggregates in AD brain, can induce both memory deficits and depressive-like phenotype in rats (Colaianna et al., 2010; Schiavone et al., 2017) and mice (Ledo et al., 2013; Ledo et al., 2016), while an acute treatment with the selective reuptake inhibitor (SSRI) fluoxetine can revert this phenotype (Ledo et al., 2013; Ledo et al., 2016; Schiavone et al., 2017). Evidence also exists that fluoxetine prevents amyloid pathology and reverses memory impairment in different AD animal models (Wang et al., 2014; Jin et al., 2016). Interestingly, a continued long-term treatment with antidepressants is known to reduce the risk to develop AD (Kessing et al., 2009; Kessing, 2012). It has been hypothesized that a chronic treatment with second-generation antidepressants can exert relevant neuroprotective effects in depressed MCI patients with a high risk to develop AD, but the molecular mechanisms underlying the neuroprotective effects of antidepressants are not yet completely understood (Caraci et al., 2018). Deficit of TGF-β1 signaling is a common pathophysiological event in both depression and AD (Caraci et al., 2018). Among SSRIs, fluoxetine increases circulating TGF-β1 levels in depressed patients (Lee and Kim, 2006; Sutcigil et al., 2007) and prevents Aβ-induced toxicity in neuronal cultures by increasing the release of TGF-β1 (Caraci et al., 2016). However, it is presently unknown whether a chronic treatment with fluoxetine or other second-generation antidepressant drugs can prevent memory deficits and depressive-like phenotype in animal models of AD. Vortioxetine is a third-generation antidepressant with a novel, multimodal, mechanism of action, directly acting on several serotonin (5-hydroxytryptamine, 5-HT) receptors (as an agonist on 5-HT1A receptor, a partial agonist on 5-HT1B, and an antagonist on 5-HT1D, 5-HT3, and 5-HT7) besides

inhibiting the serotonin transporter (SERT; MØrk et al., 2012). Several preclinical studies have clearly demonstrated robust pro-cognitive effects of vortioxetine in different animal models of depression (Pehrson et al., 2015). In particular, vortioxetine displays a superior efficacy on visuospatial memory and depressive-like behavior, than does fluoxetine, in aged mice (Li et al., 2015; Li et al., 2017). Recent clinical studies also suggest an improved efficacy of vortioxetine on specific clinical domains, where SSRIs are less effective, such as cognitive deficits associated with major depressive disorder (MDD; Thase et al., 2016), in particular in elderly patients (McIntyre et al., 2016). No studies have been conducted so far to examine the preclinical efficacy of vortioxetine compared with fluoxetine in treating depressive-like behavior and memory impairment induced by the i.c.v. injection of Aβ1-42 oligomers. The aim of the present study is to assess whether a chronic treatment with fluoxetine or vortioxetine can prevent memory deficits and depressive-like phenotype in a non-Tg model of AD obtained by i.c.v. injection of Aβ1-42 oligomers. We show that a chronic (24 days) treatment with fluoxetine or vortioxetine in young (2-month-old) C57BL/6 mice can revert both Aβ-induced depressive-like behavior and memory impairment with a key contribute played by TGF-β1.

2. Materials and Methods

Animals

Eight-week-old male C57BL/6 mice, from Envigo RMS s.r.l. laboratories (San Pietro al Natisone, Italy), were individually housed, with free access to chow and water, in an air-conditioned room, with a 12-h light–dark cycle and with constant temperature (23 ± 1°C) and humidity (57 ± 3%) conditions. Animals were left undisturbed for 1 week before beginning any behavioral procedure. All animal experiments were carried out in accordance with Italian (D.M. 116192) and EEC (O.J. of E.C.L 358/1 12/18/1986) regulations on protection of animals. Every effort has been made to minimize animal suffering and to reduce the number of animals used.

Preparation of Human Aβ1-42 Oligomers and i.c.v. Injection in Mice

Synthetic human Aβ1-42 oligomers were prepared according to the original protocol of Klein's group (Gong et al., 2003). Briefly, the Aβ1- 42 lyophilized peptide, purchased from Bachem Distribution Services GmbH (Weil am Rhein, Germany), was dissolved in trifluoroacetic acid (TFA) (1 mg/ml) and sonicated in a water bath sonicator for 10 min. Then, TFA was evaporated under a gentle stream of argon, and 1 ml of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was added to the peptide. After 1-h incubation at 37°C, the peptide solution was dried under a stream of argon and then solubilized again by adding 2 ml of HFIP. Finally, HFIP was removed by argon streaming followed by further drying

in a lyophilizer for 1 h, and then Aβ1-42 was suspended in 5 mM of anhydrous dimethyl sulfoxide (DMSO), before dilution to 100 μM in ice-cold cell culture medium Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12). Samples of Aβ1-42 at the concentration of 100 μM were incubated for 72 h at 4°C and then stored at −20°C until use. To obtain a non-transgenic (non-Tg) AD model, animals were anesthetized for 7 min with 2.5% isoflurane using a vaporizer system and gently restrained only during the injection procedure. Aβ1-42 oligomers were administered i.c.v. into the brain. Synthetic human Aβ1-42 oligomers were diluted from the stock in DMEM solution (100 μM) in sterile 0.1 M phosphate buffered saline (PBS) (pH 7.4) at a final concentration of 10 μM and then injected i.c.v. Sterile 0.1 M PBS was injected i.c.v. into control animals (vehicle). Intracerebroventricular injection was used because of its simplicity with respect to stereotaxis in mice and to ensure diffusion of Aβ1-42 in the whole brain (Maurice et al., 1996; Leggio et al., 2016). Two microliters was injected using a microsyringe with a 28-gauge 3.0-mm-long stainless steel needle; 2 μL of the 10 μM Aβ solution corresponds to 20 pmol of Aβ monomer equivalent, e.g., 0.09 μg Aβ per mouse brain (weighing around 500 mg). Assuming that soluble Aβ oligomers are freely diffusing in cerebrospinal fluid and then in the brain, their final concentration would be approximately 0.18 μg/g of tissue.

Drugs and treatment

Vortioxetine hydrobromide [purity > 98.0% (HPLC)] was provided by H. Lundbeck A/S (Denmark) according to the MTA N.417394 signed by University of Catania (Department of Drug Sciences) and H. Lundbeck A/S and Lundbeck Italia S.p.A. Fluoxetine hydrochloride [product number: F132; purity > 98.0% (TLC)] was purchased from Sigma-Aldrich (St Louis, MO). Both compounds were dissolved in DMSO and further diluted with a final concentration of 1% of DMSO. Fluoxetine was administered intraperitoneally (i.p.) at the dose of 10 mg/kg (100 μL/10 g body weight), while vortioxetine was administered i.p. at two different doses (5 and 10 mg/kg; 100 μL/10 g body weight). Control animals received the vehicle i.p. (100 μL/10 g, DMSO 1%). The fluoxetine dose and the two vortioxetine doses were selected on the basis of previous studies where these antidepressant drugs were administered in animal models of depression (Pehrson et al., 2015).

Experimental design

In order to assess the effects of fluoxetine and vortioxetine on depressive-like behavior and memory impairment induced by Aβ oligomers, three different cohorts of animals were used, according to the following experimental design. *Experiment 1 (first cohort)*: No i.c.v. injection of Aβ1-42 oligomers was performed in this cohort. Mice were randomly divided into four experimental groups (n = 7-10

mice per treatment group): vehicle, fluoxetine (FLX) 10 mg/kg, vortioxetine (VTX) 5 mg/kg, and VTX 10 mg/kg. All drugs were administered i.p. for 21 days. To assess the antidepressant activity of fluoxetine and vortioxetine, mice were tested in the forced swim test (FST) on day 22. *Experiment 2 (second cohort)*: Aβ1-42 oligomers or PBS i.c.v. injection was performed in this cohort of mice 7 days after the beginning of antidepressant treatment (day 7). The treatment with antidepressants lasted until day 26, when all behavioral tests were completed. Mice were randomly allocated to five experimental groups (n = 7-8 animals/group): PBS i.c.v. + vehicle i.p., Aβ i.c.v. + vehicle i.p., Aβ i.c.v. + FLX 10 mg/kg i.p., Aβ i.c.v. + VTX 5 mg/kg i.p., and Aβ i.c.v. + VTX 10 mg/kg i.p. Memory deficits were evaluated after 24 days of chronic treatment with FLX or VTX in the passive avoidance test (PAT), 15–17 days after Aβ injection, whereas depressive-like behavior was evaluated with FST after 26 days of treatment with antidepressant drugs. *Experiment 3 (third cohort)*: Animals received 3 weeks of treatment with antidepressants and Aβ1-42 oligomers or PBS. Intracerebroventricular injection was performed 7 days after the beginning of antidepressant treatment (day 7). Experimental groups were not only those described in Experiment 2 but also those included the following four experimental groups: vehicle, FLX 10 mg/kg, VTX 5 mg/kg, and VTX 10 mg/kg. This third cohort of animals was tested in the object recognition test (ORT), after 21 days of chronic treatment with FLX or VTX.

Forced swim test

The FST protocol employed here was adapted from Porsolt et al., (1978). Mice were placed for 6 min in a 4-L Pyrex glass beaker containing 3 L of water at 24 ± 1°C. Water was changed between animals. After a habituation period of 2 min, mobility and immobility were recorded during the last 4 min of the 6-min testing period. A trained researcher blinded to group assignment recorded immobility time using a stopwatch. An increase in immobility time indicates depressive-like behavior. A mouse was judged immobile when it floated in an upright position and displayed only small movements to keep its head above water.

Passive avoidance test

PAT was performed as previously described (Leggio et al. 2016). The apparatus for the step-through PAT was an automated shuttle-box divided into an illuminated compartment and a dark compartment of the same size by a wall with a guillotine door. In the experimental session, each mouse was trained to adapt to the step-through passive avoidance apparatus. In the adaptation trial, the animal was placed into the illuminated compartment. After 10 sec, the door between these two boxes was opened and the mouse was allowed to freely move into the dark compartment. The learning trial was similar to

the adaptation trial except that the door was closed automatically as soon as the mouse stepped into the dark compartment and an inescapable foot-shock (0.2 mA, 2 sec) was delivered through the grid floor. Following the shock, the mouse was removed and returned to its home cage. The retention of the step-through passive avoidance response was measured the day after the learning trial and the latency to re-enter into the dark compartment was recorded. In the retention test, no foot-shock was delivered. Adaptation trial, learning trial, and retention test were performed 15, 16, and 17 days after PBS or Aβ i.c.v. injections (see above for details regarding the experimental design).

Object recognition test

ORT was performed as previously described (Gulisano et al., 2018). The apparatus consisted in the arena (a plastic white box 50 × 35 × 45 cm) placed on a lab bench, a webcam connected to the computer and was fixed on the wall, objects of different colors and shapes (e.g. pyramid, cube, truncated sphere, cylinder, prism, star, etc.) designed by Solid works software and 3D printed in polylactic acid by a Prusa-inspired 3D printer of our design. Three days before training (from day 21 to day 23), mice were habituated to the new context (empty arena and arena containing one or two objects) and allowed to freely explore it for 10 min. On day 24, mice, previously treated for 24 days with i.p. injections of antidepressants or vehicle, 45 min after the last injection of FLX or VTX, underwent the first trial (T1) of ORT consisting in exploring two identical objects (randomly chosen among our collection) placed in the central part of the box, equally distant from the perimeter. T1 lasted 10 min, a time sufficient to learn the task. The second trial (T2) was performed 24 h after T1 (day 25) to test memory retention for 10 min. Mice were presented with two objects, a "familiar" (i.e. the one used for T1) and a "novel" object. The latter was placed on the left or the right side of the box in a randomly but balanced manner, to minimize potential biases due to a preference for particular locations. To avoid olfactory cues, the objects and the apparatus were cleaned with 70% ethanol after each trial. Exploration, defined as the mouse pointing its nose toward the object from a distance not > 2 cm (as marked by a reference circle), was manually evaluated by an investigator blind with respect to treatment. In particular, the following parameters were studied: (i) discrimination index (D), calculated as "exploration of novel object minus exploration of familiar object/total exploration time"; (ii) total exploration time. We excluded from the analyses mice with a total exploration time < 5 s.

Western blot

Western blot analysis was performed as previously described (Caraci et al. 2015) on hippocampi of mice from the different experimental groups (n = 4 per group). Tissues were harvested at 4 °C in

RIPA buffer, in the presence of a cocktail of protease inhibitors (Sigma-Aldrich, P2714), serine/threonine phosphatase inhibitors (Sigma-Aldrich, P0044) and tyrosine protein phosphatases inhibitors (Sigma-Aldrich, P5726), followed by sonication. Protein concentrations were determined by Bradford's method using bovine serum albumin as a standard. After blocking, membranes were incubated with the following primary antibodies, overnight at 4 °C: rabbit anti-TGF-β1 (Abcam 92486, Cambridge, UK; 1:1.000), mouse anti-GAPDH (Millipore MAB374, Burlington, MA, USA; 1:1.000), rabbit anti-PSD-95 (3450S Cell signaling Technology Inc., Danvers, MA, USA; 1:1.000), mouse anti-synaptophysin (SC-17750 Sunta Cruz Biotechnology Inc., CA, USA, 1:40.000), rabbit anti-Actin (A2066, Sigma-Aldrich, St Louis, MO 1:5.000). Secondary goat anti-rabbit labeled with IRDye 680 (Li-COR Biosciences, 1:20.000) and goat anti-mouse labeled with IRDye 800 (Li-COR Biosciences, 1:20.000) were used at room temperature for 45 min. Hybridization signals were detected with the Odyssey Infrared Imaging System (LI-COR Biosciences). Western blot data were quantified by densitometry analysis of the hybridization signals in four different blots per experiment.

Gene Expression Analysis by Real-Time RT-PCR

Gene expression analysis by quantitative qRT-PCR was performed as previously described (Caruso et al. 2019) with slight modifications. In brief, the concentration of total RNA recovered by using RNeasy Mini Kit from 10 mg hippocampus tissue was determined by measuring the absorbance at 260 nm with a Nano Drop® ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). SuperScript III First-Strand Synthesis SuperMix (Thermo Fisher Scientific) was used to carry out the reverse transcription (100 ng of total RNA for each sample), by random priming. All samples were then quantified with a NanoDrop® ND-1000, diluted to a final concentration of 25 ng/μL, and the gene expression was simultaneously measured for all the samples by using a 384-well plates and a LightCycler® 480 System (Roche Molecular Systems, Inc., Pleasanton, CA, USA). The Quanti Tect Primer Assays (Qiagen, Hilden, Germany) employed for gene expression analysis along with official name, official symbol, alternative titles/symbols, detected transcript, amplicon length, and primers catalogue number are shown in Table 1.

For each sample amplification, performed in quadruplicate, a total reaction volume of 10 μL, consisting of 6 μL of amplification mixture (5 μL PCR Master Mix + 1 μL specific primers) plus 4 μL of cDNA (100 ng), was used. Amplification conditions and fluorescence data collection included a first cycle at 95 °C (15 min) followed by 50 cycles at 94 °C (15 sec), an annealing step at 56 °C (30 sec, and a final cycle at 72 °C (30 sec). As a negative control, a reaction in absence of cDNA (no template control, NTC) was performed. The relative RNA expression level for each sample was

calculated using the $2^{-\Delta\Delta CT}$ method by comparing the threshold cycle (Lanctôt, Amatniek et al.) value of the gene of interest to the CT value of our selected internal control (GAPDH gene).

Statistics

All experiments were blind with respect to treatment. Data were expressed as mean ± standard error mean (SEM). Statistical analysis was performed using dedicated software (GraphPad Prism, La Jolla, CA; Systat 9 Software, Chicago, IL). The withingroup comparison was performed by a one-way analysis of variance (ANOVA). The post hoc Bonferroni test was used for multiple comparisons. One-sample t-test was used to compare D index with zero in ORT.

Study Approval

The study was authorized by the Institutional Animal Care and Use Committee (IACUC) of the University of Catania and by the Italian Ministry of Health (DDL 26/2014 and previous legislation; OPBA Project #266/2016). Animal care followed Italian (D.M. 116192) and EEC (O.J. of E.C.L 358/1 12/18/1986) regulations on protection of animals used for experimental and scientific purposes.

3. Results

3.1 Fluoxetine and Vortioxetine Showed Similar Antidepressant Efficacy in Young Mice

We first examined the effects of FLX and VTX on depressive-like behavior in the first cohort of mice (Experiment 1) in the FST, a well-established behavioral test used to evaluate the preclinical efficacy of antidepressant drugs (Castagné et al., 2011; Li et al., 2017). Depressive-like behavior was assessed at day 22 by scoring immobility time (expressed in seconds) for each animal (**Figure 1A**). As depicted in **Figure 1B**, both FLX and VTX, at the dose of 10 mg/kg, gave comparable results, reducing the immobility time [$p < 0.001$ and $p < 0.01$ for FLX and VTX vs. vehicle (VEH), respectively]. VTX was able to significantly reduce the immobility time also at the dose of 5 mg/kg ($p < 0.01$ vs. VEH).

3.2 Fluoxetine and Vortioxetine Prevented Memory Retention Loss and Depressive-Like Behavior Induced by Aβ Oligomers

We then investigated the effects of FLX and VTX on the memory retention loss in mice treated with Aβ oligomers (second cohort of mice, Experiment 2). The treatment with antidepressants started 7 days before Aβ1-42 oligomers or PBS i.c.v. injection, and memory deficits were evaluated in the PAT with memory retention test after 24 days of chronic treatment with FLX or VTX (i.e., 17 days after Aβ injection, **Figure 2A**). As observed in our previous studies (Leggio et al., 2016), mice treated with Aβ1-42 showed a lower latency time in PAT than did vehicle-treated controls ($p < 0.01$ vs. VEH;

Figure 2B). Interestingly, a chronic treatment with FLX (10 mg/kg) and VTX (10 mg/kg) was able to rescue Aβ-induced memory loss ($p < 0.01$ vs. Aβ + VEH and $p < 0.05$ vs. Aβ + VEH, respectively) (**Figure 2B**). Depressive-like behavior was then evaluated in FST, in the same cohort of mice, 26 days after treatment with antidepressant drugs (19 days after Aβ injection; **Figure 2C**). We show, for the first time, that Aβ injection was able to induce a long-lasting significant increase in immobility time 19 days after i.c.v. Aβ injection ($p < 0.05$ vs. VEH). Chronic i.p. treatment with VTX or FLX, administered at the same dose of 10 mg/kg for 26 days, was able to revert Aβ1-42-induced depressive-like behavior ($p < 0.001$ and $p < 0.01$ for FLX and VTX vs. Aβ + VEH, respectively). Interestingly, VTX at the low dose of 5 mg/kg was also effective in preventing depressive-like behavior in Aβ-injected mice ($p < 0.01$ vs. Aβ + VEH).

3.3 Fluoxetine and Vortioxetine Improved Object Recognition Memory in Aβ-Treated Mice

We then evaluated recognition memory by ORT, a task based on the natural tendency of rodents to explore unfamiliar objects, which depends upon integrity of the perirhinal cortex, the hippocampus, and the medial temporal lobe (Barker et al., 2007; Broadbent et al., 2009). We measured the exploration time of both the familiar and novel objects at T2, i.e., 24 h after training, in Aβ-injected mice; and we calculated the discrimination index (D = exploration of novel object minus exploration of familiar object/total exploration time) (**Figure 3A**). Aβ-injected mice, compared with vehicle-injected mice, showed an impairment of recognition memory, as they did not discriminate between the familiar and novel objects ($p < 0.05$; **Figure 3B**). Comparison of D with zero confirmed that Aβ-injected mice were not able to learn ($p > 0.05$). The chronic treatment with FLX (10 mg/kg) or VTX (10 mg/kg) was effective in rescuing Aβ-induced memory impairment ($p < 0.01$ vs. Aβ + VEH for both treatments). Results were not affected by differences in total exploration time between the animal groups (**Figure 3C**). Treatment with FLX or VTX *per se* did not modify discrimination index (**Figure 3D**) nor (**Figure 3E)** total exploration index.

3.4 Molecular Mechanisms Underlying the Antidepressant and Procognitive Effects of Fluoxetine and Vortioxetine: A Key Role of TGF-β1

Neuroinflammation plays a central role in the pathogenesis of depression (Bhattacharya et al., 2016) and AD (Businaro et al., 2018; Knezevic and Mizrahi, 2018). Previous studies have demonstrated that Aβ oligomers promote neuroinflammation and neurodegeneration in AD brain and in animal models of AD by eliciting the release of pro-inflammatory cytokines from microglia (Ledo et al., 2016; Businaro et al., 2018) and also by interfering with the synthesis of TGF-β1 (Diniz et al., 2017). We therefore examined the effects of Aβ1-42 oligomers i.c.v. injection on the mRNA levels of pro-

inflammatory cytokines (IL-1β and TNF-α) and anti-inflammatory cytokines (IL-4 and TGF-β1) in the hippocampus (**Figure 4**), a brain area of primary relevance in the pathogenesis of depression (Villa et al., 2016). Aβ injection did not affect the expression level of IL-1β and TNF-α mRNA (**Figure 4A** and **B**), and the expression level of IL-4 (**Figure 4C**), whereas it induced a statistically significant decrease in the expression level of TGF-β1 mRNA in the hippocampus of Aβ-injected mice compared with vehicle-treated controls ($p < 0.05$ vs. VEH; **Figure 4D**). Interestingly, VTX at the low dose (5 mg/kg) was able to completely rescue hippocampal TGF-β1 mRNA levels compared with those in Aβ-injected mice ($p < 0.01$ vs. Aβ + VEH), and it further increased TGF-β1 mRNA levels at the dose of 10 mg/kg ($p < 0.001$ vs. Aβ + VEH). FLX at the dose of 10 mg/kg rescued hippocampal TGF-β1 mRNA levels with an efficacy comparable with that of VTX 5 mg/kg ($p < 0.05$ vs. Aβ + VEH). These antidepressant drugs *per se* did not increase hippocampal TGF-β1 mRNA (**Figure 4E**). TGF-β1 is an anti-inflammatory cytokine whose final activity is regulated not only at a transcriptional level but also at a post-transcriptional level and primarily regulated through the conversion of latent TGF-β1 to active TGF-β1 by a variety of proteases (Annes et al., 2003). Interestingly, western blot analysis carried out in the hippocampus of these mice confirmed that i.c.v. Aβ injection was able to induce a significant decrease of active TGF-β1 levels ($p < 0.05$ vs. PBS + VEH) and, most importantly, that both FLX and VTX (at both doses) were able to completely rescue hippocampal TGF-β1 levels when compared with those in Aβ-injected mice treated with vehicle ($p < 0.01$ vs. Aβ + VEH for FLX and VTX at 5 mg/kg; $p < 0.001$ vs. Aβ + VEH for VTX at 10 mg/kg; **Figure 5A** and **B**). Since it is known that TGF-β1 protects synapses against Aβ oligomers toxicity (Diniz et al., 2017), we examined the expression levels of two established synaptic protein markers, synaptophysin and PSD-95, in the hippocampus of Aβ-injected mice. Aβ injection significantly decreased both synaptophysin (**Figure 5C** and **D**) and PSD-95 levels (**Figure 5E** and **F**) ($p < 0.05$ vs. PBS + VEH); and, interestingly, both FLX and VTX (at 10 mg/kg) rescued hippocampal synaptophysin ($p < 0.01$ vs. Aβ + VEH for FLX and VTX at 10 mg/kg) and PSD-95 ($p < 0.05$ vs. Aβ + VEH for FLX and $p < 0.01$ vs. Aβ + VEH for VTX at 10 mg/kg) levels when compared with those in Aβ-injected mice treated with vehicle.

4. Discussion

In this paper, we have demonstrated for the first time that a long-term treatment with fluoxetine (10 mg/kg/day) or with the multimodal antidepressant vortioxetine (5 and 10 mg/kg/day) was able to prevent the loss of memory and the Aβ1-42 oligomer-induced depressive-like phenotype with a key contribute played by TGF-β1 in the mouse hippocampus.

We have used a non-Tg model of AD obtained by i.c.v. injection of Aβ1-42 oligomers, known to play a primary role in synaptic loss and progressive cognitive decline in AD (Ferretti et al., 2012; Klein, 2013). Synthetic human Aβ1-42 oligomers were prepared according to the original protocol of Klein's group as modified and characterized in Giuffrida et al. (2009). An open question in the field remains to establish whether Aβ1-42 oligomers can induce transient or long-term memory deficits in mice (Balducci and Forloni, 2014; Epelbaum et al., 2015). Different groups have demonstrated that, in the field of translational neuropharmacology, this model represents a simple and reliable paradigm, useful to investigate the molecular mechanisms through which Aβ oligomers interfere with cognitive processes and finally to test the efficacy of new therapeutic approaches (Balducci and Forloni, 2014). We have adopted this non-Tg AD model because we know from our previous work that i) the amount of injected oligomers reaches a cerebral concentration comparable with the concentration of soluble Aβ observed in AD brains, e.g., close to 1 μg/g (Leggio et al., 2016); and ii) i.c.v. injection of Aβ induces a memory deficit that persists for 14–21 days, as assessed by using two well-validated tasks in AD field, the passive avoidance task and the object recognition test (Leggio et al., 2016). We used this non-Tg model of AD to study the neurobiological links between depression and AD and the role of Aβ oligomers in the pathophysiology of amyloid-related depression, a recently identified clinical phenotype characterized by a low response to "monoaminergic antidepressants in depressed patients with an high risk to develop AD" (Li et al., 2017). Mimicking this clinical phenotype in rodents is a difficult challenge (Nyarko et al., 2019) but also an essential step to improve drug discovery processes in AD and explore the disease-modifying potential of antidepressant drugs in AD (Caraci et al., 2018). Previous studies have been conducted in rodents where a depressive-like phenotype was detected by FST only 7 days after a single Aβ injection in rats (Colaianna et al., 2010; Schiavone et al., 2017) or 24 h after Aβ infusion in mice (Ledo et al., 2016). In the present work, we demonstrate for the first time that Aβ injection can induce a long-lasting depressive-like phenotype, with a significant reduction in immobility time detectable with FST until 19 days after Aβ injection (**Figure 2C**). Interestingly, this depressive-like phenotype co-exists in our Aβ-injected mice with a severe impairment of reference memory (assessed by PAT) (**Figure 2B**) and object recognition memory (assessed by ORT) (**Figure 3A** and **B**). In the present work, only one memory test was conducted in each cohort of mice (second and third) to minimize potential effect of behavioral testing on FST. Future studies should be conducted in the same model to assess whether depressive-like phenotype precedes the onset of cognitive deficits as recently observed in late-life depressed patients with an increased risk to develop AD (Chung et al., 2015; Yasuno et al., 2016).

In the present work, we measured the antidepressant-like efficacy of fluoxetine and vortioxetine in FST, in the second cohort of Aβ-injected mice, after a 26-day treatment. Drug doses for both

fluoxetine and vortioxetine were chosen to reach a reliable occupancy of SERT in brain, as reported in previous studies (Pehrson et al., 2015). For the present study, we selected these specific antidepressants because fluoxetine is a SSRI known to revert cognitive deficits in different transgenic animal models of AD (Wang et al., 2014; Jin et al., 2016; Ma et al., 2017; Sun et al., 2017), and it is also able to rescue memory deficits in MCI patients (Mowla et al., 2007), while vortioxetine is a novel multimodal antidepressant endowed with strong pro-cognitive effects in preclinical models of depression (Pehrson et al., 2015) with a high clinical efficacy in the treatment of elderly patients with late-life depression and cognitive symptoms, a clinical subgroup that shows an increased risk to develop AD (Lauriola et al., 2018).

Interestingly, when comparing the effects of a chronic treatment (26 days) of fluoxetine and vortioxetine in our non-Tg AD model, we found for the first time that these two drugs have a similar preclinical efficacy at a dose of 10 mg/kg/day in preventing memory deficits, as assessed by PAT and ORT. Other studies have shown that fluoxetine can impair recognition memory in rats (Valluzzi and Chan, 2007) and in middle-aged mice (Castañé et al., 2015; Li et al., 2017), whereas vortioxetine does not affect object recognition memory in middle-aged mice (Li et al., 2017) but significantly improves the performance in this task in different animal models of cognitive dysfunction (Westrich et al., 2015; Pehrson et al., 2018). Surprisingly, 5 mg/kg vortioxetine exerted a significant antidepressant effect as detected in FST (without a further increase at a dose of 10 mg/kg), which was comparable with that of fluoxetine 10 mg/kg. Considering that vortioxetine at the dose of 5 mg/kg nearly saturates all 5-HT3 receptors, but only partially occupies the SERT (Sanchez et al., 2015), these data seem to suggest an increased, and probably SERT-independent, antidepressant efficacy of vortioxetine compared with fluoxetine in our model of amyloid-related depression. We cannot exclude that the young age of our cohorts of mice can affect our results in behavioral tests, but we should also consider that in this study we have adopted a secondary prevention strategy to prevent the onset of amyloid-related depression, starting the treatment with antidepressants 7 days before Aβ injection. This approach was also settled moving from the evidence that second-generation antidepressants, such as fluoxetine, exert relevant neuroprotective effects *in vitro* in experimental models of Aβ-induced neurodegeneration (Caraci et al., 2016; Caraci et al., 2018). We also believe that this approach might be helpful in the future to assess the disease-modifying efficacy of antidepressants in animal models of AD, independently from their symptomatic efficacy against the depressive-like phenotype.

To understand the molecular mechanisms underlying the precognitive and antidepressant effects of vortioxetine and fluoxetine, we focused on neuroinflammatory phenomena in the hippocampus of Aβ-injected mice, because previous studies in the same model found aberrant TNF-α signaling with

increases in hippocampal levels of TNF-α 24 h after Aβ infusion (Ledo et al., 2016). In order to correlate the preclinical efficacy of antidepressants with the effects on neuroinflammatory phenomena, we examined the mRNA levels of different pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-4 and TGF-β1) cytokines in the hippocampus of the second cohort mice only after completing behavioral tests (26 days). We did not detect a significant increase in hippocampal levels of TNF-α and IL-1β (**Figure 4A** and **B**), but we found a significant decrease in hippocampal levels of TGF-β1 (**Figure 4D**), further confirmed by western blot analysis (**Figure 5A** and **B**). Our data are in accordance with a previous study conducted in 3-month-old male Swiss mice, where reduced TGF-β1 levels were found in the hippocampus 24 h after Aβ injection (Diniz et al., 2017). Interestingly, we found that the deficit of hippocampal TGF-β1 is a long-lasting molecular marker associated with depressive-like phenotype and memory deficits in our non-Tg model of AD. TGF-β1 is an anti-inflammatory cytokine that exerts neuroprotective effects in different models of amyloid-induced neurodegeneration (Caraci et al., 2008; Caruso et al., 2019a; reviewed by Caraci et al., 2011). We have recently identified a key role for TGF-β1 in recognition memory formation, demonstrating that it is essential for the transition from early to late long-term potentiation (Caraci et al., 2015). Deficit of TGF-β1 signaling is a primary event in AD pathogenesis, and a reduced expression of type 2 TGF-β1 receptor specifically correlates with cognitive decline in early AD patients (Tesseur et al., 2006). TGF-β1 plays a key role in synaptic plasticity (Caraci et al., 2015), and it also protects synapses against Aβ oligomers toxicity (Diniz et al., 2017). Interestingly, we found, in our non-Tg model of AD, a significant reduction of the synaptic proteins synaptophysin and PSD-95 paralleling the deficit of TGF-β1 detected in the hippocampus of Aβ-injected mice. Aβ oligomers are known to exert synaptotoxic effects (Musardo and Marcello, 2017), and our data are in accordance with previous studies where i.c.v. Aβ injection in mice caused both memory deficits and a significant decrease of PSD-95 and synaptophysin levels in the hippocampus (Morroni et al., 2016; Wu et al., 2018). In the present work, for the first time, we found a correlation between the synaptotoxic effects of Aβ oligomers and the deficit of TGF-β1 in the hippocampus of Aβ-injected mice.

The deficit of TGF-β1 signaling has been hypothesized to contribute to inflammaging and cognitive decline in both depression and AD (Caraci et al., 2018). The +10 CC genotype of TGF-β1 gene, which affects the levels of expression of TGF- β1, is associated with depressive symptoms in AD (>5-fold risk) (Caraci et al., 2012), and an impairment of TGF-β1 signaling can promote the onset of a depressive-like phenotype in mice (Depino et al., 2011). TGF-β1 plasma levels are reduced in MDD patients, correlate with depression severity, and significantly contribute to treatment resistance in MDD patients (Musil et al., 2011; Caraci et al., 2018), a clinical subgroup with an increased risk to develop AD (Chung et al., 2015; Li et al., 2017).

Our work identified for the first time a selective deficit of TGF-β1 in a non-Tg model of AD that mimics what was observed in AD brain and, most importantly, showed that vortioxetine (5 mg/kg) and fluoxetine (10 mg/kg) completely rescue hippocampal TGF-β1 levels. Interestingly, fluoxetine and vortioxetine completely rescued hippocampal synaptophysin and PSD-95 levels in Aβ-injected mice only at the dose of 10 mg/kg, suggesting a protective effect of these drugs against the synaptotoxic effects of Aβ oligomers. Fluoxetine was known to induce TGF-β1 release from cortical astrocytes (Caraci et al., 2016), but this is the first demonstration that a chronic treatment with the multimodal antidepressant vortioxetine promotes TGF-β1 synthesis at hippocampal level in an animal model of amyloid-related depression. Future studies should be conducted in transgenic animal models of AD to assess whether fluoxetine or vortioxetine can prevent amyloid-induced depression and cognitive deficits by rescue of TGF-β1 signaling.

Overall, our data, obtained in a non-Tg model of AD, indicate that a deficit in TGF-β1 might represent one of the neurobiological links between depression and AD and also that rescue of TGF- β1 signaling with second-generation antidepressants might represent a new pharmacological strategy to prevent both amyloid-induced depression and cognitive decline in AD.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript and the supplementary files.

ETHICS STATEMENT

The study was authorized by the Institutional Animal Care and Use Committee (IACUC) of the University of Catania and by the Italian Ministry of Health (DDL 26/2014 and previous legislation; OPBA Project #266/2016). Animal care followed Italian (D.M. 116192) and EEC (O.J. of E.C.L 358/1 12/18/1986) regulations on protection of animals used for experimental and scientific purposes.

AUTHOR CONTRIBUTIONS

FC gave substantial contributions to the conception and design of the work. ST, FG, MT, MG, AF, NM, GS, and GC performed the experiments. FC, SS, DP, and GL analyzed the data. GL, FT, AP, SS, DP, and FD participated in the design of the study. FC and GL drafted the work. All authors approved the version to be published.

FUNDING

This research was conducted with the unrestricted support of Lundbeck.

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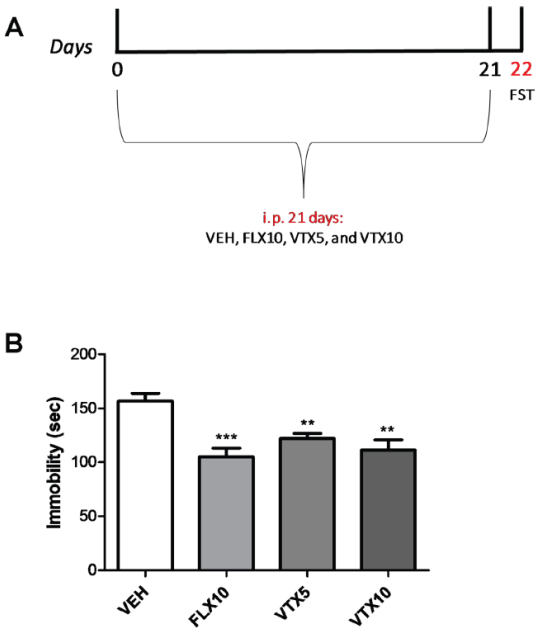
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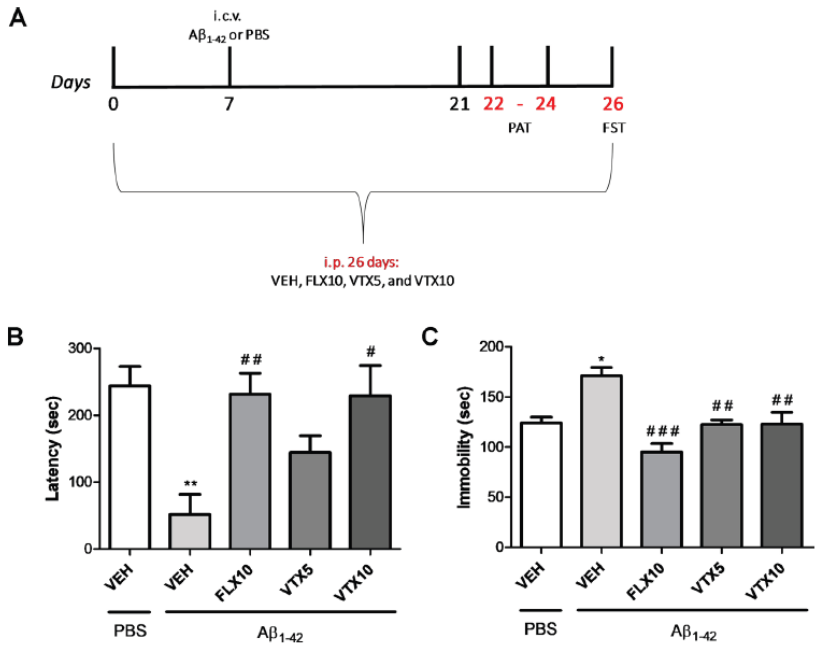

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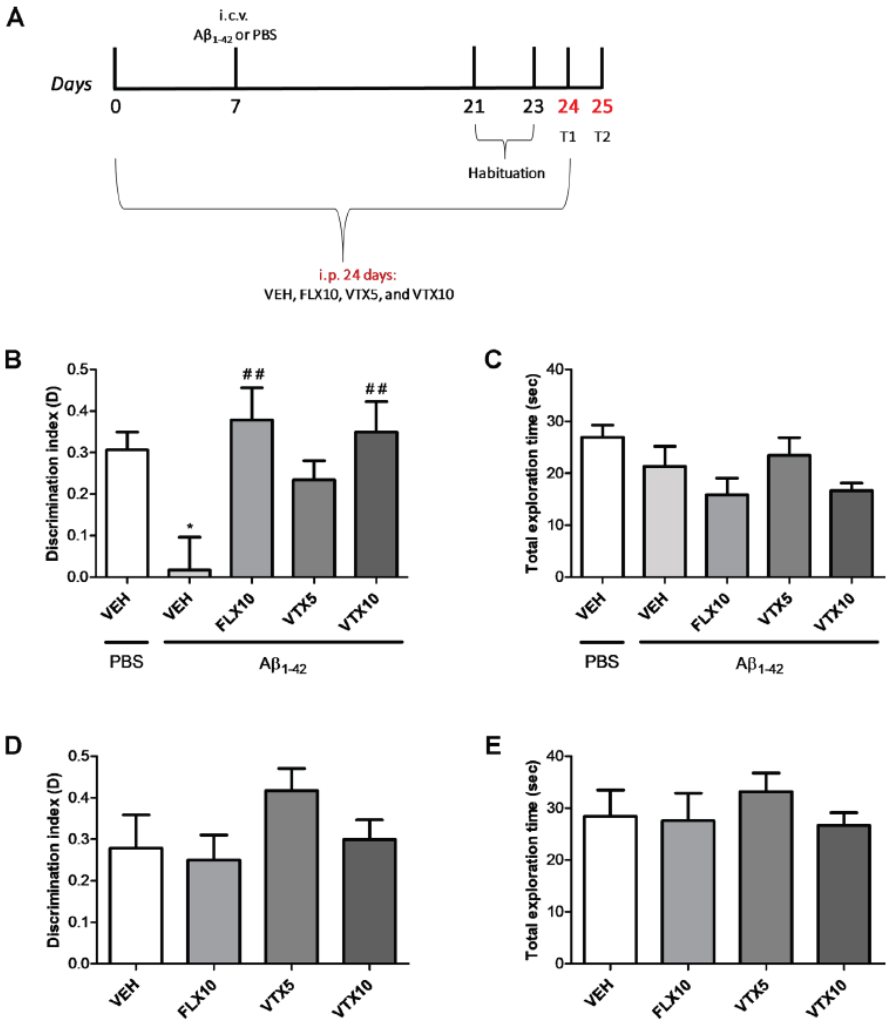

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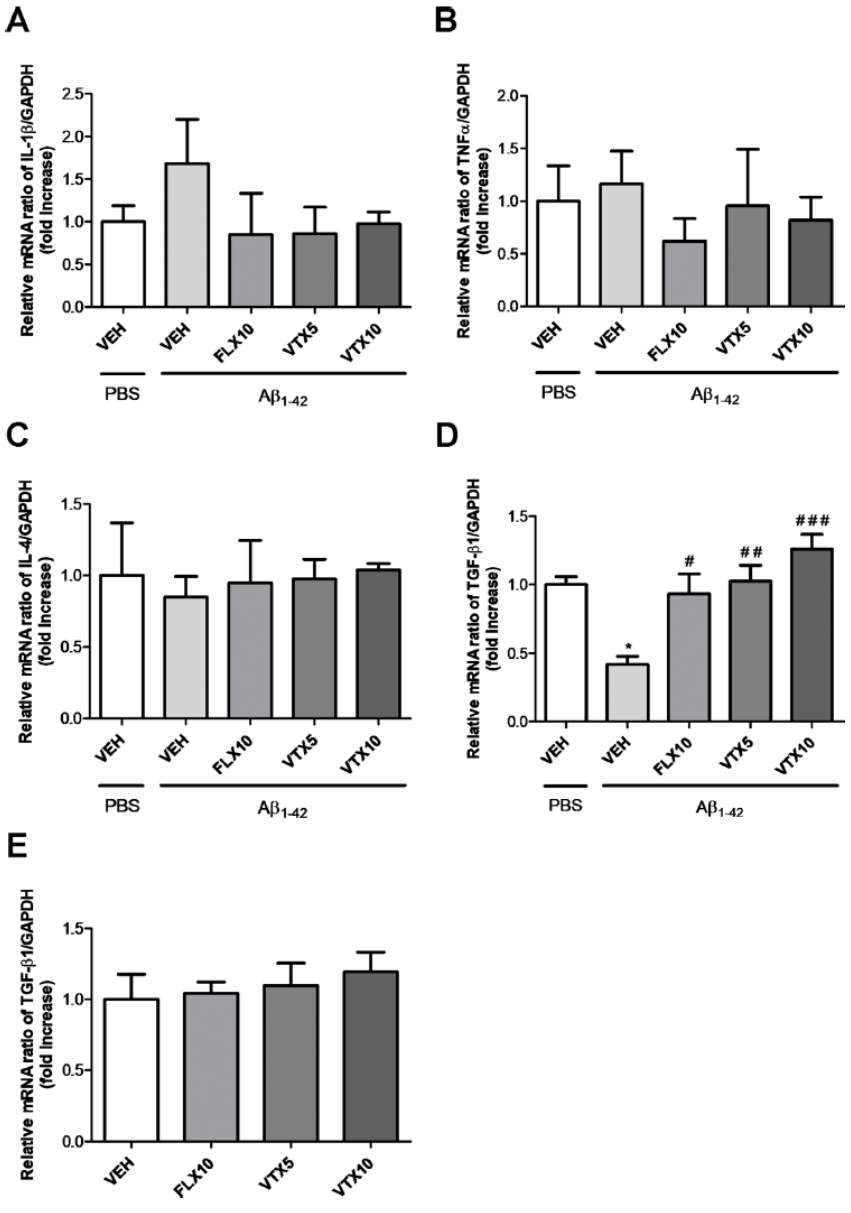

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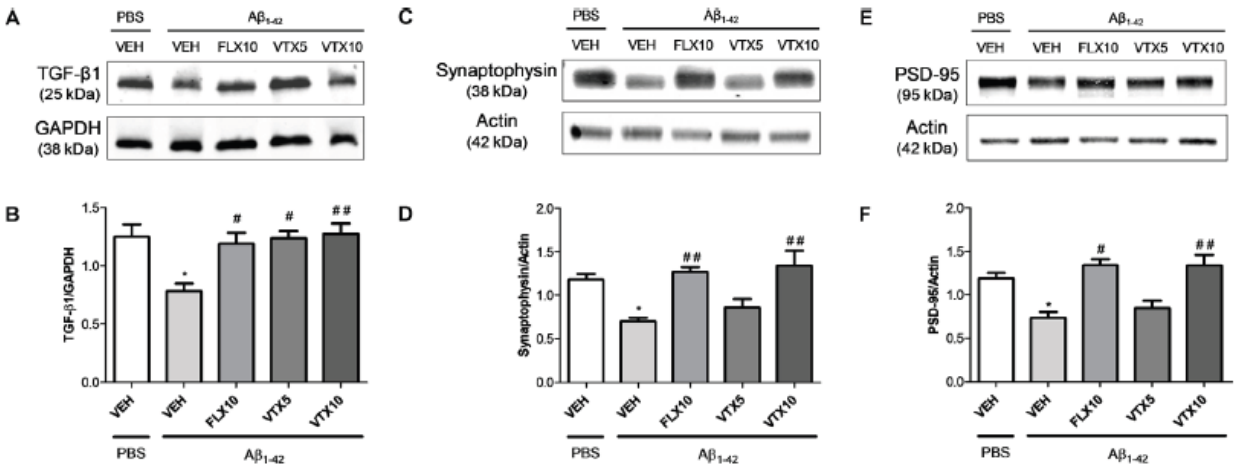

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Figure legends

Fig. 1. Vortioxetine decreases depressive-like behavior in a concentration-dependent manner. Forced swim test (FST), carried out to evaluate the depressive-like behavior, was performed the day after the last injection. VEH = Vehicle (i.p.), FLX10 = Fluoxetine 10 mg/Kg, (i.p.), VTX5 = Vortioxetine 5 mg/Kg (i.p.), and VTX10 = Vortioxetine 10 mg/Kg (i.p.) were administered chronically for 21 days. i.p. = Intraperitoneal injection. **(A)** Schematic representation of the experimental design. **(B)** Immobility time displayed by FLX10 (n = 9), VTX5 (n = 10), and VTX10 (n = 7) treated-groups was significantly reduced if compared to vehicle-treated group (n = 9) over 4-min test period. Immobility time measures are expressed in seconds (sec). Data are shown as mean ± S.E.M. ** $p < 0.01$, *** $p < 0.001$ vs. VEH; ANOVA among all: $F_{(3,31)} = 10.04$.

Fig. 2. Vortioxetine decreases depressive-like behavior and memory impairment $A\beta_{1-42}$-induced. Forced swim test (FST) and passive avoidance test (PAT) were used to evaluate depressive-like behavior and memory impairment, respectively. VEH, FLX10, VTX5, and VTX10 were administered chronically for 26 days. Sterile PBS or $A\beta_{1-42}$ were administered i.c.v. 7 days after the first i.p. injection. i.c.v. = Intracerebroventricular injection. **(A)** Schematic representation of the experimental design. **(B)** Latency time to re-enter the dark box during the retention test is expressed in seconds (sec). **(C)** Immobility time measures are expressed in seconds (sec). PBS + VEH (n = 8), $A\beta_{1-42}$ + VEH (n = 5), $A\beta_{1-42}$ + FLX10 (n = 7), $A\beta_{1-42}$ + VTX5 (n = 7), and $A\beta_{1-42}$ + VTX10 (n = 6). FST and PAT were performed on the same experimental animal groups. Data are shown as mean ± S.E.M. * $p < 0.05$, ** $p < 0.01$ vs. PBS + VEH; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. $A\beta_{1-42}$ + VEH; $F_{(4,28)} = 10.44$ for (B), and $F_{(4,28)} = 5.59$ for (C).

Fig. 3. Vortioxetine reduces the $A\beta_{1-42}$-induced impairment of recognition memory. Object recognition test (ORT) was used to evaluate recognition memory by assessing the discrimination index (D). **(A)** Schematic representation of the experimental design. **(B)** The impairment of recognition memory induced by i.c.v. administration of $A\beta_{1-42}$ ($t_{(9)} = 0.221$, $p > 0.05$ for $A\beta_{1-42}$ group vs. zero) is completely rescued by FLX10 and VTX10 treatments (ANOVA among all: $F_{(5,54)} = 4.4$; Bonferroni's $p < 0.05$ between PBS + VEH vs. $A\beta_{1-42}$ + VEH; $p < 0.01$ between $A\beta_{1-42}$ + VEH vs. VTX10 and vs. FLX10;) **(C)** Total exploration time is similar among the different conditions (ANOVA among all: $F_{(5,54)} = 2.274$). PBS + VEH (n = 14), $A\beta_{1-42}$ + VEH (n = 10), $A\beta_{1-42}$ + FLX10 (n = 7), $A\beta_{1-42}$ + VTX5 (n = 11), and $A\beta_{1-42}$ + VTX10 (n = 11). **(D)** FLX10, VTX5, or VTX10 treatments *per se* do not modify discrimination index ($F_{(3,28)} = 1.409$) nor **(E)** total exploration time ($F_{(3,28)} = 0.467$). ** $p < 0.05$ vs. PBS + VEH, ## $p < 0.01$ vs. $A\beta_{1-42}$ + VEH.

Fig. 4. Fluoxetine and vortioxetine increase the expression of TGF-β1 mRNA. Effects induced by i.c.v. administration of $A\beta_{1-42}$ ($A\beta_{1-42}$ + VEH) in absence or presence of FLX10, VTX5, or VTX10 on IL-1β **(A)**, TNF-α **(B)**, IL-4 **(C)**, and **(D)** TGF-β1 mRNAs expression examined by qRT-PCR (Experiment 2). **(E)** Effects of drugs on TGF-β1 mRNA expression in absence of $A\beta_{1-42}$ treatment (Experiment 3). The abundance of each mRNA of interest was expressed relative to the abundance of GAPDH-mRNA, as an internal control. As a negative control, a reaction in absence of cDNA (no template control, NTC) was performed. qRT-PCR amplifications were performed in quadruplicate. Data are shown as mean ± S.E.M. * $p < 0.05$ vs. PBS + VEH, # $p < 0.05$ vs. $A\beta_{1-42}$ +VEH, ## $p < 0.01$ vs. $A\beta_{1-42}$ + VEH, ### $p < 0.001$ vs. $A\beta_{1-42}$ + VEH; $F_{(4,14)} = 0.86$ for (A), $F_{(4,10)} = 0.35$ for (B), $F_{(4,10)} = 0.06$ for (C), $F_{(4,15)} = 10.23$ for (D), and $F_{(3,19)} = 0.35$ for (E).

Fig. 5. Fluoxetine and vortioxetine rescue TGF-β1, synaptophysin and PSD-95 levels in $A\beta_{1-42}$-treated mice. Effects induced by i.c.v. administration of $A\beta_{1-42}$ ($A\beta_{1-42}$ + VEH) in absence or presence of FLX10, VTX5, or VTX10 on TGF-β1, synaptophysin and PSD-95 levels examined by western blot. **(A)** Representative immunoblots of active TGF-β1 (about 25 kDa) in total protein extracts from hippocampus tissues. **(B)** Histograms refer to the means ± S.E.M. of the densitometric values of active TGF-β1 bands normalized against GAPDH. Each experiment was repeated four times. * $p < 0.05$ vs. PBS + VEH, # $p < 0.05$ vs. $A\beta_{1-42}$ +VEH, ## $p < 0.01$ vs. $A\beta_{1-42}$ +VEH; $F_{(4,15)} = 5.91$ for (B). **(C)** Representative immunoblots of synaptophysin (about 38 kDa) in total protein extracts from hippocampus tissues. **(D)** Histograms refer to the means ± S.E.M. of the densitometric values of synaptophysin bands normalized against Actin. Each experiment was repeated four times. * $p < 0.05$ vs. PBS + VEH, ## $p < 0.01$ vs. $A\beta_{1-42}$ +VEH; $F_{(4,17)} = 7.91$ for (D). **(E)** Representative immunoblots of PSD-95 (about 95 kDa) in total protein extracts from hippocampus tissues. **(D)** Histograms refer to the means ± S.E.M. of the densitometric values of PSD-95 bands normalized against Actin. Each experiment was repeated four times. * $p < 0.05$ vs. PBS + VEH, # $p < 0.05$ vs. $A\beta_{1-42}$ +VEH, ## $p < 0.01$ vs. $A\beta_{1-42}$ +VEH; $F_{(4,17)} = 8.21$ for (F).

Table 1. List of primers used for quantitative real-time PCR (qRT-PCR).

Official name[#]	Official symbol	Alternative titles/symbols	Detected transcript	Amplicon Length	Cat. No.[§]

interleukin 1 beta	Il1b	Il-1b; IL-1beta; IL-1β	NM_008361 XM_006498795	150 682	QT01048355
tumor necrosis factor	Tnf	DIF; Tnfa; TNF-a; TNFSF2; Tnlg1f; Tnfsf1a; TNFalpha; TNF-alpha; TNF-α	NM_013693 NM_001278601	112 bp 112 bp	QT00104006
interleukin 4	IL4	Il-4; BSF-1	NM_021283	132 bp	QT02418311
transforming growth factor, beta 1	Tgfb1	Tgfb; Tgfb-1; TGFbeta1; TGF-beta1	NM_011577	145 bp	QT00145250
glyceraldehyde-3-phosphate dehydrogenase	Gapdh	Gapd	NM_008084 XM_001003314 XM_990238 NM_001289726	144 bp	QT01658692

[#]https://www.ncbi.nlm.nih.gov/gene/
[§]https://www.qiagen.com/it/shop/pcr/real-time-pcr-enzymes-and-kits/two-step-qrt-pcr/quantitect-primer-assays/

Chapter 3.

Antioxidant activity of fluoxetine and vortioxetine in a non-transgenic animal model of Alzheimer's disease

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Abstract

Depression is a risk factor for the development of Alzheimer's disease (AD). A neurobiological and clinical continuum exists between AD and depression, with neuroinflammation and oxidative stress being involved in both diseases. Second-generation antidepressants, in particular selective serotonin reuptake inhibitors (SSRIs), are currently investigated as neuroprotective drugs in AD. By employing a non-transgenic AD model, obtained by intracerebroventricular (i.c.v.) injection of amyloid-β (Aβ) oligomers in 2-month-old C57BL/6 mice, we recently demonstrated that the SSRI fluoxetine (FLX) and the multimodal antidepressant vortioxetine (VTX) reversed the depressive-like phenotype and memory deficits induced by Aβ oligomers rescuing the levels of transforming growth factor-β1 (TGF-β1). Aim of our study was to test FLX and VTX for their ability to prevent oxidative stress in the hippocampus of Aβ-injected mice, a brain area strongly affected in both depression and AD. The chronic intraperitoneal (i.p.) administration of FLX (10 mg/Kg) and VTX (5 and 10 mg/Kg) for 24 days, starting 7 days before Aβ injection, was able to prevent the over-expression of inducible nitric

oxide synthase (iNOS) and NADPH oxidase 2 (Nox2) induced by Aβ oligomers. Antidepressant pre-treatment was also able to rescue the mRNA expression of glutathione peroxidase 1 (Gpx1) antioxidant enzyme. FLX and VTX also prevented Aβ-induced neurodegeneration in mixed neuronal cultures treated with Aβ oligomers. Our data represent the first evidence that the long-term treatment with the antidepressants FLX or VTX can prevent the oxidative stress phenomena related to the cognitive deficits and depressive-like phenotype observed in a non-transgenic animal model of AD.

Keywords: oxidative stress, Alzheimer's disease, depression, amyloid-β, vortioxetine, fluoxetine, TGF-β1, neuroprotection.

* *Published in Front. Pharmacol. 2021 Dec 24;12:809541.*

1. Introduction

Alzheimer's disease (AD) represents a type of dementia affecting memory, global cognitive function, and behavior, severe enough to interfere with activities of daily living (Kumar et al., 2021). This disease also presents neuropsychiatric symptoms, such as depression, along with neurodegeneration, neuroinflammation, and oxidative stress phenomena (Caruso et al., 2021). The latter occurs when the homeostatic equilibrium between pro-oxidants species and antioxidants is missing, with the pro-oxidants being in excess (Caruso et al., 2019a). With regard to depression, a neurobiological and clinical continuum has been demonstrated between this disease and AD (Caraci et al., 2018b). In fact, depression represents a risk factor for AD development, while the occurrence of depressive symptoms significantly increases the conversion from mild cognitive impairment (MCI) into AD (Petersen et al., 2014).

It is now well-known that amyloid-β (Aβ), the peptide involved in the pathogenesis of AD, can undergo aggregation, starting with soluble monomers and forming species characterized by higher molecular weight such as oligomers, protofibrils, and mature fibrils (Brorsson et al., 2010). Among the above species, oligomers represent the most toxic species of Aβ, leading to synaptic loss and neuronal death in AD brain (Klein 2013). It has been shown that oxidative stress plays a crucial role in mediating the toxicity Aβ oligomers; in fact, neurodegeneration and neuroinflammation as well as the impairment of synaptic plasticity are, at least in part, due to the oxidative stress Aβ oligomers-induced (Varadarajan et al., 2000; Gelain et al., 2012). Oxidative stress is able to promote Aβ oligomerization (Zhao and Zhao, 2013b) and β- and γ-secretase activation, the two enzymes involved in the formation of the different Aβ species. (Zhao and Zhao, 2013a). Markers of oxidative stress have been found in AD animal models before plaques deposition as well as in brain, plasma, and erythrocytes from MCI and AD patients (Glenner and Wong, 1984; Minati et al., 2009), suggesting

that redox imbalance and oxidative damage play a key role in an early stage of AD pathophysiology, as well as in the disease progression (Cheignon et al., 2018).

Different groups are currently studying antidepressants in AD (Lozupone et al., 2018). Second-generation antidepressants have been associated with a reduced risk of developing AD, but there are still no clear findings demonstrating the ability of these drugs to counteract the progression of this disease (Correia and Vale, 2021). Positive outcomes have been observed by using selective serotonin reuptake inhibitors (SSRIs) (Dafsari and Jessen, 2020). Long-term SSRI treatment (>4 years) was significantly associated with a delayed progression from MCI to AD (Bartels et al., 2018). The immune regulatory effect of antidepressants observed in depressed patients is attributable to their ability to decrease the levels of pro-inflammatory cytokines (e.g tumor necrosis factor-α (TNF-α)) and increase those of anti-inflammatory cytokines, such as transforming growth factor-β1 (TGF-β1) (Szałach Ł et al., 2019). Antidepressant drugs could also exert their therapeutic effect by suppressing the production of reactive oxygen and nitrogen species, ROS and RNS respectively, and/or rescuing the antioxidant defense (Behr et al., 2012; Wu et al., 2013). The SSRI fluoxetine (FLX) is able to revert the brain oxidative damage by reducing lipid peroxidation at hippocampal level, also increasing the activity of antioxidants, such as superoxide dismutase (SOD) and catalase (CAT), in different animal models of depression (Chung et al., 2010; Moretti et al., 2012). Evidence also exists that FLX prevents amyloid pathology, also reverting memory impairment in different animal models of AD (Wang et al., 2014; Jin et al., 2017). Furthermore, FLX exerts neuroprotection in an established in vitro model of Aβ-induced neurodegeneration via a paracrine signaling mediated by TGF β1 (Caraci et al., 2016). Acute and long-term chronic treatments with the new multimodal antidepressant vortioxetine (VTX) improve cognitive function in preclinical models of depression (Bennabi et al., 2019). This drug also exhibits an increased efficacy, compared to FLX, in aged mice in counteracting depressive-like behavior and memory deficits (Li et al., 2017; Bennabi et al., 2019). At clinical level, VTX has proven more effective than SSRIs in the treatment of specific clinical domains, such as cognitive deficits in elderly depressed patients (McIntyre et al., 2016; Thase et al., 2016), underlining its therapeutic potential for the treatment of cognitive impairment in depression (Bennabi et al., 2019). Interestingly, VTX exerts antioxidant activity and anti-inflammatory effects in human monocytes/macrophages stimulated with phorbol 12-myristate 13-acetate (PMA), also inducing the shift of macrophages from M1 (pro-inflammatory) to M2 (anti-inflammatory) phenotype (Talmon et al., 2018).

Intracerebroventricular (i.c.v.) injection of Aβ oligomers in mice has been used to obtain a non-transgenic (non-Tg) AD model characterized by memory deficits and depressive-like phenotype (Ledo et al., 2016; Ledo et al., 2020). An equivalent outcome has been observed in rats that underwent

i.c.v. injection of Aβ oligomers (Colaianna et al., 2010; Schiavone et al., 2017). By using this non-Tg Ad AD model, we have recently demonstrated that FLX or VTX revert the behavioral and memory alterations induced by Aβ oligomers (Torrisi et al., 2019). In the same study we also detected a significant reduction of the synaptic proteins synaptophysin and PSD-95 paralleled by a significant deficit of TGF-β1 at hippocampal level that was completely rescued by the long-term chronic treatment with FLX or VTX.

Starting from these grounds, we hypothesized that the i.c.v. injection of Aβ oligomers could also induce oxidative stress in the hippocampus of our non-Tg model of AD and that a long-term chronic treatment with FLX or VTX could prevent this phenomenon by regulating the subtle equilibrium between pro- and antioxidant factors.

2. Materials and Methods

Materials

All chemicals and reagents used in this study were of analytical grade and obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA) or Thermo Fisher Scientific Inc. (Pittsburgh, PA, USA) unless specified otherwise.

Establishment of the non-Tg AD mouse model

The cohorts of animals whose tissues were used for gene and protein analysis are the same described in Torrisi et al. (Torrisi et al., 2019).

Eight-week-old male C57BL/6 mice, obtained from Envigo RMS s.r.l. laboratories (San Pietro al Natisone, Italy), were maintained and used as previously described (Torrisi et al., 2019), following procedures in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

As previously described, in order to obtain the non-Tg AD mouse model, 2 μL of the 10 μM Aβ oligomers solution (Bachem Distribution Services GmbH, Weil am Rhein, Germany), prepared according to the original protocol of Klein's group (Gong et al., 2003), were i.c.v. injected by using a microsyringe with a 28-gauge stainless-steel needle 3.0-mm-long . The injection of this Aβ solution corresponds to 20 pmol of Aβ monomer equivalent, giving a final concentration of approximately 0.18 μg/g tissue.

Drug treatment

Vortioxetine hydrobromide [purity > 98.0% (HPLC)] was obtained from H. Lundbeck A/S (Denmark) according to the MTA N.417394 signed by University of Catania (Department of Drug and Health Sciences) and H. Lundbeck A/S and Lundbeck Italia S.p.A. FLX and VTX were prepared and administered i.p. (FLX at 10 mg/Kg; VTX at 5 or 10 mg/Kg) daily for a total of 24 days starting from 7 days before Aβ i.c.v injection as previously described in details (Torrisi et al., 2019). Control animals received the vehicle i.p. A total of five groups of animals were employed in this study and are indicated as follows: 1) **control group** (phosphate-buffered saline (PBS + vehicle (VEH)); 2) **Aβ group** (Aβ + VEH); 3) **FLX10 group** (Aβ + FLX 10 mg/kg); 4) **VTX5 group** (Aβ + VTX 5 mg/kg); 5) **VTX10 group** (Aβ + VTX 10 mg/kg). PBS and VEH were injected i.c.v. and i.p., respectively, while to test the drug activity *per se*, so in absence of Aβ, drugs were administered i.p. for a total of 21 days.

Gene expression analysis by quantitative real-time PCR (qRT-PCR)

Gene expression analysis by qRT-PCR was carried out on hippocampal samples. The protocol employed for these experiments is the same previously described (Caruso et al., 2019c; Fidilio et al., 2021) with slight modifications. Briefly, NanoDrop® ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the RNA concentrations, while Qubit® 3.0 Fluorometer (Thermo Fisher Scientific) was employed to assess the quality of RNA (Fresta et al., 2020a). The reverse transcription was obtained by using the SuperScript III First-Strand Synthesis SuperMix kit. The quantification of all the cDNA samples obtained and loaded in a 384-well plate was measured through a LightCycler® 480 System (Roche Molecular Systems, Inc., Pleasanton, CA, United States). The information relative to the Quanti Tect Primer Assays (Qiagen, Hilden, Germany) used is reported in Table 1. Sample amplification, fluorescence data collection, and sample quantification is the same previously described elsewhere (Caruso et al., 2019c; Fidilio et al., 2021). The relative RNA expression level for each sample was calculated using the $2^{-\Delta\Delta CT}$ method in which the threshold cycle value of the target gene is compared to the CT value of the selected internal control (GAPDH gene in our case). The number of samples analyzed obtained by each animal group is indicated in the pertinent Figure legend.

Protein expression analysis by Western Blot (WB)

WB analysis was performed on hippocampal samples following the previously described procedure (Caraci et al., 2015). Briefly, once the protein concentration in tissue homogenate was determined (Pierce™ BCA protein assay kit), 30 μg of total proteins were denatured, separated by gel electrophoresis, and transferred to nitrocellulose membranes. The membranes were incubated

overnight (4 °C) with the following primary antibodies: rabbit anti-iNOS (Abcam ab136918, 1:1000), rabbit anti-Nox2/gp91phox (Abcam ab80508, 1:4000), rabbit anti-Gpx1 (Cell Signaling Technology 3206, 1:500), mouse anti-β-actin (Sigma Aldrich A4700, 1:1000). Secondary goat anti-rabbit labeled with IRDye 800 (Li-COR Biosciences; 1:15.000) and goat anti-mouse labeled with IRDye 680 (Li-COR Biosciences; 1:15.000) were used at room temperature in the dark for 1 h after three washes in tris-buffered saline (TBS)/Tween 20X 0.1%. Hybridization signals were detected by the Odyssey Infrared Imaging System (LI-COR Biosciences) and the densitometry analysis was performed by using Image J software. The number of samples analyzed obtained by each animal group is indicated in the pertinent Figure legend.

Mixed neuronal cultures

Mixed neuronal cultures consisting of 35–40% neurons and 60–65% glial cells (astrocytes and microglia) were obtained from rats at embryonic day 15 (Harlan Laboratories, Italy) as previously described (Caraci et al., 2016). Cells were grown into DMEM/F12 (1:1) (American Type Culture Collection (ATCC), Manassas, VA, USA) supplemented with 10% horse serum, 10% fetal calf serum, 2 mM glutamine, and 6 mg/ml glucose. After 7-10 days *in vitro*, to avoid the proliferation of non-neuronal elements, cytosine-D-arabinoside (10 μM) was added, for a total of 3 days. Cells were then moved into a maintenance medium in absence of serum. Mixed neuronal cultures were treated with Aβ oligomers (2 μM) for 48 h both in absence or presence of FLX (1 μM) or increasing concentrations of VTX (100 nM, 250 nM, and 1 μM) (pre-treatment of 1 h). The Aβ oligomers-induced toxicity was quantitatively assessed by trypan blue exclusion assay. Cell counts were performed in three to four random microscopic fields/well.

Statistics

Data are reported as mean ± standard error of the mean (S.E.M.) except in the case of cell experiments in which standard deviation (S.D.) was showed. One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test were used for multiple comparisons. The version 8.0 of GraphPad Prism software® (GraphPad, La Jolla, CA, USA) was used to perform all the analyses. Only two-tailed *p* values < 0.05 were considered statistically significant.

Study approval

The study was authorized by the Institutional Animal Care and Use Committee (IACUC) of the University of Catania and by the Italian Ministry of Health (DDL 26/2014 and previous legislation;

OPBA Project #266/2016). Animal care followed Italian (D.M. 116192) and EEC (O.J. of E.C.L 358/1 12/18/1986) regulations on protection of animals used for experimental and scientific purposes.

3. Results

3.1 Fluoxetine and vortioxetine decreased the expression of iNOS and Nox2 mRNAs

Oxidative stress and neuroinflammation play a significant role in the pathogenesis of depression (Bhattacharya and Drevets, 2017; Caruso et al., 2019a) and AD (Huang et al., 2016; Knezevic and Mizrahi, 2018). During the inflammation process, both inducible nitric oxide synthase (iNOS), responsible for nitric oxide production (Aktan, 2004; Metto et al., 2013), and NADPH oxidase 2 (Nox2), responsible for superoxide production (de Campos et al., 2015), are over-activated in immune cells including microglia (Siegel et al., 2019). When the above-mentioned enzymes are simultaneously activated, they synergistically promote neuronal cell death by generating peroxynitrite (Beckman and Crow, 1993). We therefore examined the effects of Aβ oligomers on the mRNAs levels of the pro-oxidant enzymes iNOS and Nox2 in the hippocampus (**Figure 1**), a brain area strongly affected in depression and AD (Villa et al., 2016; Setti et al., 2017). The i.c.v. injection of Aβ oligomers induced a statistically significant increase in the expression level of iNOS mRNA in the hippocampus compared with vehicle-treated controls ($p < 0.05$ vs. PBS + VEH; Figure 1A). Long-term i.p. treatment with FLX or VTX, administered at the same dose of 10 mg/Kg, was able to abolish the over-expression of iNOS Aβ-induced ($p < 0.05$ *vs.* Aβ oligomers), whereas the lower dose of VTX (5 mg/Kg) did not reach a statistically significant difference, even though a trend in iNOS mRNA enzyme expression decrease was observed. More robust effects were observed when measuring the variation of Nox2 mRNA expression levels under our experimental conditions. In fact, as shown in **Figure 1B**, the expression level of Nox2 mRNA was significantly increased in the hippocampus of Aβ-injected mice compared with vehicle-treated controls ($p < 0.001$ *vs.* PBS + VEH). Chronic i.p. treatment with FLX (10 mg/Kg) or VTX, at both doses (5 or 10 mg/kg), was able to completely counteract the over-expression of this enzyme ($p < 0.001$ *vs.* Aβ oligomers for all of them). It is worth mentioning that treatment with FLX or VTX *per se* did not significantly modify the mRNA expression levels of iNOS and Nox2 enzymes (**Supplementary Figure 1**).

3.2 Fluoxetine and vortioxetine decreased the expression of iNOS and Nox2 proteins

We then carried out WB experiments in order to corroborate with protein data the results obtained by using qRT-PCR in which the expression levels of iNOS and Nox2 mRNA was measured. WB analysis confirmed that the i.c.v injection of Aβ oligomers was able to induce a significant increase of iNOS protein expression at hippocampal level ($p < 0.05$ *vs.* PBS + VEH) and, most importantly, that the

treatment with FLX and VTX, was able to completely abolish Aβ-induced iNOS expression ($p < 0.001$ *vs.* Aβ oligomers for both of them; **Figure 2A**). A very similar profile was observed when measuring the variation of Nox2 protein levels. **Figure 2B** shows that the expression level of Nox2 protein was significantly increased in the hippocampus of Aβ-injected mice compared with vehicle-treated controls ($p < 0.05$ *vs.* PBS + VEH). Treatment with FLX at the dose of 10 mg/Kg or with the lower dose of VTX (5 mg/kg) abolished Aβ oligomers induction ($p < 0.05$ *vs.* Aβ oligomers), while a stronger decrease was observed in the case of the higher dose of VTX (10 mg/kg) ($p < 0.01$ *vs.* Aβ oligomers). As expected based on the qRT-PCR results, the treatment with FLX or VTX *per se* did not significantly modify the protein expression levels of iNOS and Nox2 enzymes (**Supplementary Figure 2**).

3.3 Fluoxetine or vortioxetine rescued the expression of glutathione peroxidase 1 at gene but not at protein level

As previously mentioned, oxidative stress, a key factor in the progression of AD (Zhao and Zhao, 2013a; Bajpai et al., 2014), reflects the imbalance between the production and quenching of reactive species in the biological system. This imbalance could also depend on the reduced activity of antioxidant enzymes such as glutathione peroxidase 1 (Gpx1) (Marcus et al., 1998; Katrenčíková et al., 2021). The lack of Gpx1 has been related to the exacerbation of Aβ-mediated neurotoxicity in cortical neurons (Crack et al., 2006). With this in mind, we investigated the effects of Aβ oligomers, in absence or presence of FLX10, VTX5, or VTX10, on the mRNA levels of Gpx1 in the hippocampus. The results depicted in Figure 3A show that the expression level of Gpx1 mRNA was significantly decreased in the hippocampus of Aβ-injected mice compared with vehicle-treated controls ($p < 0.05$ vs. PBS + VEH), while the treatment with FLX was able to completely rescue restore Gpx1 mRNA levels ($p < 0.05$ vs. Aβ oligomers). A more significant effect was observed in the case of both doses of VTX ($p < 0.001$ vs. Aβ oligomers). Since it has been shown that Gpx1 enzyme oxidative stress can induce multimerization of Gpx1 enzyme by forming complexes via oxidative linkage between subunitsis able to form multimers (Park et al., 2004; Sultan et al., 2018), we performed WB analysis by measuring both monomeric and dimeric forms. However, the results obtained by WB showed that either the monomeric nor the dimeric form of Gpx1 protein in the hippocampus were significantly affected by Aβ treatment as compared to vehicle-treated controls (**Figure 3B-C**). The presence of antidepressants during Aβ treatment did not modulate the expression levels of either enzyme isoforms. The treatment with FLX or VTX *per se*, in the absence of Aβ

treatment, did not significantly modify the mRNA (**Supplementary Figure 1**) and protein (**Supplementary Figure 2**) levels of Gpx1 enzyme.

3.4 Fluoxetine and vortioxetine exert neuroprotection against the toxicity induced by Aβ oligomers

The neuroprotective activity of FLX in mixed cultures of cortical cells treated with Aβ oligomers, representing an established experimental model of Aβ-induced neurodegeneration (Caruso et al., 2019c), has been already reported (Caraci et al., 2016). However, it is presently unknown whether a treatment with VTX can prevent the neuronal cell death due to Aβ treatment. We then investigated the neuroprotective activity of increasing concentrations of VTX in mixed cultures of cortical cells treated with Aβ oligomers (2 μM) for 48 hours. In this set of experiments, FLX at the concentration of 1 μM was used as a gold standard. Since Aβ is known to promote glutamate release and toxicity (Caraci et al., 2011), the experiments were performed in the presence of a cocktail of ionotropic glutamate receptor antagonists [MK-801 (10 μM) and DNQX (30 μM)] to exclude the contribution of endogenous excitotoxicity to the overall process of neuronal death. The treatment of mixed cultures of cortical cells with Aβ oligomers for 48 h led to a significant increase (about 300%) in the number of trypan blue positive cells (dead neurons) compared to untreated (CTRL) cells ($p < 0.001$) (**Figure 4**). VTX, starting at a concentration of 250 nM, significantly prevented Aβ toxicity in mixed neuronal cultures ($p < 0.05$ *vs.* Aβ), though not completely. The maximal neuroprotective effect was observed in the case of VTX 1 μM ($p < 0.001$ *vs.* Aβ), with a number of dead cells comparable to that observed for untreated cells or for FLX-treated cells (positive control).

4. Discussion

Reactive species such as ROS and RNS play a crucial role in numerous human pathophysiological processes. These species, when produced at physiological concentration, are able to regulate growth, apoptosis, and complex functions such as blood pressure, immune and cognitive functions. When overproduced, they can contribute to a well-known and deleterious phenomenon called oxidative stress (Estévez and Jordán, 2002). During acute oxidative stress, the components of the antioxidant system are able to counteract the increased levels of pro-oxidants, such as ROS and RNS, resetting them to the physiological levels. Whenever ROS and RNS levels overcome the antioxidant defense, chronic oxidative stress takes place, as it has been observed in neuropsychiatric disorders such as depression and AD (Caruso et al., 2019d). Both ROS and RNS are involved in the pathogenesis of depression by influencing neuronal processes such as neurogenesis and neuroplasticity, also inducing neuroinflammation and neurodegeneration (Cheignon et al., 2018; Solleiro-Villavicencio and Rivas-

Arancibia, 2018). When considering AD, the over-production of ROS and RNS, and then oxidative stress, has been related to increased Aβ production and/or aggregation, which in turn exacerbates neuronal oxidative damage, contributing to neuronal death in AD brain (Cheignon et al., 2018).

In the present work we employed a non-Tg model of AD, obtained by i.c.v. injection of Aβ oligomers, representing a simple and reliable paradigm, usefulallowing to investigate the molecular mechanisms throughby which the oligomeric form of Aβ oligomers causesinterfere with cognitive processesdysfunction, and finally to test novel pharmacological approaches (Balducci and Forloni, 2014). As previously demonstrated, this non-Tg AD model is characterized by cerebral concentrations of soluble Aβ oligomers comparable to those observed in the AD brain, sufficient to induce a memory deficit that persist for 2-3 weeks (Leggio et al., 2016). By adopting this model, we have recently demonstrated that both FLX and VTX are ablepossess the ability to reverse the depressive-like phenotype and memory deficits induced by the i.c.v. injection of Aβ oligomers in mice, also rescuing the levels of the synaptic proteins synaptophysin and PSD-95 as well as of TGF-β1, the deficit of which has been shown to contribute to inflammation and cognitive decline both in depression and AD (Caraci et al., 2018b; Torrisi et al., 2019). In order to correlate the above-mentioned preclinical efficacy of these two second-generation antidepressants with an antioxidant activity of these drugs, we examined the mRNAs levels of iNOS and Nox2, two pro-oxidant enzymes synergistically able to promote neuronal death through the production of ROS and RNS (Beckman and Crow, 1993), in the hippocampus of Aβ-injected mice, a brain area strongly affected in both depression and AD (Villa et al., 2016; Setti et al., 2017). Our results show that the i.c.v. injection of Aβ oligomers in mice induced a significant increase in iNOS and Nox2 enzymes in the hippocampus, both at mRNA (**Figure 1**) and protein (**Figure 2**) level, compared with vehicle-treated controls. These findings are in agreement with an in vivo study carried out by Medeiros et al. in which the i.c.v. injection of Aβ1–40 induced iNOS protein expression in hippocampus and prefrontal cortex of mice that was paralleled by marked deficits of learning and memory (Medeiros et al., 2007), emphasizing the deleterious effects of aberrant expression of NOS isoforms in AD brain (Lüth et al., 2002). Our findings are also in line with other studies showing that AD is characterized by inflammatory processes in which the production of NO from iNOS and/or superoxide from Nox2 is strongly increased (Murphy, 2000; Brown and Bal-Price, 2003; Zekry et al., 2003). In the present study we showed for the first time that a chronic i.p. treatment with FLX or VTX was able to abolish the over-expression of iNOS and Nox2 induced by Aβ oligomers (Figures 1 and 2). These results are also consistent with previous findings, showing the ability of antidepressant drugs to exert immune-regulatory effects by reducing the levels of pro-inflammatory cytokines, also decreasing the production of reactive species and/or enhancing key elements of the antioxidant machinery. Along

this line, FLX has been shown to revert brain oxidative damage by reducing lipid peroxidation and increasing the activity of the antioxidant enzymes (i.e., CAT and SOD) in the hippocampus of an animal model of depression (Moretti et al., 2012). In a study carried out by Talmon et al. the multimodal antidepressant VTX was able tosignificantly reduced the PMA-induced oxidative burst induced by PMA in monocytes and in macrophages (M1 and M2), also leading to causing a concomitant shift of macrophages polarization from the pro-inflammatory (M1) to the anti-inflammatory (M2) phenotype. The above reduction of oxidative stress was also paralleled by a decrease of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) translocation and TNF-α release (Talmon et al., 2018). The present data on the antioxidant activity of FLX and VTX in vivo, reinforce the above-described previous findings obtained in translational models of inflammation. Our findings on the antioxidant activity of FLX and VTX might be therapeutically relevant in the context of depression and AD, when considering that depression acts as a risk factor for AD and, most importantly, that oxidative stress processes play a key role in the pathophysiology of cognitive deficits in depression (Scapagnini et al., 2012).

About 30% of depressed patients show a poor response to conventional antidepressants associated with a significant cognitive impairment and a poor quality of life, a clinical phenotype classified as treatment-resistant depression (TRD) (Caraci et al., 2018a). It has been recently shown how TRD is characterized by increased oxidative stress coupled to inflammation (Sowa-Kućma et al., 2018). Furthermore, plasma levels of Coenzyme Q10 (CoQ10), a strong antioxidant with anti-inflammatory activity, are lower in TRD patients compared to responders depressed patients (Maes et al., 2009) and CoQ10 (200 mg/die) has been proposed as an adjuvating agent for the treatment of depression (Mehrpooya et al., 2018). According to this scenario second-generation antidepressant drugs, endowed with antioxidant activity, may display an increased clinical efficacy in TRD patients; studies in animal models are useful to test this hypothesis. As previously mentioned, antioxidants play a significant role in maintaining redox homeostasis. It is also worth mentioning that an increase in Nox2 protein expression and the related oxidative stress has also been observed in sleep deprivation known to induce memory impairments, serotoninergic system dysfunction, and depression in mice (Wang et al., 2020). In our experimental model of amyloid-related depression the presence of depressive-like behavior and memory deficit was paralleled by an increase in Nox2 protein levels in Aβ-injected mice that was rescued by the long-term chronic treatment with FLX at 10 mg/kg and more significantly by VTX, being effective at the lowest dose of 5 mg/kg, suggesting that the antidepressant activity of these drugs is not simply related to the inhibition of serotonin transporter (SERT), but it is also includes antioxidants effects.

In our study we also measured the mRNA and protein levels of Gpx1, an antioxidant enzyme known to play a protective role against Aβ-toxicity and the related ROS accumulation at intracellular level (Barkats et al., 2000), and the deficit of which has been related to increased Aβ-mediated neurotoxicity (Crack et al., 2006). Our data show that the treatment with FLX or VTX is needed to rescue the expression of Gpx1 at mRNA, but not at protein level. We cannot exclude a post-trascriptional regulation of Gpx1 gene expression. In our case, the discrepancy observed when comparing mRNA and protein levels within the same time frame might be due to a compensatory response of the antioxidant system with an additional role played by FLX or VTX, which remains to be elucidated.

We know from our previous work in the non-Tg model of AD that FLX and VTX not only counteract oxidative stress, but also rescue the hippocampal TGF-β1 levels. This neurobiological link could be of utmost importance since it has already been demonstrated that other multimodal drugs that counteract oxidative stress can also rescue TGF-β1 levels (Caruso et al., 2019b; Fresta et al., 2020b). In two very recent research studies it has also demonstrated that the ability of TGF-β1 to protects retinal ganglion cells from against oxidative stress via through the modulation of the HO-1/Nrf2 pathway (Chen et al., 2020) and chondrocytes via FOXO1-autophagy axis (Kurakazu et al., 2021), strongly suggesting a key role of TGF-β1 in counteracting oxidative stress induced by Aβ.

In the AD brain, oxidative stress promotes the generation of ROS that contribute to neurodegeneration (Shimohama et al., 2000; Abramov and Duchen, 2005). During the last decade, based on their ability to exert neuroprotection, the use of antidepressants to reduce the risk to develop AD has been proposed (Kessing et al., 2009; Kessing, 2012; Yuste et al., 2015; Bartels et al., 2018). By using a well-established in vitro model of Aβ-induced neurodegeneration consisting of mixed cultures of cortical cells challenged with Aβ oligomers, we were able to compare, for the first time, the well-known neuroprotective activity of FLX (Caraci et al., 2016), with the protective effects of VTX. Interestingly we found that VTX pre-treatment started to exert significant neuroprotective effects at nanomolar concentrations (250 nM) with a maximal effect at 1 μM, similar to that observed for FLX pre-treatment-treated and untreated cells (**Figure 4**). The neuroprotective effects exerted in vitro, as well as the antidepressant effects exerted in vivo by FLX and VTX could be related to the anti-amyloidogenic and anti-aggregant activity of these drugs. In particular, SSRIs including FLX have shown the potential to prevent Aβ aggregation by direct binding and could be beneficial to AD patients (Tin et al., 2019). FLX also possesses a recognized ability to revert soluble Aβ-induced depressive phenotype with a specific "Aβ-lowering" effect (Schiavone et al., 2017). Furthermore, it cannot be excluded that both FLX and VTX could exert their therapeutic potential by enhancing the release of TGF-β1 from microglial cells, as observed in our *in vivo* experiments, then rescuing the

antioxidant system through the activation of TGF-β1 signaling. The results observed in our experimental models with therapeutic concentrations of VTX also stimulate further studies both in rodents and MCI patients with amyloid-related depression.

All together the data presented in this study, obtained by using a non-Tg model of AD, demonstrated that oxidative stress, taking place as a consequence of pro-oxidant enzymes (i.e., iNOS and Nox2) activation, along with the previously showed deficit of TGF-β1, represents one of the neurobiological links between depression and AD. We also showed for the first time how a chronic administration of the antidepressants fluoxetine and vortioxetine, able to rescue the TGF-β1 pathway, can also contribute to prevent amyloid-induced depression and cognitive decline by counteracting Aβ-induced oxidative stress.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

GC and FC gave substantial contributions to the conception and design of the work. GC, MG, AF, SAT, NM, FG, MRT, and AP performed the experiments. GC, MG, AF, and FC analyzed the data. FT, DP, SS, GML, FD, and FC participated in the design and coordination of the study. GC, MG, AF, and GML drafted the work. All authors revised the work and approved the version to be published.

Funding

This research was funded by University of Catania, PIAno InCEntivi per la RIcerca di Ateneo UniCT2020–2022-Linea 3 Project Asclepio and also conducted with a partial unrestricted support of Lundbeck.

Acknowledgments

This research was al also supported by PRIN2017 (Program of Relevant National interest-2017AY8BP4_004) from the Italian Ministry of University and Research. The authors would like to thank the BRIT laboratory at the University of Catania (Italy) for the valuable technical assistance and use of their laboratories.

Supplementary Material

Supplementary Material contains the following Figures: 1) **Supplementary Figure 1**. Fluoxetine and vortioxetine do not influence the basal expression levels of iNOS, Nox2, and Gpx1 mRNAs; 2) **Supplementary Figure 2**. Fluoxetine and vortioxetine do not influence the basal expression levels of iNOS, Nox2, and Gpx1 proteins.

Data Availability Statement

All datasets generated for this study are included in the manuscript and the supplementary files.

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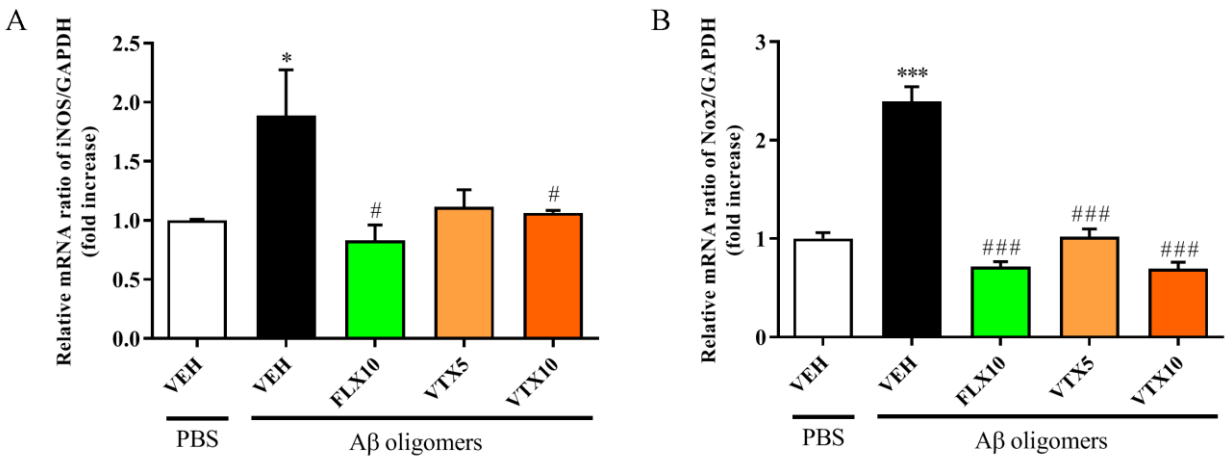


Figure 1.

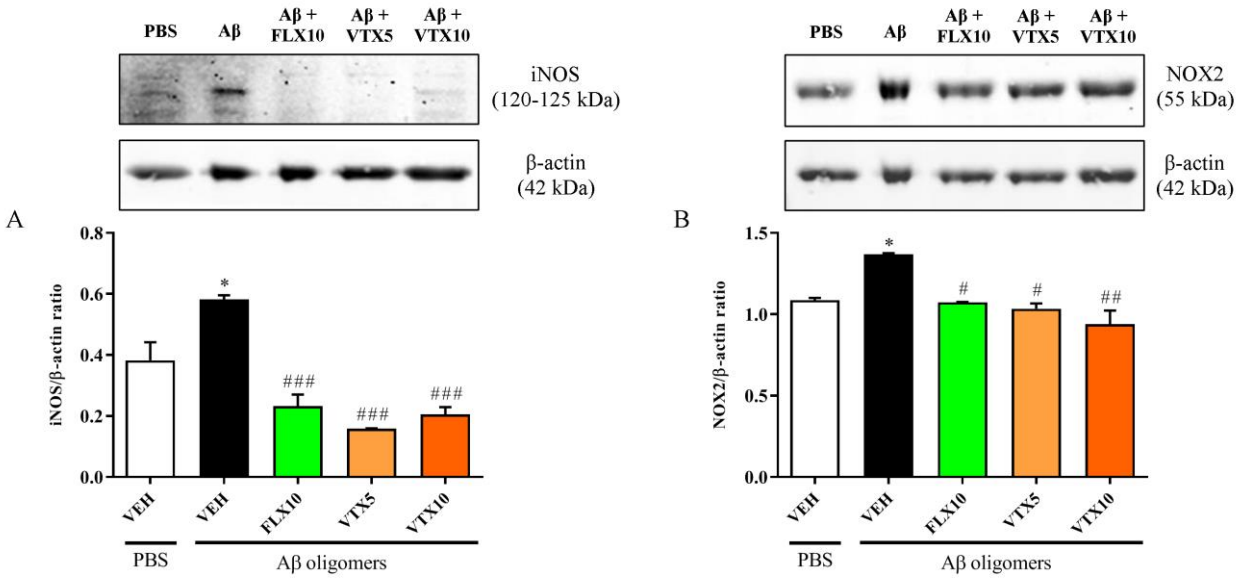


Figure 2.

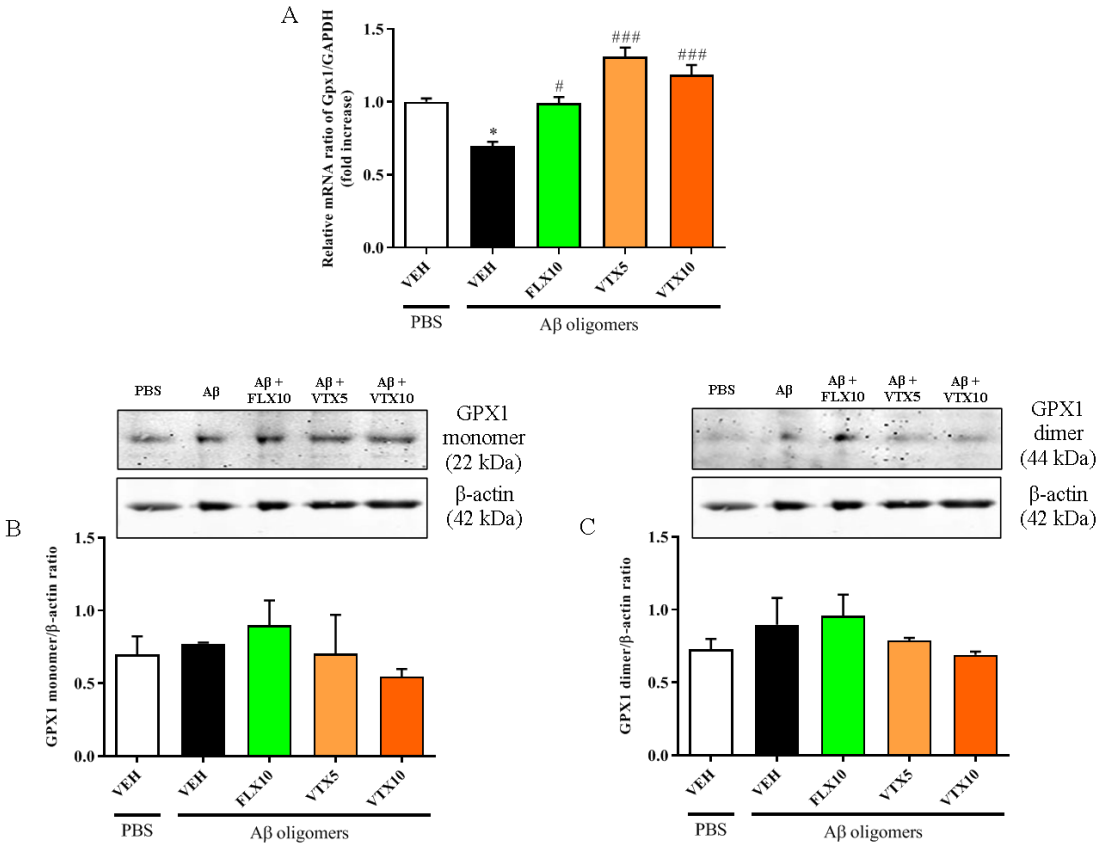


Figure 3.

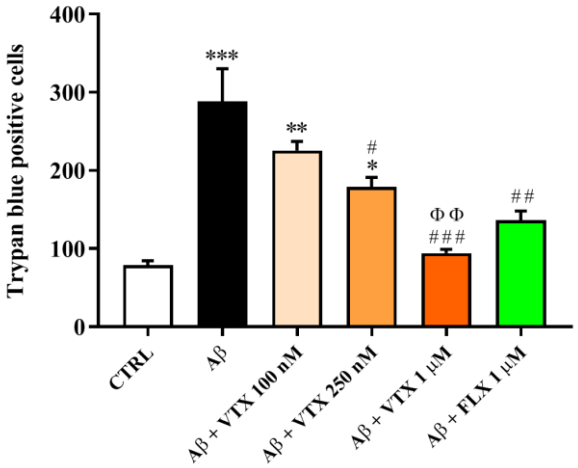
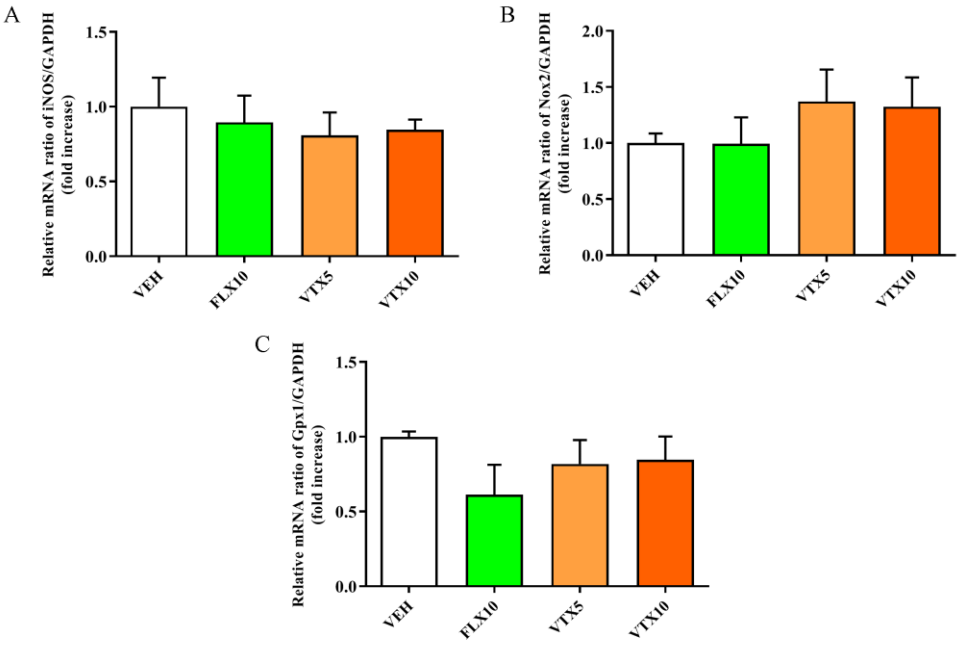
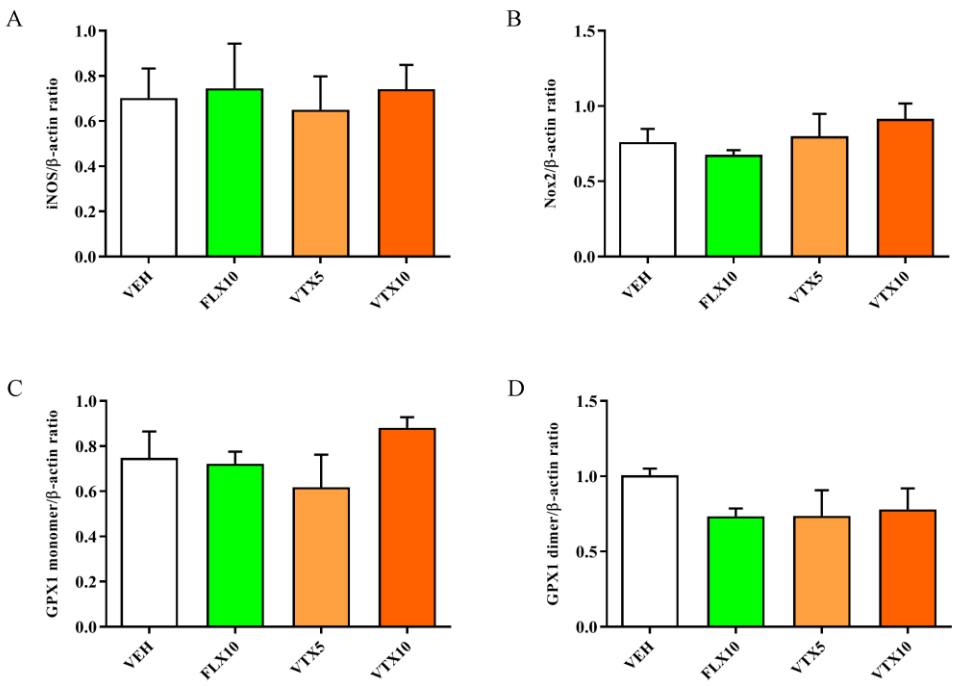


Figure 4.



Supplementary Figure 1.



Supplementary Figure 2.

Figure legends

Fig 1 – **Fluoxetine and vortioxetine decrease the expression of iNOS and Nox2 mRNAs**. Effects induced by i.c.v. administration of Aβ oligomers (Aβ + VEH) in absence or presence of FLX10, VTX5, or VTX10 on A) iNOS and B) Nox2 mRNAs expression measured by qRT-PCR. The abundance of each mRNA of interest was expressed relative to the abundance of GAPDH-mRNA, as an internal control. As a negative control, a reaction in absence of cDNA (no template control, NTC) was performed. qRT-PCR amplifications were performed at least in triplicate (mean of three to six determinations). Data are shown as mean ± S.E.M. $*p < 0.05$ *vs.* PBS + VEH, $^{\#}p < 0.05$ *vs.* Aβ oligomers + VEH, $^{\#\#}p < 0.01$ *vs.* Aβ oligomers + VEH.

Fig 2 – **Fluoxetine and vortioxetine decrease the expression of iNOS and Nox2 proteins.** Effects induced by i.c.v. administration of Aβ oligomers (Aβ + VEH) in absence or presence of FLX10, VTX5, or VTX10 on B) iNOS and D) Nox2 protein levels measured by WB. A) and C) show the representative immunoblots of iNOS (120-125 kDa) and Nox2 (55 kDa), respectively, in total protein extracts from hippocampal tissue. Histograms refer to the means ± S.E.M. of the densitometric values of iNOS or Nox2 bands normalized against β-actin. Each experiment was repeated three times. $*p < 0.05$ *vs.* PBS + VEH, $^{\#}p < 0.05$ *vs.* Aβ oligomers + VEH.

Fig 3 – **The treatment with fluoxetine or vortioxetine is needed to rescue the expression of glutathione peroxidase 1 at gene but not at protein level**. Effects induced by i.c.v. administration of Aβ oligomers (Aβ + VEH) in absence or presence of FLX10, VTX5, or VTX10 on A) Gpx1 mRNA expression measured by qRT-PCR; B) monomer and D) dimer of Gpx1 protein levels measured by WB. C) and E) show the representative immunoblots of Gpx1 monomer (22 kDa) and Gpx1 dimer (44 kDa) in total protein extracts from hippocampal tissue. In the case of gene expression measurements, the abundance of Gpx1 mRNA was expressed relative to the abundance of GAPDH-mRNA, as an internal control. As a negative control, a reaction in absence of cDNA (no template control, NTC) was performed. qRT-PCR amplifications were performed at least in triplicate (mean of three to five determinations). In the case of protein expression measurements, histograms refer to the means ± S.E.M. of the densitometric values of Gpx1 monomer or Gpx1 dimer bands normalized against β-actin. Each experiment was repeated three times. $*p < 0.05$ *vs.* PBS + VEH, $**p < 0.01$ *vs.* PBS + VEH, $^{\#}p < 0.05$ *vs.* Aβ oligomers + VEH, $^{\#\#\#}p < 0.001$ *vs.* Aβ oligomers + VEH.

Fig 4 – **Fluoxetine and vortioxetine exert neuroprotection against the toxicity induced by Aβ oligomers.** Primary mixed neuronal cultures were treated with Aβ oligomers (2 μM) for 48 h both in absence or presence of FLX (1 μM) or increasing concentrations of VTX (100 nM, 250 nM, and 1 μM) (pre-treatment of 1 h). The toxicity of Aβ oligomers in mixed neuronal cultures was assessed by cell counting after trypan blue staining. Cell counts was performed in three random microscopic fields/well. Data are the mean of 3 to 4 determinations. S.D. are represented by vertical bars. $*p <$

0.05 *vs.* CTRL, ***p* < 0.01 *vs.* CTRL, ****p* < 0.001 *vs.* CTRL, $^{\#}$p < 0.05 *vs.* Aβ, $^{\#\#}$p < 0.01 *vs.* Aβ, $^{\#\#\#}$*p* < 0.001 *vs.* Aβ, $^{\theta\theta}$p < 0.01 *vs.* VTX 100 nM.

Table 1. List of primers used for quantitative real-time PCR (qRT-PCR).

Official name[#]	Official symbol	Alternative titles/symbols	Detected transcript	Amplicon Length	Cat. No.[§]
nitric oxide synthase 2, inducible	Nos2	iNOS; Nos-2; Nos2a; i-NOS; NOS-II; MAC-NOS	NM_010927	118 bp	QT00100275
cytochrome b-245, beta polypeptide	Cybb	Cgd; Cyd; Nox2; C88302; gp91-1; gp91phox; CGD91-phox	NM_007807 XM_006527565	146 bp	QT00139797
glutathione peroxidase 1	Gpx1	Gpx; CGPx; GPx-1; GSHPx-1; AI195024; AL033363	NM_008160	133 bp	QT01195936
glyceraldehyde-3-phosphate dehydrogenase	Gapdh	Gapd	NM_008084 XM_001003314 XM_990238 NM_001289726	144 bp	QT01658692

[#]https://www.ncbi.nlm.nih.gov/gene/

[§]https://www.qiagen.com/it/shop/pcr/real-time-pcr-enzymes-and-kits/two-step-qrt-pcr/quantitect-primer-assays/

Chapter 4.

Carnosine Prevents Aβ-Induced Oxidative Stress and Inflammation in Microglial Cells: A Key Role of TGF-β1

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Abstract

Carnosine (β-alanyl-L-histidine), a dipeptide, is an endogenous antioxidant widely distributed in excitable tissues like muscles and the brain. Carnosine is involved in cellular defense mechanisms against oxidative stress, including the inhibition of amyloid-beta (Aβ) aggregation and the scavenging of reactive species. Microglia play a central role in the pathogenesis of Alzheimer's disease, promoting neuroinflammation through the secretion of inflammatory mediators and free radicals. However, the effects of carnosine on microglial cells and neuroinflammation are not well understood. In the present work, carnosine was tested for its ability to protect BV-2 microglial cells against oligomeric Aβ1-42-induced oxidative stress and inflammation. Carnosine prevented cell death in BV-2 cells challenged with Aβ oligomers through multiple mechanisms. Specifically, carnosine lowered the oxidative stress by decreasing NO and $O2^-$ intracellular levels as well as the expression of iNOS and Nox enzymes. Carnosine also decreased the secretion of pro-inflammatory cytokines such as IL-1β, simultaneously rescuing IL-10 levels and increasing the expression and the release of TGF-β1. Carnosine also prevented Aβ-induced neurodegeneration in mixed neuronal cultures challenged with Aβ oligomers, and these neuroprotective effects were completely abolished by SB431542, a selective inhibitor of the type-1 TGF-β receptor. Our data suggest a multimodal mechanism of action of carnosine underlying its protective effects on microglial cells against Aβ toxicity with a key role of TGF-β1 in mediating these protective effects.

Keywords: carnosine; microglia; Alzheimer's disease; neurodegeneration; neuroinflammation; reactive oxygen and nitrogen species; oxidative stress; TGF-β1

* *Published in Cells 2019, 8, 64.*

1. Introduction

Carnosine (β-alanyl-L-histidine) is a natural dipeptide widely distributed in mammalian tissues [1,2] and exists at particularly high concentrations (millimolar order) in the brain as well as in skeletal and cardiac muscles (up to 20 mM). Carnosine has been shown to be neuroprotective through different mechanisms: the prevention of oxidative stress [3], reduction of intraneuronal amyloid-βeta (Aβ) accumulation, mitochondrial dysfunctions and cognitive deficits in 3xTg-AD mice [4], as well as the inhibition of Aβ aggregation [5], modulation of macrophage nitric oxide (NO) production and pro-/anti-inflammatory (M1/M2) ratio [6]. Furthermore, it is also able to scavenge the superoxide ion (O2-) [7] and other reactive species [8]. Aβ is a 42 amino acids long (Aβ 1-42) peptide physiologically present in the brain and cerebrospinal fluid of human beings [9]. Together with marked inflammation [10], both the extracellular deposition of insoluble aggregates of this peptide in the brain and its blood vessels [11,12] and the formation of neurofibrillary tangles composed by the highly phosphorylated form of tau protein [13] represent the neuropathological hallmarks of Alzheimer's disease (AD). Aβ

peptide can undergo aggregation through a step-by-step process, starting with soluble monomers and evolving to the formation of oligomers, protofibrils, and mature fibrils [14], with the oligomeric structures representing the more toxic species [15]. Microglia are a subtype of brain glial cells and constitute up to 10% of all cells in the healthy human cortex [16]. These cells are in intimate contact with neurons and are involved in many basic physiological processes [17]. Microglial cells, often found near Aβ plaques in AD patients [18], are able to produce various neurotrophic and anti-inflammatory factors essential for cell growth and protection; they can also release different cytotoxic substances, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), and pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α [17]. All the above substances are strongly connected to AD pathogenesis and amyloid-related neurodegeneration [19]. NO (RNS species) and O2- (ROS species) are part of the natural aerobic metabolism of cells and are involved in many physiological and pathological processes [20,21]. Peroxynitrite, the reaction product between NO and O2-, can react and consequently damage fatty acids, proteins, DNA, and mitochondria leading to oxidative and nitrosative stress [22], inflammation [23], and then neurodegenerative phenomena [24,25]. During the inflammation process, both inducible nitric oxide synthase (iNOS), responsible for NO production [26,27], and NADPH oxidase (Nox), responsible for O2- production [28], are overactivated in immune cells such as macrophage and microglia [20,29,30]. When simultaneously activated, iNOS and Nox act synergistically to promote neuronal death through the generation of peroxynitrite, which is a more dangerous species if compared with NO and/or O2- [31]. Mediators of cytotoxicity released from activated microglia also include arachidonic acid, glutamate, and histamine [32]. Furthermore, it has been shown that macrophages and microglia play a crucial role in several diseases characterized by oxidative stress and inflammation and also that the modulation of their pro-/anti-inflammatory (M1/M2) ratio and secretion products might represent a novel pharmacological approach for the treatment of these disorders [33,34]. Neuroinflammation is a widely accepted factor associated with the pathogenesis of AD [35]. Different cell types, including microglia, shift from the resting to activated state during the neuroinflammation process [36], producing a higher amount of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [19]. Activated microglia have been shown to contribute to the development and progression of neurodegenerative disorders [37] and the presence of both reactive microglia and astrocytes has been observed in association with amyloid accumulation in AD brain [38]. However, activated microglia are also able to produce anti-inflammatory mediators [39]. Among these molecules, the multifunctional cytokine TGF-β1 has been shown to play a pivotal role in AD, exerting neuroprotective effects against Aβ-induced neurodegeneration [40,41]. Furthermore, the secretion of TGF-β1 from peripheral blood mononuclear cells in the circulation [42] along with the levels of TGF-β1 in the plasma [43] are reduced in AD

subjects. Lastly,Wyss-Coray et al. showed that TGF-β1 promoted microglial Aβ clearance and the reduction of plaque burden in AD mice and enhanced Aβ clearance by BV-2 microglial cells [44], suggesting a link between microglia activity, TGF-β1 release, and the neuroprotective activity of this neurotrophic factor against Aβ-induced toxicity. In the present study, we first investigated the toxicity and the production of NO and O2- induced by different concentrations of Aβ1-42 oligomers, in the absence or in the presence of carnosine, in BV-2 cells, an established experimental model for mimicking neuroinflammation in primary microglia [45]. Additionally, in order to understand the molecular mechanisms underlying the ability of carnosine in decreasing the production of molecules related to oxidative and nitrosative stresses, we studied the expression of iNOS and Nox enzymes along with the expression and secretion of pro- and anti-inflammatory cytokines in BV-2 cells challenged with Aβ1-42 oligomers. Lastly, the protective activity of carnosine, as well as the role played by TGF-β1 in preventing Aβ-induced neuronal death, was evaluated in mixed neuronal cultures. The evidence that carnosine exerts protective effects by decreasing Aβ1-42-induced toxicity in microglial cells, counteracting the oxidative stress and the inflammation status, is presented.

2. Material and Methods

2.1. Materials and Reagents

Microglial BV-2 cells (ICLC ATL03001) were purchased from Interlab Cell Line Collection (ICLC, Genova, Italy). HFIP-treated amyloid _-peptide (1-42) was obtained from Bachem Distribution Services GmbH (Weil am Rhein, Germany). DMEM/F12 (1:1) medium, RPMI-1640 medium, phenol red-free RPMI-1640 medium, trypsin-EDTA solution (0.25% Trypsin/0.53 mM EDTA in HBSS without calcium or magnesium), fetal bovine serum (FBS), and penicillin–streptomycin antibiotic solution were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). L-Carnosine, anhydrous dimethyl sulfoxide (DMSO), trypan blue solution, glucose, cytosine-D-arabinoside, sodium dodecyl sulfate (SDS), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium salt, and phosphate-buffered saline (PBS) were all supplied by Sigma Aldrich (St. Louis, MO, USA). Agilent DNA 1000 Kit was obtained from Agilent (Santa Clara, CA, USA). The 4-amino-5-methylamino-2’,7’-difluorofluorescein diacetate (DAF-FM DA) and MitoSOX Red probes were purchased from Life Technologies (Carlsbad, CA, USA). Platinum Taq DNA Polymerase, SuperScript™ II Reverse Transcriptase, SuperScript III First-Strand Synthesis SuperMix, dNTP Set, TE buffer, GlutaMAX Supplement, 25 and 75 mL polystyrene culture flasks, 12-, 48-, and 96-well plates, ethanol (95%), sodium hydroxide, boric acid, hydrochloric acid, horse serum, fetal calf serum (FCS), and C-Chip disposable hemocytometers were obtained from Thermo

Fisher Scientific (Thermo Fisher Waltham, MA, USA). QuantiTect SYBR Green PCR Kits, RNeasy Mini Kit, QuantiTect Primer Assays, and Custom Multi-Analyte ELISArray Kit were purchased from Qiagen (Hilden, Germany). Eppendorf LoBind 1.5 ml Microcentrifuge Tubes PCR Clean and PCR tubes were both supplied by Eppendorf (Hamburg, Germany). Polyethersulfone membrane (3 kDa) centrifuge filters were purchased from VWR International (West Chester, PA, USA). The specific inhibitor of type1 TGF-_1 receptor 4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1Himidazol-2-yl]benzamide (SB431542) was obtained from the R&D system (Minneapolis, MN, USA). Polydimethylsiloxane (PDMS) microdevices were prepared from a Sylgard 184 elastomer kit (Ellsworth Adhesives, Germantown, WI, USA). Highest Grade Mica Sheets V1 were purchased from Ted Pella Inc (Redding, CA, USA). All water used was ultrapure (18.3 MW cm) (Milli-Q Synthesis A10, Millipore, Burlington, MA, USA).

2.2. Preparation of Aβ 1-42 Oligomers

Aβ 1-42 oligomers (oAβ1-42) were prepared as previously described in details elsewhere [46]. In brief, the lyophilized HFIP-treated Aβ1-42 monomers were first suspended in DMSO and then diluted in an ice-cold cell culture medium DMEM/F12 (1:1) at the final concentration of 100 µM. Next, the Aβ1-42 samples (100 µM) were incubated in the absence (oAβ1-42) or presence (Aβ1-42 + Car (co-inc.)) of carnosine at the final concentration of 1 mM for 72 h at 4 °C. After this incubation step, the two (without or with carnosine) Aβ1-42 samples were immediately used or aliquoted and stored at -20 °C until use.

2.3. Atomic Force Microscope (AFM)

Amyloid oligomer formation was verified by AFM (Supplementary Figure S1A). AFM images were collected by using dynamic scanning force microscopy in the air, using a WITec ALPHA300 RS Confocal Raman AFM combined microscope (LOT-QuantumDesign GmbH, Darmstadt, Germany) and etched-silicon probes (Nanosensors, Neuchâtel, Switzerland) with a pyramidal-shaped tip having a radius of curvature <10 nm and a nominal internal angle of 35°. A total of 5 µL of each individual Aβ1-42 sample (oAβ1-42 or Aβ1-42 + Car (co-inc.)) were adsorbed onto the mica and analyzed directly by sensing the adsorbed material with a microfabricated silicon tip attached to a sensitive cantilever. The resulting relief map was subsequently converted into a visual image.

2.4. Cell Culture and Preparation

2.4.1. BV-2 Cells

BV-2 cells were cultured in an RPMI-1640 medium enriched with heat-inactivated FBS (10% v/v), L-glutamine (2 mM), streptomycin (0.3 mg mL-1), and penicillin (50 IU mL-1). The cells were cultured in 75 mL polystyrene culture flasks at a density of 5x106 cells/flask, maintained in a humidified environment at 37 °C and 5% CO2/95% air atmosphere, and passaged every 3–5 days depending on the cell confluence in order to avoid cell overgrowth. The day prior to treatment, cells were harvested using a 2.5 mL of trypsin-EDTA solution, counted with a C-Chip disposable hemocytometer, and seeded in 5 mL culture flasks, 12-, or 48-well plates at the appropriate density. Prior to the beginning of each experiment, the exact number of live BV-2 cells necessary for cell seeding was determined by using the trypan blue exclusion assay. For each cell count, 50 µL of cell suspension was diluted 1:2 to 1:5 (based on cell density) with a 0.4% trypan blue solution.

2.4.2. Mixed Neuronal Cultures

Mixed neuronal cultures were obtained from rats at embryonic day 15 (Harlan Laboratories, Italy) as previously described [46,47]. Cells were grown into DMEM/F12 (1:1) and enriched with 10% horse serum, 10% FCS, 2 mM glutamine, and 6 mg/ml glucose. After 7–10 days in vitro, to avoid the proliferation of non-neuronal elements, cytosine-D-arabinoside (at the final concentration of 10 µM) was added to the cultures for 3 days. Cells were then moved into a maintenance serum-free medium. As soon as the right confluence was reached, cells were treated with Aβ oligomers (2 µM) for 48 h both in the presence or in the absence of increasing concentrations of carnosine (1, 5, and 10 mM). The possible neuroprotective activity against Aβ1-42-induced toxicity played by TGF-β1 was indirectly investigated by using the specific inhibitor of type-1 TGF-β receptor, SB431542, at 10 µM as previously accomplished [46].

2.5. Measurement of Cell Viability and Cell Death by the MTT and Trypan Blue Exclusion Assays

The effect on the BV-2 cells viability of the treatment with different concentrations (1, 5, and 10 µM) of Aβ1-42 oligomers for 24 h as well as the possible protective effects of carnosine in counteracting Aβ1-42-induced toxicity (Aβ1-42 + Car (co-inc.) or BV-2 cells simultaneously treated with already formed Aβ1-42 oligomers and carnosine (oAβ1-42 + Car (co-treat.)) were measured through the MTT assay as previously reported [48,49]. Briefly, BV-2 cells were seeded in 48-well plates at the density of 1.5 x 10^5 cells/well. A total of 24 h after cell treatment the medium from each well was removed and the MTT solution (1 mg/mL in RPMI-1640 medium) was added. Following 2 h of incubation at 37 °C and 5% CO2/95% air atmosphere, the MTT solution was removed and the formed crystals were dissolved with DMSO. Lastly, 200 µL of each well were transferred to a 96-well plate and the absorbance at 569 nm was read using a plate reader (Spectra Max M5, Molecular Devices, Sunnyvale,

CA, USA). Resting (untreated) cells were used as controls. The toxicity induced in mixed neuronal cultures 48 h after Aβ1-42 oligomers treatment was quantitatively assessed by trypan blue exclusion assay [46,47]. Cell counts were performed in three to four random microscopic fields/well.

2.6. NO and O2- Production Determination Using DAF-FM DA and MitoSOX Red Probes

The experiments carried out to investigate the production of NO and O2- were performed as described previously [50] with slight modifications. BV-2 cells previously seeded in 5 mL culture flasks (5x106 cells) were treated for 24 h. At the end of the cell treatment, in order to analyze intracellular NO and O2- production, the cells were washed three times with cold PBS (0.01 M, pH 7.4) and then incubated with a phenol red free RPMI-1640 medium containing DAF-FM DA or MitoSOX Red probes previously prepared in 99% sterile DMSO for 1 h. During the incubation time, each flask was covered with aluminum foil to minimize any photobleaching of the probes. Next, the BV-2 cells were harvested, counted, and centrifuged (1137 x g for 4 min). The obtained cell pellet was washed three times with cold PBS (0.01 M, pH 7.4), lysed using 50 µL of pure ethanol, centrifuged (18.690 x g for 10 min), and filtered with a polyethersulfone) membrane (3 kDa) centrifuge filter. Then 10 µL of each filtered cell lysate was added to a 90 µL solution consisting of 10 mM boric acid and 7.5 mM SDS at pH 9.2 and transferred to a 96-well plate where the fluorescence was read using a plate reader (Spectra Max M5). Resting cells were used as controls. In order to detect the real fluorescence due to the reaction between the probes (DAF-FM DA or MitoSOX Red) and the molecules of interest (NO or O2-), and to discriminate our compounds from (if any) other fluorescent side products, at least one sample for each experimental condition was run using microchip electrophoresis with laser-induced fluorescence (ME-LIF). The fabrication of PDMS microdevices [51,52], as well as the experimental conditions (sample injection, separation, and detection), data acquisition, and data analysis employed to carry out the ME-LIF experiments, have been described previously [6]. Briefly, a 4” diameter silicon wafer was coated with SU-8 10 negative photoresist to a thickness of 15 mm with a Cee 100 spincoater (Brewer Science Inc., Rolla, MO, USA). The obtained wafer was soft baked in two steps (65 °C for 2 min and 95 °C for 5 min) using a programmable hotplate (Thermo Scientific, Asheville, NC, USA). Microchip designs were drawn with AutoCAD (Autodesk Inc., San Rafael, CA, USA) and printed onto a transparency film (Infinite Graphics Inc., Minneapolis MN, USA). The coated wafer was covered with a transparency film mask and exposed to UV light (ABM Inc., San Jose, CA, USA). The wafer was then post-baked in two steps (65 °C for 2 min and 95 °C for 10 min). After the post-bake, the wafer was developed in SU-8 developer, rinsed, and dried. Lastly, the wafer underwent a hard bake at 180–200 °C for 2 h. The final silicon master contained 15 mm thick and 40 mm wide microchannels. In order to complete the final hybrid PDMS-glass microchip device, the PDMS layer

was sealed to a borofloat glass plate. Prior to each cell lysate analysis, the PDMS-glass device was flushed with NaOH (0.1 M for 5 min) and a running buffer (10 mM boric acid, 7.5 mM SDS at pH 9.2 for 5 min). Each separation was performed using a 30 kV high voltage power supply (Ultravolt, Ronkonkoma, NY, USA). A total of +2400 V and +2200 V were applied to the running buffer reservoir and sampling reservoir, respectively. The sample was introduced into the separation channel using a 1-s gated injection. To avoid the presence of any residual sample on the channels, the system was flushed for 60 s with a running buffer after each sample analysis. Excitation, detection, data acquisition, and data analysis were carried out using the same technologies and programs already described [6]. A schematic representation of the different steps of the chip manufacturing process, the various components needed for ME-LIF experiments, as well as a representative electropherogram, obtained running a cell sample lysate for NO and O2- detection, are shown in Supplementary Figure S2.

2.7. Gene Expression Analysis by Quantitative Real-Time PCR (qRT-PCR)

The total RNA was extracted using the commercial RNeasy Mini Kit according to the manufacturer's recommendations. The concentration of total RNA recovered from 3.5 x 10^5 cells (previously seeded in 12-well plates) treated for 6 h was determined by measuring the absorbance at 260 nm with a Varioskan®Flash spectrophotometer (Thermo Fisher Scientific,Waltham, MA, USA). Reverse transcription was performed using 100 ng of total RNA, RNaseH reverse transcriptase, and random primer hexamers (Superscript II, Thermo Fisher Scientific). Next, each sample was quantified, diluted to a final concentration of 25 ng/µL, and used for qRT-PCR analysis (LightCycler®480 System, Roche Molecular Systems, Inc., Pleasanton, CA, USA). The QuantiTect Primer Assays (Qiagen) employed for the gene expression analysis along with the official name, official symbol, alternative titles/symbols, detected transcript, amplicon length, and primers catalog number are shown in Table1. qRT-PCR amplifications were performed in quadruplicate using a mixture of SYBR Green PC Master Mix (Thermo Fisher Scientific), cDNA samples (100 ng), and specific primers (total reaction volume of 10 µL). Amplification conditions included a first cycle at 95 °C (10 min) followed by 50 cycles at 95 °C (10 seconds), and a final cycle at 60 °C (30 seconds). As a negative control, a reaction in the absence of cDNA (no template control, NTC) was performed and verified by using an Agilent Bioanalyzer 2100 with Agilent DNA 1000 Kit. The relative RNA expression level for each sample was calculated using the 2-DDCT method [53,54] by comparing the threshold cycle value of the gene of interest to the CT value of our selected internal control (GAPDH gene).

2.8. Cytokine Secretion

Cytokines quantification in cell culture supernatants was carried out by using a Custom Multi-Analyte ELISArray Kit according to the manufacturer's instructions. Briefly, BV-2 cells previously seeded in 48-well plates at the density of 1.5 x 10^5 cells/well were treated for 24 h and the supernatant from each well was collected, centrifuged at 1000 x g for 10 min in order to remove any particulate material, and assayed immediately or stored at -80 °C. A total of 50 µL of assay buffer and 50 µL of samples or control samples were added into the appropriate wells of the ELISArray plate and incubated for 2 h at room temperature (RT). After washing 3 times with Wash Buffer, 100 µL of Detection Antibody Solution was added to each well pursued by the following steps: 1 h incubation, 3 washes, the addition of 100 µL Avidin-HRP Conjugate, 30 min incubation at RT, 4 washes, the addition of a 100 µL of Development Solution, and 15 min incubation at RT under the dark. As a final step, 100 µL of Stop Solution was added to each well and the absorbance at 450 nm was read using a Synergy H1 Hybrid Multi-Mode Microplate Reader (Biotek, Shoreline, WA, USA) within 30 minutes of stopping the reaction. As suggested by the vendor, in order to detect the real absorbance, wavelength correction was applied, subtracting the readings at 570 nm from the reading at 450 nm.

2.9. Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad software, San Diego, CA, USA). The within-group comparison was performed by the one-way analysis of variance (ANOVA). The post hoc Tukey test was used for multiple comparisons.

2.10. Study Approval

The study in mixed neuronal cultures was authorized by the Institutional Animal Care and Use Committee (IACUC) of the University of Catania (OPBA Project #169/2015). Animal care followed Italian (D.M.116192) and EEC (O.J. of E.C. L 358/1 12/18/1986) regulations on the protection of animals used for experimental and scientific purposes.

3. Results

3.1. Carnosine Protects BV-2 Cells Against Aβ1-42 Oligomers-Induced Cell Death

The first aim of the present study was to evaluate the toxicity induced by increasing concentrations of Aβ1-42 oligomers (oAβ1-42) (1, 5, and 10 µM) on microglial BV-2 cells. Data illustrated in Figure 1 show that the treatment of BV-2 cells for 24 h with increasing concentrations of oAβ1-42 provoked a dose-dependent decrease in cell viability.

Unlike 1 μM oAβ1-42 (−3% in cell viability, not significant), the treatment with 5 μM oAβ1-42 led to a significant toxic effect (−19% in cell viability, $p < 0.01$ compared to the resting cells). As expected, the stronger decrease in cell viability (−36%, $p < 0.001$ compared to the resting cells) was observed after the treatment with 10 μM oAβ1-42. In order to examine the protective effects of carnosine, BV-2 cells were treated simultaneously with oAβ1-42 and carnosine (oAβ1-42 + Car (co-treat.)). Figure 1 clearly shows that the BV-2 cells' viability significantly increased in the presence of carnosine when compared to treatment with the increasing concentrations of oAβ1-42 (1, 5, and 10 μM). A maximal protective effect was observed for cells treated simultaneously with oAβ1-42 10 μM and carnosine (+18% in cell viability, $p < 0.001$ compared to the corresponding treatment with no carnosine). As a part of our toxicity studies, we also challenged BV-2 cells with a solution consisting of Aβ1-42 monomers previously incubated with carnosine during the oligomerization process (Aβ1-42 + Car (co-inc.)). This set of experiments was purposely designed in order to determine whether the well-know anti-aggregation property of carnosine contributed to increasing the cell viability counteracting oAβ1-42 formation and then preventing Aβ toxicity. Considering the presence of carnosine during the oligomerization process, both treatments 5 μM Aβ1-42 + Car (co-inc.) and 10 μM Aβ1-42 + Car (co-inc.) showed cell viability values significantly higher (+15%, $p < 0.01$ and + 29%, $p < 0.001$, respectively) compared to the corresponding treatment with no carnosine.

3.2. Carnosine Decreases Aβ1-42-Induced NO Production in Cultured Microglial Cells

Figure 2 shows the effect of Aβ1-42 treatment on the intracellular NO production in BV-2 cells. This increase in NO production was significant in the case of both 5 μM oAβ1-42 (+19%, $p < 0.001$ compared to resting cells) and 10 μM oAβ1-42 (+60%, $p < 0.001$ compared to resting cells) treatments. The addition of carnosine to the resting BV-2 cells did not cause any significant change in the basal microglia NO production. To test the effect of carnosine on NO production in stimulated BV-2 cells, carnosine was added along with three different concentrations of oAβ1-42. The amount of NO production was essentially the same for cells treated with 1 μM oAβ1-42, in the presence or absence of carnosine. A slight, but not significant, decrease (−5%) was measured in 5 μM oAβ1-42 + Car (co-treat.) compared to cells stimulated in the absence of carnosine. The production due to the treatment with 10 μM oAβ1-42 was significantly lowered by the presence of carnosine (−29%, $p < 0.001$). As for 1 μM oAβ1-42 + Car (co-treat.), the production of NO for Aβ1-42 1 μM + Car (co-inc.) treatment was comparable to the one detected in the resting cells. The presence of carnosine during the oligomerization process strongly decreased the effect of Aβ1-42 in inducing NO production. In fact, both 5 μM Aβ1-42 + Car (co-inc.) (−14%, $p < 0.05$) and 10 μM Aβ1-42 + Car (co-inc.) (−49%, $p < 0.001$) treatments showed a significant decrease in NO production when

compared with the corresponding treatment in the absence of carnosine. Interestingly, the production of NO for the 10 μM Aβ1-42 + Car (co-inc.) treatment was significantly lower than 10 μM oAβ1-42 + Car (co-treat.) (−20 %, $p < 0.001$).

3.3. Carnosine Decreases Aβ1-42-Induced O2−• Production in Cultured Microglial Cells

Figure 3 depicts the effect of A β1-42 treatment on the intracellular O2−• production in BV-2 cells. As observed in the case of NO production, the stimulation of the cells with increasing concentration of oAβ1-42 caused a dose-dependent increase in O2−• production. This increase was significant in the case of both 5 μM oAβ1-42 (+43%, $p < 0.001$ compared to resting cells) and 10 μM oAβ1-42 (+78%, $p < 0.001$ compared to resting cells) treatments. A slight, but not significant, increase (+9%) compared to resting cells was measured in cells treated with 1 μM oAβ1-42. The addition of carnosine to resting BV-2 cells did not cause any significant change in the basal microglia O2−• production. To test the effect of carnosine on O2−• production in stimulated BV-2 cells, carnosine was added along with three different concentrations of oAβ1-42. The amount of O2−• production was essentially the same for cells treated with 1 μM oAβ1-42, in the presence or absence of carnosine. The co-treatment with carnosine was able to counteract O2−• production in cell stimulated with 5 μM oAβ1-42 (−29%, $p < 0.001$) as well as 10 μM oAβ1-42 (−53%, $p < 0.001$). As for 1 μM oAβ1-42 + Car (co-treat.), the production of O2−• was essentially the same of the controls for Aβ1-42 1 μM + Car (co-inc.). Once again, as previously observed for NO production, the presence of carnosine during the oligomerization process strongly decreased the effects of Aβ1-42 in inducing O2−• production. In fact, both 5 μM Aβ1-42 + Car (co-inc.) (−34%, $p < 0.001$) and 10 μM Aβ1-42 + Car (co-inc.) (−60%, $p < 0.001$) treatments showed a significant decrease in O2−• production when compared with the corresponding treatment in the absence of carnosine. A slight, but not significant, decrease (−9%) was measured for 1 μM Aβ1-42 + Car (co-inc.) compared to the 1 μM oAβ1-42 treatment.

It is also worth underlining that in our experimental model the treatment of BV-2 cells with oAβ1-42 produced more evident effects on O2−• production than those detected for NO production, at all concentrations (1, 5, or 10 μM) tested.

By combining the information obtained from the first three sets of experiments (Figures1–3), we selected the optimal oAβ1-42 concentration (10 μM) able to generate the strongest response in BV-2 cells, then used it, in the absence or presence of carnosine, to analyze mRNA expression and protein secretion.

3.4. Carnosine Decreases Aβ1-42-Induced mRNA Expression Level of iNOS, Nox1, and Nox2 and Increases TGF-β1 mRNA Expression in Cultured Microglial Cells

Since the treatment of BV-2 cells with carnosine decreased the oAβ1-42-induced production of NO and $O2^{-\bullet}$ (Figures 2 and 3, respectively), we assessed the ability of carnosine to modulate the expression of iNOS and Nox subunits as well as the synthesis and the release of several cytokines related to Aβ-induced inflammation in microglial cells. As expected, the expression level of iNOS mRNA was significantly increased (3.45 folds) following oAβ1-42 treatment ($p < 0.001$ compared to resting cells) (Figure 4A).

Both oAβ1-42 + Car (co-treat.) and Aβ1-42 + Car (co-inc.) treatments were able to counteract Aβ1-42-induced iNOS activation ($p < 0.001$ compared to oAβ1-42-treated cells). The strongest inhibitory effect was observed for the Aβ1-42 + Car (co-inc.) treatment (from 3.45 folds to 1.09 folds) compared to the simple co-treatment with carnosine. The addition of carnosine to resting BV-2 cells did not cause any significant change in the expression level of iNOS mRNA. A slight, but not significant, increase (1.07 folds) was measured for Nox1 mRNA following oAβ1-42 treatment, while values lower than the control were observed for oAβ1-42 + Car (co-treat.) (0.91 folds) and Aβ1-42 + Car (co-inc.) (0.87 folds) treatments (Figure 4B). As observed for iNOS mRNA expression, the stimulation of BV-2 cells with oAβ1-42 significantly increased the expression of Nox2 mRNA (1.96 folds, $p < 0.001$) (Figure 4C). Both oA β1-42 + Car (co-treat.) and Aβ1-42 + Car (co-inc.) treatments gave values significantly lower than cells treated with Aβ1-42 in the absence of carnosine ($p < 0.001$ compared to oAβ1-42). Figure 4D shows the effect of Aβ1-42 treatment, alone or in combination with carnosine, on the expression level of TGF-β1 mRNA in BV-2 cells. A slight, but not significant, decrease (0.85 folds) was measured for TGF-β1 mRNA following oAβ1-42 treatment. The co-treatment with carnosine increased the expression level to 1.09 folds ($p < 0.05$ compared to oAβ1-42-treated cells). For Aβ1-42 + Car (co-inc.) treatment the expression of TGF-β1 measured was equal to 1.30 folds ($p < 0.01$ compared to resting cells; $p < 0.001$ compared to oAβ1-42-treated cells). Interestingly, carnosine per se provoked a significant increase (1.85 folds) in the expression level of TGF-β1 mRNA in resting BV-2 cells ($p < 0.001$ compared to all other treatments). At this time point (6 h), the expression level of IL-6 mRNA in BV-2 cells did not significantly change in all the experimental conditions (data not shown).

3.5. Carnosine Modulates the Release of Pro- and Anti-Inflammatory Cytokines in Cultured Microglial Cells

The analysis of cytokines in cell supernatants indicated an up-regulation (+15%) of the pro-inflammatory cytokine IL-1β induced by oAβ1-42 treatment compared to resting cells (Figure 5A).

The presence of carnosine along with Aβ1-42 down-regulated the IL-1β production, with Aβ1-42 + Car (co-inc.) having a stronger effect (–36%, $p < 0.001$ compared to oAβ1-42-treated cells) than that

of oAβ1-42 + Car (co-treat.) (–27%, $p < 0.05$ compared to oAβ1-42-treated cells). The addition of carnosine to resting BV-2 cells did not cause any significant change in the release of IL-1β. The treatment of BV-2 cells with oAβ1-42, alone or in co-treatment with carnosine, did not lead to any significant change in the production of the pro-inflammatory cytokine IL-6 (Figure5B). On the contrary, both A β1-42 + Car (co-inc.) and carnosine alone treatments down-regulated IL-6 production ($p < 0.05$ compared to resting and oAβ1-42-treated cells and $p < 0.05$ compared to resting, respectively). oAβ1-42 treatment up-regulated IFN-γ (+16%) in BV-2 cells (Figure5C). Both treatments with carnosine down-regulated the IFN-γ production compared to the corresponding treatment with no carnosine, with oAβ1-42 + Car (co-treat.) treatment showing a stronger and significant effect (−33%, $p < 0.05$ compared to oAβ1-42-treated cells) than that of Aβ1-42 + Car (co-inc.) (−23%). The treatment with carnosine alone lowered IFN-γ by 10%. Figure5D,E shows the modulation in the release of the two major anti-inflammatory cytokines (IL-10 and TGF-β1) by Aβ1-42 in the absence or presence of carnosine. oAβ1-42 treatment strongly down-regulated IL-10 production (−38%, $p < 0.05$ compared to resting cells). oAβ1-42 + Car (co-treat.) treatment slightly increased the production of IL-10 (+12%) while Aβ1-42 + Car (co-inc.) treatment rescued the IL-10 release to the values detected in resting cells, significantly higher than that of oAβ1-42 alone (+41%, $p < 0.05$) (Figure 5D). As in the case of IL-1 β, the addition of carnosine to resting BV-2 cells did not cause any significant change in the release of IL-10. The level of TGF-β1 was very similar between resting and oAβ1-42-treated BV-2 cells (Figure 5E). The treatment with carnosine along with oAβ1-42 markedly up-regulated TGF-β1 (+40%, $p < 0.001$ compared to resting and oAβ1-42-treated cells) while the Aβ1-42 + Car (co-inc.) treatment led to an increase of TGF-β1 production equal to +66% compared to resting and oAβ1-42-treated cells ($p < 0.001$) and +26% compared to oAβ1-42 + Car (co-treat.) treatment ($p < 0.05$). Once again, as already observed for the expression level of TGF-β1 mRNA, the addition of carnosine per se to resting BV-2 cells strongly up-regulated the release of TGF-β1 (+93%, $p < 0.05$ compared to Aβ1-42 + Car (co-inc.)-treated cells, $p < 0.001$ compared to all other treatments).

3.6. Carnosine Prevents oAβ1-42-Induced Toxicity in Mixed Neuronal Cultures via TGF-β1

Finally, we examined the neuroprotective activity of carnosine in mixed neuronal cultures containing both neurons (35–40%) and glial cells (astrocytes and microglia; 60–65%) challenged with oAβ1-42 (2 μM). We have previously demonstrated that mixed neuronal cultures treated with oAβ1-42 represent an established experimental model of Aβ-induced neurodegeneration, where oAβ1-42 show a faster kinetics compared to pure neuronal cultures, with a substantial increase in the number of dead neurons (about 100%) being detected after 48 h of exposure to Aβ oligomers [55].

Figure 6A clearly shows the neuroprotective effects of carnosine against oA β1-42-induced toxicity. Carnosine decreased the oAβ1-42 toxic effect in mixed neuronal cultures in a dose-dependent manner, with 10 mM carnosine (oAβ1-42 + 10 mM Car) having a stronger protective effect (-55% in cell death, $p < 0.001$ compared to oAβ1-42 and oAβ1-42 + 1 mM Car). The presence of carnosine at the final concentration of 1 or 5 mM reduced oAβ1-42-induced cell death by 34% and 46%, respectively ($p < 0.001$ compared to oAβ1-42-treated cells). As shown in Figure 6B, the specific inhibitor of type-1 TGF-β receptor, SB431542, prevented the neuroprotective activity of carnosine that was directly applied to mixed neuronal cultures challenged with oAβ1-42 ($p < 0.001$ compared to oAβ1-42 + 10 mM Car) suggesting that TGF-β1 release and activation of TGF-β1 signaling play a central role in mediating the neuroprotective effects of carnosine. The percentage of toxicity due to the presence of oAβ1-42 decreased by 53% (47% of cell death) in the presence of carnosine, but it increased again to a value very similar to oAβ1-42 treatment in the presence of SB431542 (96% of cell death). The treatment with SB431542 (10 μM) as well as with carnosine (10 mM) alone had no significant effects per se on neuronal death in mixed neuronal cultures in the absence of oAβ1-42 treatment (Figure 6B, insert).

4. Discussion

Oligomers, the most toxic species of Aβ1-42 aggregated forms, cause neuronal dysfunction and death in AD brains [56]. Microglial cells and neuronal cultures challenged with synthetic analogs of human oligomers of Aβ1-42 provide a widely accepted and reliable model of neuroinflammation and neurodegeneration occurring in AD [47,57,58]. In this scenario, oxidative stress plays a central role in Aβ-induced neurodegeneration [59,60].

Oxidative stress is a process referring to an imbalance between pro-oxidants, such as ROS and RNS, and antioxidants in favor of pro-oxidants. A wide body of literature supports the negative impact and key role played by this phenomenon in the pathogenesis of AD [61], preceding the appearance of the two hallmarks of this disease represented by the abnormal deposition of Aβ (senile plaques) and the intracellular accumulation of hyperphosphorylated tau protein (neurofibrillary tangles) [62]. In particular, on one hand the oligomeric form of Aβ peptide impairs synaptic plasticity, and promotes neurodegeneration and neuroinflammation through oxidative stress [63,64]; on the other hand oxidative stress favors Aβ oligomerization [65].

In the present study, we first explored the toxicity induced by different concentrations (1, 5, and 10 μM) of Aβ1-42 oligomers on BV-2 microglial cells and then examined the correlation between Aβ toxicity and the production of NO and $O2^{-}\bullet$, two well-known reactive species that significantly contribute to neurodegeneration in AD [66,67]. This order of magnitude (μM) is physiologically relevant since from one hand under normal physiological conditions and in AD patients the

concentration of Aβ peptide in brain extracellular fluid is low (pM to nM levels) [68–70]; on the other hand in vitro studies suggest that the critical concentration for spontaneous aggregation (e.g., oligomer formation) of Aβ peptide is in the μM range [71,72]. Accordingly, Aβ concentrations in vivo would have to increase by at least 3 to 4 orders of magnitude (e.g., close to the amyloid plaques) for spontaneous aggregation to be feasible in the extracellular space. When monitoring the change in cell viability, we observed that oAβ1-42 decreased BV-2 cell viability in a dose-dependent manner (Figure1). BV-2 cells were able to counteract amyloid-induced cell toxicity only at low concentrations (1 or 5 μM) while at the highest Aβ concentration (10 μM) the well-known ability of microglia in amyloid clearance [73] was overcome by Aβ toxicity. Figure1also shows the protective effects of carnosine co-treatment in all the conditions tested. We hypothesized that these protective effects could be due to the ability of carnosine in counteracting both oxidative stress and inflammation in microglial cells [52,74]. In fact, as showed by Fleisher-Berkovich et al., carnosine as well as its acetylated form are able to decrease LPS-induced microglial oxidative stress and inflammation [74]. Furthermore, carnosine has been shown to protect neurons against oxidative stress by modulating the MAPK cascade signaling [3]. The neuroprotective effects exerted by carnosine have also been demonstrated by Lopachev et al. by using the primary culture of rat cerebellar cells under oxidative stress [75]. Our hypothesis was also strongly corroborated by the direct measurement of NO and O2−• intracellular levels as well as the expression of iNOS and Nox enzymes in Aβ-stimulated cells. The levels of these reactive species increased in a dose-dependent manner by Aβ1-42 oligomers, whereas they significantly diminished in the presence of carnosine (Figures 2 and 3) in accordance to its antioxidant activity and the ability of this peptide to directly interact with these species, decreasing their availability [6,76]. In particular, part of the observed decreased toxicity could be due to an increased uptake of carnosine by immune cells under stress conditions [77], to the ability of this dipeptide to convert NO into its not-toxic end-product nitrite [6], and/or carnosine capability to disassemble aggregate structures already formed [78,79]. In accordance to the viability and NO and O2−• results, Figure 4A–C shows that the decrease in reactive species depends not only on the scavenging activity of carnosine [80] but also on the ability of this peptide to decrease the expression Aβ-induced enzymes related to oxidative and nitrosative stress.

As a part of our study, instead of co-treating with carnosine and Aβ1-42 oligomers already formed, we challenged BV-2 cells with a solution consisting of Aβ1-42 monomers previously incubated in the presence of carnosine during the oligomerization process (indicated in each figure as Aβ1-42 + Car. (co-inc.)). Our aim was to determine whether the well-known anti-aggregation property of carnosine [81–83] contributed to decreasing the cell toxicity and oxidative stress by preventing the formation of Aβ1-42 toxic species. As expected, carnosine, by inhibiting oligomers formation

(confirmed by AFM analysis (Supplementary Figure S1B), protected microglial cells (Figure1), reduced NO (Figure2) and O 2−• (Figure3) intracellular levels, and inhibited iNOS and Nox up-regulation (Figure 4A–C). These results are in accordance with Corona et al. which showed that carnosine supplementation in 3xTg-AD mice, a transgenic model of AD, led to a strong reduction in the hippocampal intraneuronal accumulation of Aβ, completely rescuing AD and aging-related mitochondrial dysfunctions [4]. It is worth underlining that when considering the different protective effects observed between oAβ1-42 + Car. (co-treat.) and Aβ1-42 + Car. (co-inc.) treatments, the latter gave always slightly stronger effects. The increased protective effect observed with carnosine in microglial cells after the co-incubation of Aβ monomers with a millimolar concentration of carnosine suggests that the anti-aggregation properties of carnosine [5,78,79] significantly contribute to increase the overall protective effects of carnosine against Aβ1-42 toxicity in addition to the antioxidant activity of this peptide. Furthermore, our data obtained with AFM suggest that carnosine might preserve Aβ monomers, which are essential for neuronal survival and maintaining neuronal glucose homeostasis [84,85], and it can also promote the dissociation of Aβ oligomers. Future studies are needed to assess whether carnosine can act as a monomer stabilizer, preventing the transition from Aβ monomers to Aβ oligomers.

Activated microglia and astrocytes are the main source of cytokines in the brain [86], and elevated markers of microglial activation (measured by translocator protein binding in vivo with PET) have been found in AD patients [87]. Aβ oligomers promote neuroinflammation and neurodegeneration in AD brains by eliciting the release of pro-inflammatory cytokines from microglia cells [88].

In the present study, we adopted an experimental model of neuroinflammation, where BV-2 microglial cells were challenged with a micromolar concentration of Aβ oligomers [57,58]. Aβ-induced inflammation in these cells is also known to strictly correlate with oxidative stress and an increase in ROS formation [89,90].

In our experimental model, Aβ oligomers (24 h treatment) significantly reduced the secretion of anti-inflammatory cytokines such as IL-10 (Figure 5D), whereas no statistically significant effect was observed for IL-1β and IFN-γ secretion (Figure 5A,C) as well as for IL-6 and TGF- β1 release (Figure 5B,E). With particular regard to IL-6, it is important to underline that differently from the gene expression analysis (6 h treatment), where the expression level of IL-6 mRNA was not changed by carnosine, cytokine secretion experiments (24 h treatment) demonstrate the ability of carnosine to decrease IL-6 levels. This suggests that under our experimental conditions: i) carnosine may require more than 6 h to directly modulate IL-6 gene expression; ii) carnosine could decrease IL-6 at post-translational level by direct interaction and/or modulating signal transduction pathways connected to its production such as phospholipases C and D [91]. Interestingly carnosine rescued IL-10 levels in

Aβ-treated BV-2 cells and also reduced IL-1β, IL-6, and IFN-γ levels as assessed by the ELISA assay (Figure5). Previous studies conducted in vivo have demonstrated that carnosine reduces both oxidative stress and microglial activation in animal models of subcortical ischemic vascular dementia and subarachnoid hemorrhage model [92–95], but no studies have been yet conducted in animal models of AD to examine the effects of carnosine on microglia activation. As discussed above, it is known from previous in vitro studies that carnosine can prevent LPS-induced microglial inflammation and oxidative damage [74], but our in vitro study is the first evidence that carnosine can counteract in microglial cells both oxidative stress and the release of pro-inflammatory cytokines induced by Aβ oligomers. Furthermore, carnosine showed the ability to rescue IL-10 levels, an anti-inflammatory cytokine whose deficit in AD patients seems to play a key role in promoting neuroinflammation and cognitive deficits [96]. Future studies are needed to establish whether carnosine can exert this effect on IL-10 production in vivo in animal models of AD.

Interestingly we found that carnosine exerted a specific effect on the expression of TGF-β1, the only cytokine whose mRNA levels were significantly affected by carnosine with a relevant increase at 6 h (Figure4D) followed by a strong increase in TGF- β1 release at 24 h (Figure5E). We focused our attention on this anti-inflammatory cytokine because it is well-known that TGF-β1 exerts strong anti-inflammatory and neuroprotective effects in experimental models of AD [97]. It also plays a constitutive role in the suppression of inflammation, controlling the degree of microglial activation in the central nervous system [98] and stimulating Aβ clearance by microglia [99]. It has also been recently demonstrated that microglial activation induced by Aβ1-42 oligomers results in the inhibition of TGF-β-regulated gene expression in primary rat microglia [100]. When considering this evidence it is relevant to note that in our experimental model of Aβ-induced inflammation carnosine was able to promote both the synthesis and the release of TGF-β1 from microglial cells. This effect of carnosine is relevant when taking into account the role of TGF-β1 in the pathophysiology of AD [101]. The selective impairment of the TGF-β1 signaling pathway has been demonstrated in the early phase of AD pathogenesis [102] and this deficit of TGF-β1 contributes to neuroinflammation and cognitive decline in AD [103]. Therefore, the rescue of TGF-β1 signaling represents a new pharmacological strategy to yield neuroprotection in AD and second-generation antidepressants such as fluoxetine increase the release of TGF-β1 from astrocytes and exert relevant neuroprotective effects in experimental models of AD [46].

Starting from this evidence, we examined the neuroprotective effects of carnosine in mixed neuronal cultures challenged with Aβ oligomers, an established an experimental model of Aβ-induced neurodegeneration [46,47]. Interestingly we found that carnosine started to prevent Aβ toxicity at 1 mM, with a further relevant increase of its neuroprotective efficacy at 10 mM (with 55% of neuronal

rescue) (Figure 6A). For our knowledge, this is the first evidence that carnosine can prevent Aβ-induced neuronal death in an in vitro model of Aβ-induced neurodegeneration. The protection observed following carnosine treatment was in part expected since our results (Figures 4D and 5D,E) show that this dipeptide is able to enhance the ability of microglial cells to produce anti- inflammatory mediators (e.g., IL-10 and TGF-β1). According to this scenario, it is expected that the high percentage of glial cells (60–65%) in our co-culture model heavily contribute to neuronal protection. The ability of SB431542, a selective inhibitor of the type-1 TGF-β receptor, to completely prevent the effects of carnosine (Figure 6B) suggests that TGF- β1 release and activation of Smad-dependent TGF-β1 signaling play key roles in mediating the neuroprotective efficacy of carnosine against Aβ toxicity. Future studies should be conducted in transgenic animal models of AD to assess whether carnosine can prevent amyloid-related cognitive deficits by the rescue of TGF-β1 signaling.

5. Conclusions

In the present study, we reported for the first time that carnosine prevents Aβ-induced oxidative stress in BV-2 microglial cells by decreasing the expression of inducible nitric oxide synthase and NADPH oxidase and the concentrations of nitric oxide and superoxide anion. We demonstrated for the first time that, in an established model of Aβ-induced inflammation, carnosine was able to decrease the secretion of pro-inflammatory cytokines such as IL-1β, simultaneously rescuing IL-10 levels and increasing the synthesis and the release of TGF-β1. We then validated the protective activity of carnosine in mixed neuronal cultures challenged with Aβ1-42 oligomers, where carnosine prevented Aβ-induced neurodegenerative phenomena via the activation of TGF-β1 signaling.

The inhibition of Aβ oligomer-mediated inflammation and rescue of TGF-β1 signaling have been recently considered effective strategies for protecting against neurodegeneration and disease progression in AD. Carnosine, through its multimodal mechanism of action, might represent a new pharmacological tool to yield neuroprotection in AD.

Supplementary Materials: Supplementary materials can be found athttp://www.mdpi.com/2073-4409/8/1/ 64/s1. Figure S1: AFM analysis of Aβ1-42 samples, Figure S2: A schematic representation of chip manufacturing process, ME-LIF setup, and representative electropherograms.

Author Contributions: Conceptualization, G.C., G.L. and F.C.; Validation, G.C., C.G.F. and N.M.; Formal Analysis, G.C., C.G.F., N.M., M.G. (Mariaconcetta Giambirtone), M.G. (Margherita Grasso), S.F.S. and S.M.; Investigation, G.C., C.G.F., N.M., M.G. (Mariaconcetta Giambirtone), M.G. (Margherita Grasso), S.F.S. and S.M.; Resources, M.A.S., F.D., S.M.L., G.L. and F.C.; Data

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Funding: Part of this study was supported by the National Science Foundation, grant number CHE-1411993, and National Institutes of Health (NIH), grant number COBRE P20GM103638. GC received support from the American Heart Association (Midwest Affiliate Postdoctoral Research Fellowship), grant number NFP0075515, while FC received support from the Neuropsychopharmacology Research Program 2017, grant number RC-06-05. GC and FC would also like to acknowledge the support received from the Italian Ministry of Health Research Program 2018, grant number RC: 2635256.

Acknowledgments: We would like to thank Dr. Gino Mongelli from BRIT for his valuable technician assistance during qRT-PCR and ELISA experiments and Dr. Vanna Torrisi from BRIT for help with AFM samples analysis.

Conflicts of Interest: The authors declare no conflict of interest.

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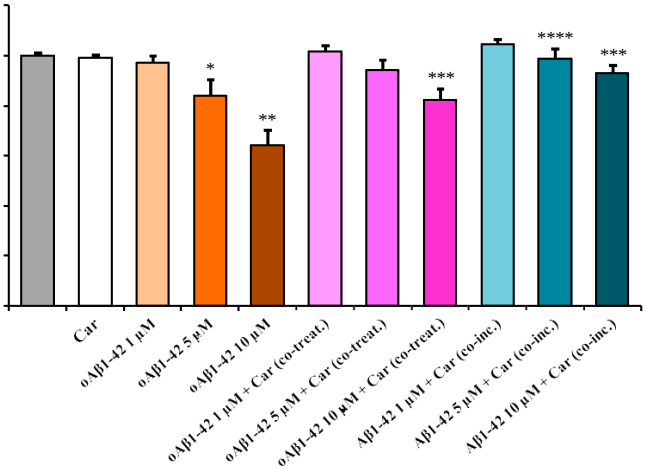

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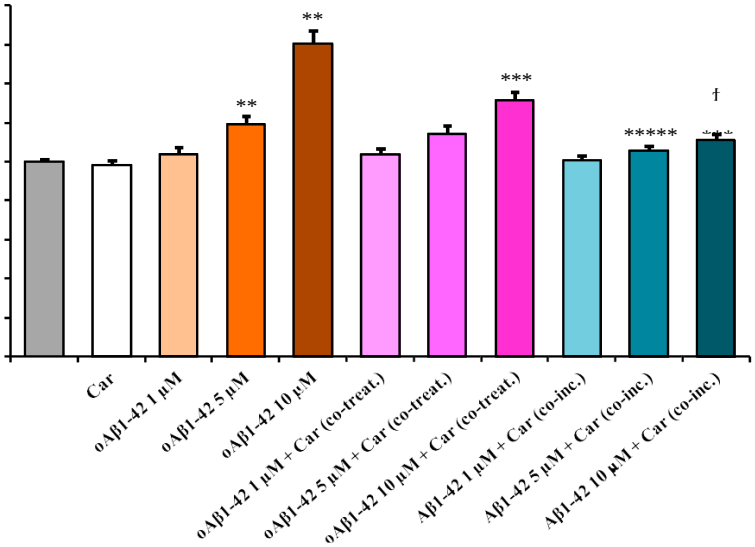

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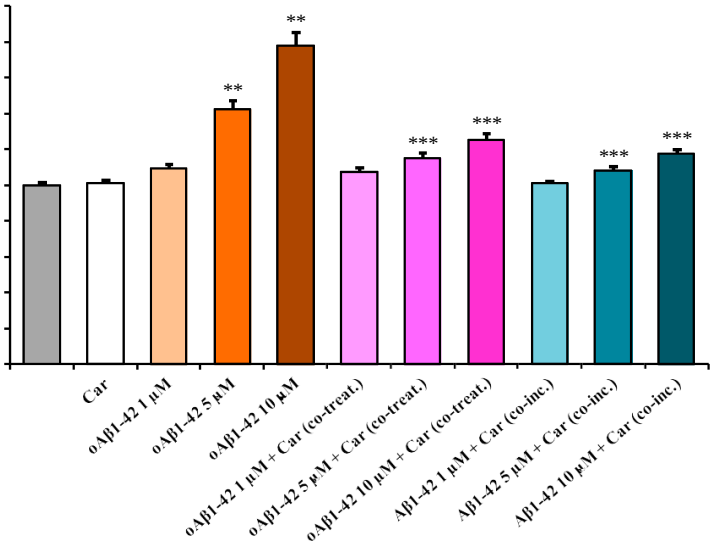
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Car
oAβ1-42 1 µM
oAβ1-42 5 µM
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oAβ1-42 1 µM + Car (co-treat.)
oAβ1-42 5 µM + Car (co-treat.)
oAβ1-42 10 µM + Car (co-treat.)
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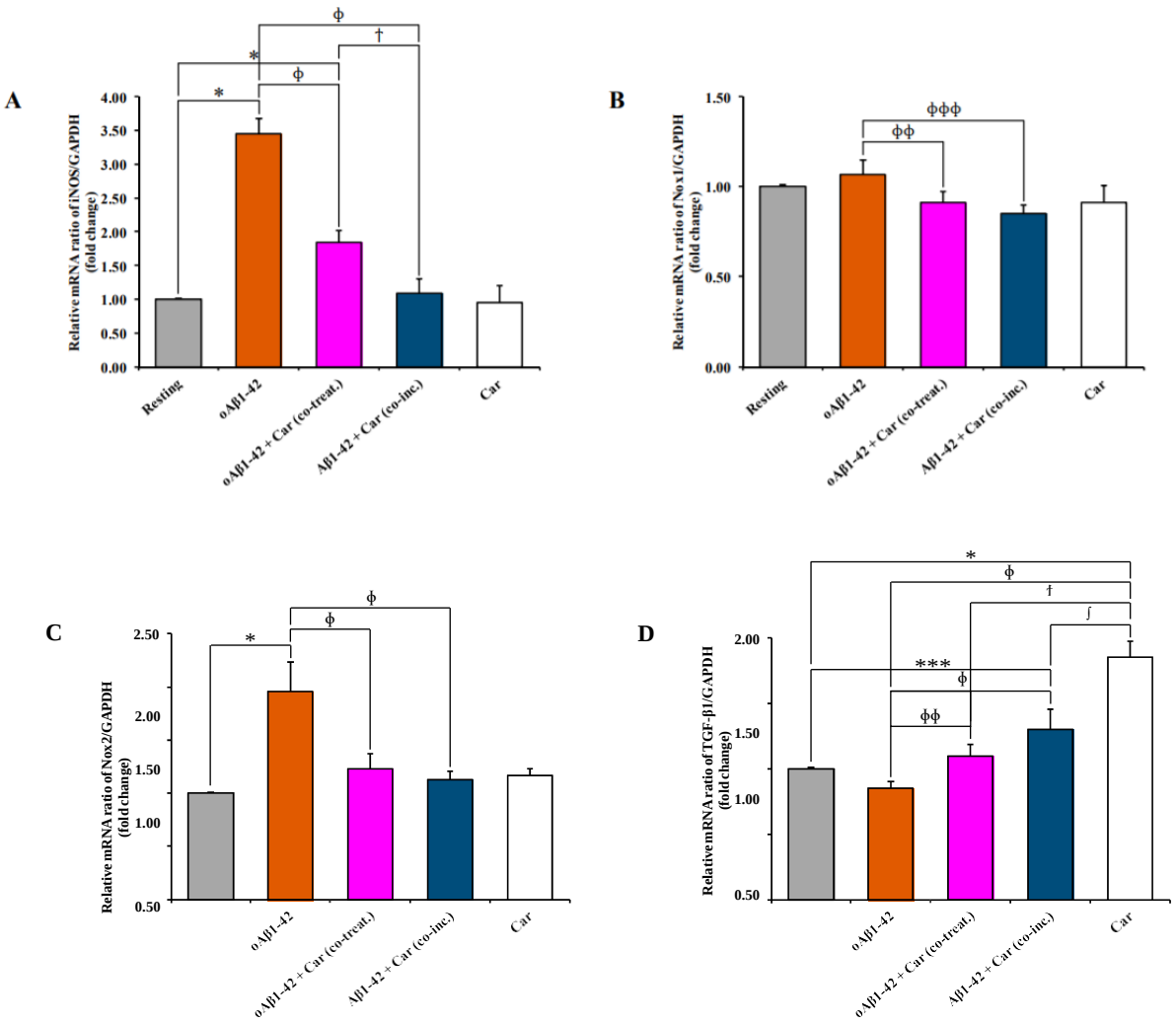
Figure 3.


A
Relative mRNA ratio of iNOS/GAPDH (fold change)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Resting
oAβ1-42
oAβ1-42 + Car (co-treat.)
Aβ1-42 + Car (co-inc.)
Car
B
Relative mRNA ratio of Nox1/GAPDH (fold change)
1.50
1.00
0.50
0.00
C
Relative mRNA ratio of Nox2/GAPDH (fold change)
2.50
2.00
1.50
1.00
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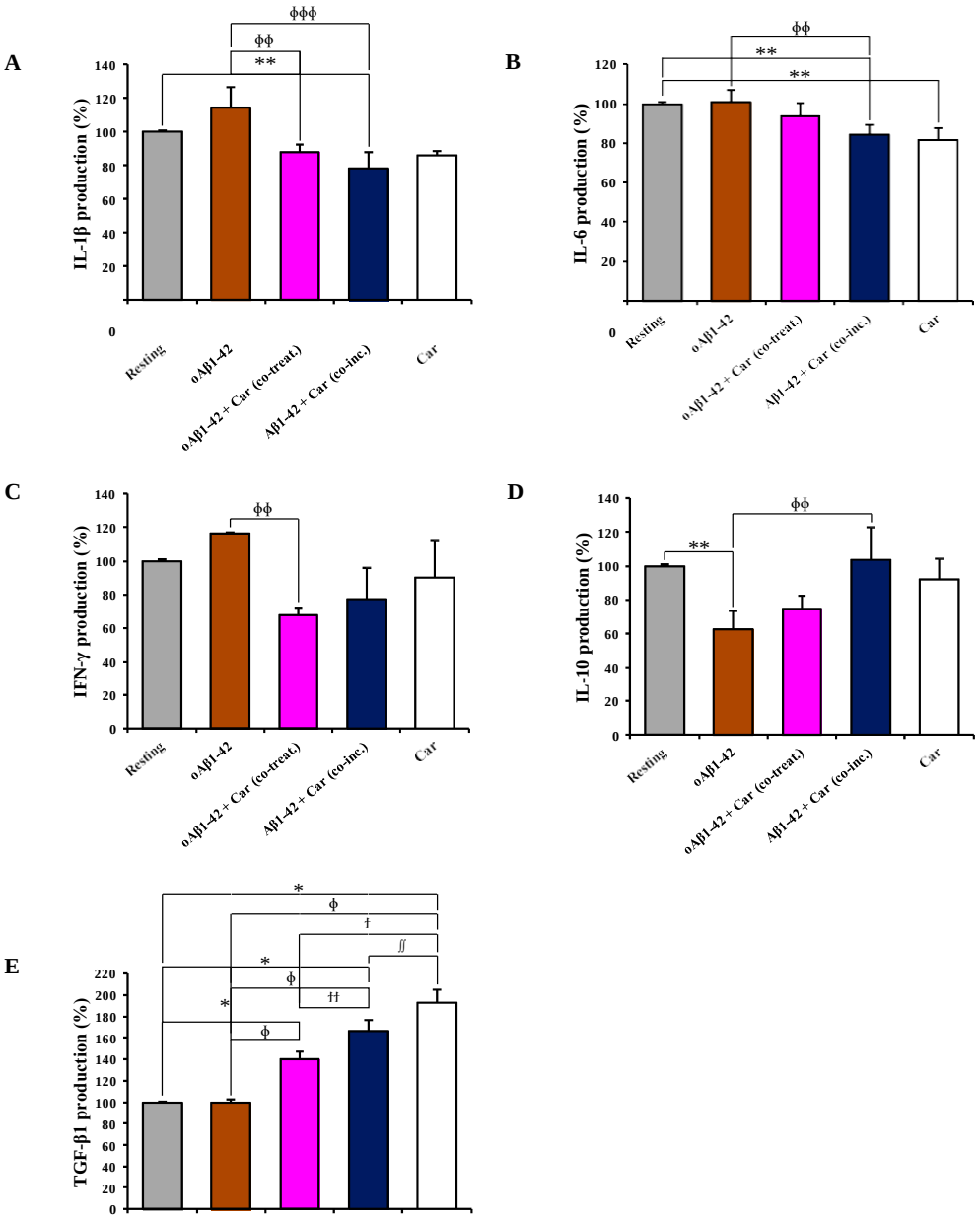
D
Relative mRNA ratio of TGF-β1/GAPDH (fold change)
2.00
1.50
1.00
0.50

Figure 4.


A
IL-1β production (%)
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**
Resting
oAβ1-42
oAβ1-42 + Car (co-treat.)
Aβ1-42 + Car (co-inc.)
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B
IL-6 production (%)
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IFN-γ production (%)
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D
IL-10 production (%)
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Figure 5.

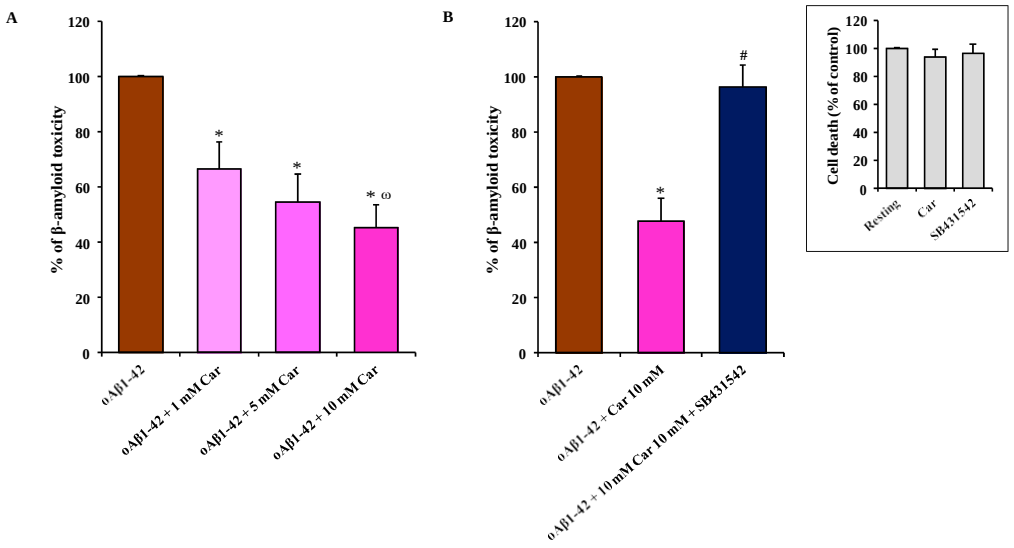

Figure 6.

Table 1. The list of primers used for quantitative real-time PCR (qRT-PCR).

Official Name #	Official Symbol	Alternative Titles/Symbols	Detected Transcript	Amplicon Length	Cat. No. §
nitric oxide synthase 2, inducible	Nos2	iNOS; Nos-2; Nos2a; i-NOS; NOS-II; MAC-NOS	NM_010927	118 bp	QT00100275
NADPH oxidase 1	Nox1	MOX1; NOH1; NOH-1; NOX1a; Nox-1; GP91-2; NOX1alpha	NM_172203 XM_006528515	180 bp	QT00140091
cytochrome b-245, beta polypeptide	Cybb	Cgd; Cyd; Nox2; C88302; gp91-1; gp91phox; CGD91-phox	NM_007807 XM_006527565	146 bp	QT00139797
transforming growth factor, beta 1	Tgfb1	Tgfb; Tgfb-1; TGFbeta1; TGF-beta1	NM_011577	145 bp	QT0014525
interleukin 6	Il6	Il-6	NM_031168	128 bp	QT00098875
glyceraldehyde-3-phosphate dehydrogenase	Gapdh	Gapd	NM_008084 XM_001003314 XM_990238 NM_001289726	144 bp	QT01658692

https://www.ncbi.nlm.nih.gov/gene/.

§ https://www.qiagen.com/it/shop/pcr/real-time-pcr-enzymes-andkits/two-step-qrt-pcr/quantitect-primer-assays/.

Figure Legends

Figure 1. The change in the cell viability caused by challenging BV-2 cells with oAβ1-42 and the protective effects of carnosine. BV-2 cells were treated for 24 h with increasing oAβ1-42 concentrations (1, 5, or 10 μM), in the absence or presence of carnosine (Car, 1 mM) (oAβ1-42 + Car (co-treat.), or with a solution consisting of Aβ1-42 monomers previously incubated in the presence of carnosine (1 mM) during the oligomerization process (Aβ1-42 + Car (co-inc.)); for more details see "Material and Methods" section. Data are the mean (n = 4) of 5 independent experiments and are expressed as the percent variation with respect to the viability recorded in resting (control) cells. Standard deviations are represented by vertical bars. * Significantly different from resting cells, $p < 0.01$, ** significantly different from resting cells, $p < 0.001$, *** significantly different from corresponding treatment with no carnosine, $p < 0.001$, **** significantly different from corresponding treatment with no carnosine, $p < 0.01$.

Figure 2. The change in the NO production induced by treating BV-2 cells with Aβ1-42 and effects of carnosine. BV-2 cells were treated for 24 h with increasing oAβ1-42 concentrations (1, 5, or 10 μM), in the absence or presence of carnosine (Car, 1 mM) (oAβ1-42 + Car (co-treat.), or with a solution consisting of Aβ1-42 monomers previously incubated in the presence of carnosine (1 mM) during the oligomerization process (Aβ1-42 + Car (co-inc.)). Data are the mean of 5 independent experiments and are expressed as the percent variation with respect to the NO production recorded in resting cells. Standard deviations are represented by vertical bars. ** Significantly different from resting cells, $p < 0.001$, *** significantly different from corresponding treatment with no carnosine, $p < 0.001$, ***** significantly different from corresponding treatment with no carnosine, $p < 0.05$, ŧ significantly different from cells treated with 10 μM oAβ1-42 + Car (co-treat), $p < 0.001$.

Figure 3. The change in $O2^{-\bullet}$ production induced by treating BV-2 cells with Aβ1-42 and effects of carnosine. BV-2 cells were treated for 24 h with increasing oAβ1-42 concentrations (1, 5, or 10 μM), in the absence or presence of carnosine (Car, 1 mM) (oAβ1-42 + Car (co-treat.), or with a solution consisting of Aβ1-42 monomers previously incubated in the presence of carnosine (1 mM) during the oligomerization process (Aβ1-42 + Car (co-inc.)). Data are the mean of 5 independent experiments and are expressed as the percent variation with respect to the nitric oxide production recorded in resting cells. Standard deviations are represented by vertical bars. ** Significantly

different from resting cells, $p < 0.001$, *** significantly different from corresponding treatment with no carnosine, $p < 0.001$.

Figure 4. Carnosine suppresses the Aβ1-42-induced mRNA expression levels of iNOS, Nox1, and Nox2 and increases the expression of TGF-β1 mRNA. Effects of Aβ1-42 and carnosine (Aβ1-42 + Car (co-treat.) and Aβ1-42 + Car (co-inc.)) on (A) iNOS, (B) Nox1, (C) Nox2, and (D) TGF-β1 mRNAs expression were examined by qRT-PCR. The abundance of each mRNA of interest was expressed relative to the abundance of GAPDH-mRNA, as an internal control. As a negative control, a reaction in the absence of cDNA (no template control, NTC) was performed. qRT-PCR amplifications were performed in quadruplicate. Standard deviations are represented by vertical bars. * significantly different from resting cells, $p < 0.001$, ** significantly different from resting cells, $p < 0.05$, *** significantly different from resting cells, $p < 0.01$, F significantly different from oAβ1-42-treated cells, $p < 0.001$, FF significantly different from oAβ1-42-treated cells, $p < 0.05$, FFF significantly different from oAβ1-42-treated cells, $p < 0.01$, ǂ significantly different from oAβ1-42 + Car (co-treat.)-treated cells, $p < 0.001$, significantly different from Aβ1-42 + Car (co-inc.)-treated cells, $p < 0.001$.

Figure 5. The modulation of cytokines secretion by carnosine. Supernatants from resting and BV-2 cells stimulated with oAβ1-42 in the absence or presence of carnosine (oAβ1-42 + Car (co-treat.) and Aβ1-42 + Car (co-inc.)) were analyzed using a Custom Multi-Analyte ELISArray Kit. Each treatment was analyzed in triplicate. The production of each cytokine is expressed as the percent variation with respect to the production recorded in resting cells. (A) IL-1β, (B) IL-6, (C) IFN-γ, (D) IL-10, and E) TGF-β1. Standard deviations are represented by vertical bars. * Significantly different from resting cells, $p < 0.001$, ** significantly different from resting cells, $p < 0.05$, F significantly different from oAβ1-42-treated cells, $p < 0.001$, FF significantly different from oAβ1-42-treated cells, $p < 0.05$, FFF significantly different from oAβ1-42-treated cells, $p < 0.01$, ǂ significantly different from oAβ1-42 + Car (co-treat.)-treated cells, $p < 0.001$, ǂǂ significantly different from oAβ1-42 + Car (co-treat.)-treated cells, $p < 0.05$, significantly different from Aβ1-42 + Car (co-inc.)-treated cells, $p < 0.001$, significantly different from Aβ1-42 + Car (co-inc.)-treated cells, $p < 0.05$.

Figure 6. The neuroprotective effects of carnosine against oAβ1-42-induced toxicity are mediated by TGF-β1. (A) Mixed neuronal cultures were challenged with oAβ1-42 (2 μM) for 48 h in the absence or presence of increasing concentrations of carnosine (1, 5, and 10 mM). (B) Effect of

SB431542 (specific inhibitor of type-1 TGF-β receptor) treatment (10 μM) on the neuroprotective activity of carnosine against oAβ1-42-induced toxicity. oAβ1-42 toxicity in mixed neuronal cultures was assessed by cell counting after trypan blue staining. Cell counts were performed in three to four random microscopic fields/well. Data are the mean of 6 (A) or 5 (B) determinations and are expressed as the percent variation with respect to the cell death recorded in oAβ1-42-treated cells. Standard deviations are represented by vertical bars. * Significantly different from oAβ1-42-treated cells, $p < 0.001$, ω significantly different from oAβ1-42 + Car 1 mM, $p < 0.001$, # significantly different from oAβ1-42 + Car 10 mM.

Chapter 5.

The multimodal MOPr/DOPr agonist LP2 reduces allodynia in chronic constriction injured rats by rescue of TGF-β1 signalling

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Abstract

Neuropathic pain is one of the most disabling forms of chronic pain and it is characterized by hyperalgesia and allodynia linked to an aberrant processing of pain transmission and to neuroinflammation. Transforming growth factor-β1 (TGF-β1) is an anti-inflammatory cytokine, which protects against neuroinflammation. It has

been demonstrated that TGF-β1 and opioid receptors signalling crosstalk results in an improvement of endogenous opioid analgesia, but it is not known whether mu opioid peptide receptor (MOPr) or delta opioid peptide receptor (DOPr) agonists can positively modulate TGF-β1 pathway. In the present study, we examined the correlation between anti-allodynic effect of LP2, a dual-target MOPr/DOPr agonist, and TGF-β1 signalling in the chronic constriction injury (CCI) model. We detected a significant decrease of active TGF-β1 and of its type II receptor TGFβ-R2 levels in the spinal cord from CCI rats and a selective deficit of TGF-β1 in microglia cells both at days 11 and 21 post-ligature, as assessed by immunofluorescence analysis. LP2, when administered from the 11 days post-ligature to 21 days, was able to reduce CCI-induced mechanical allodynia by rescue of TGF-β1 and TGFβ-R2 levels. Our data suggest that the rescue of TGF-β1 signalling by dual-target MOPr/DOPr agonist LP2 could be mediated by DOPr activation in spinal microglia, thus the dual-target approach could represent a novel pharmacological approach to increase the analgesic efficacy of MOPr agonists.

Keywords: Neuropathic pain, Transforming Growth Factor-β1 (TGF-β1), dual target MOPr/DOPr agonist, analgesia, microglia

* *Published in Front. Pharmacol. 2021 Oct 6;12:749365.*

1. Introduction

Neuropathic pain, one of the most complex and disabling forms of chronic pain, is currently defined as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system” (Treed et al., 2008). It is often associated with different pathological conditions such as diabetes mellitus, cancer, vascular and infectious diseases. It is characterized by typical symptoms such as an exaggerated pain perception of noxious stimuli (more commonly known as hyperalgesia) as well as non-noxious stimuli (allodynia), linked to an aberrant processing of pain

transmission (Costigan, et al., 2009). The underlying mechanisms include complex interactions between neurons, glia, and cells of the immune system through neurotransmitters, cytokines, and inflammatory mediators (Mika et al., 2013), so that neuropathic pain may now be considered as a "neuroimmune disorder" (Malcangio, 2019). Glia cells, which represent about 70% of the cells in the central nervous system (CNS), and microglia activation, with a shift in the balance between pro-inflammatory and anti-inflammatory cytokines, play a key role in sensitization processes (Caraci et al., 2019; Gwak et al., 2017**)**. Pro-inflammatory cytokines such as Interleukin (IL)-1, IL-6, IL-15, IL-17, IL-18, tumor necrosis factor-alfa (TNF-α), and interferon-gamma (IFN-γ) play a key role in the processes of central sensitization, while the induction of anti-inflammatory cytokines (IL-10, IL-4, or transforming growth factor-β1 (TGF-β1)) can exert a protective role against neuroinflammatory events underlying neuropathic pain (Caraci et al., 2019; Chen et al., 2013; Uçeyler et al., 2007). Thus, anti-nociceptive strategies include inhibition of pro-inflammatory cytokines and use of anti-inflammatory cytokines (Shubayev et al., 2010). It has been hypothesized that a deficit of TGF-β1 signalling might contribute to the pathophysiology of chronic pain (Lantero et al., 2012). TGF-β1 can counteract the development of chronic neuropathic pain (Lantero et al., 2014) and exert anti-allodynic and analgesic effects in animal models of neuropathic pain (Lantero et al., 2014; Echeverry et al., 2009). Moreover, TGF-β1 is involved in the pathogenesis of depressive disorders, which often occur in comorbidity with chronic pain (Caraci et al., 2018). Echeverry et al. also observed that recombinant TGF-β1, delivered into the spinal cord of rats, was effective not only in preventing, but also in reversing the hypersensitivity evoked by damage to peripheral nerve through different mechanisms, i.e. blocking microglial cells proliferation, inhibiting spinal microglial activation, and reducing the expression of pro-inflammatory cytokines (Echeverry et al., 2009). Furthermore, Lantero et al. demonstrated that pre- and post-synaptic modulation of the endogenous opioid

system by TGF-β1 signalling can prevent the development of allodynia and improve the analgesic efficacy of both endogenous and exogenous opioid agonists both in inflammatory and neuropathic pain conditions (Lantero et al., 2014).

Available therapies with mu opioid peptide receptor (MOPr) agonists often provide incomplete pain relief and treatment-related side effects are common such as respiratory depression, constipation, and tolerance (Chou et al., 2015). A novel drug discovery strategy in chronic pain is the development of MOPr/delta opioid peptide receptor (DOPr) dual-target compounds able to activate MOPr as well as DOPr (Martínez-Navarro et al., 2018). The MOPr/DOPr simultaneous targeting is supported by the co-expression of both receptors in areas involved in pain modulation and by the crucial role of MOPr activation in the regulation of DOPr trafficking (Scherrer et al., 2009). Moreover, activation of DOPr leads to fewer typical opioid side effects.

We previously reported that the dual-target benzomorphan-based compound LP2 was able to simultaneously bind and activate MOPr (K_i = 1.08 nM, IC_{50} = 21.5 nM) and DOPr (K_i = 6.6 nM, IC_{50} = 4.4 nM) (Pasquinucci et al., 2017). In tail-flick and formalin test LP2 was found to produce significant anti-nociceptive (ED_{50} = 0.9 mg/kg i.p.) and anti-inflammatory (ED_{50} = 0.88 and 0.79 mg/kg i.p., phases I and II of formalin test) effects (Pasquinucci et al., 2019). In rats subjected to unilateral sciatic nerve chronic constriction injury (CCI), we already showed that LP2 significantly inhibits the development of mechanical allodynia and prevents CCI-induced Cx43 alterations and pro-apoptotic signalling in the CNS (Vicario et al., 2019a). These findings prompted us to further examine the molecular mechanisms underlying the analgesic effects of the MOPr/DOPr agonist LP2 in the CCI model. Presently, it is not known whether MOPr and/or DOPr agonists can positively modulate TGF-β1 pathway and whether the rescue of TGF-β1 signalling can contribute to increase the analgesic effects of MOPr/DOPr compounds. Thus, in the present study we examined the hypothesis that CCI can induced an impairment of

the TGF-β1 pathway and that the anti-allodynic effects of LP2 in the CCI rat model can be mediated by an increased expression of TGF-β1 and its type 2 receptor (TGFβ-R2) at spinal cord level along the time course of neuropathic pain.

2. Material and Methods

Animal model of Neuropathic Pain

Experiments were carried out on male Sprague-Dawley rats (Envigo Laboratories), weighing 180-200 gr. Animals were set at a constant temperature (23-25 °C) between 9:00 am and 15:00 pm. This study was performed according to the European Communities Council directive and Italian regulations (EEC Council 2010/63/EU and Italian D. Lgs. no. 26/2014) and approved by Italian Ministry of Health (OPBA Project 946/2018-PR) in order to replace, reduce, and refine the use of laboratory animals. The model used to induce neuropathic pain was the CCI model, according to Bennet and Xie (Bennet and Xie, 1988), with secondary (minor) modifications (Parenti, et al., 2013). Animals were put in a chamber, anesthetized with isoflurane inhalation (4% induction, 2% for maintenance), and an incision was made underneath the hipbone, parallel to the common sciatic nerve, which was exposed. Later, 4 ligatures (4/0 chromic silk, Ethicon) were tied firmly around the nerve, proximal to the trifurcation of the nerve at about 1 mm spacing, observing a twitch in the respective hind limb. In the SHAM rats, the sciatic nerve was exposed, but there were not made any ligatures. Then, rats were randomly assigned to three different groups: SHAM-vehicle, CCI-vehicle, and CCI + LP2. Starting from 11 days post-ligatures (dpl), after the measurements of allodynic thresholds, CCI groups received a daily intraperitoneal (i.p.) injection of either vehicle or LP2 (0.9 mg/kg) up to 21 dpl.

Evaluation of Mechanical allodynia

Allodynia, already measured in the present CCI model in Vicario et al. (2019a), has been reproduced in a new cohort of rats. Rats were allocated and allowed to acclimate for 20 minutes in a wire mesh bottom test chamber. The ventral surface of the hind paw was mechanically stimulated from below with an ascending series of calibrated Von Frey's filaments, which bending forces ranging from 0.02 to 30 g. The "up-down" method was used to evaluate the withdrawal threshold, increasing and decreasing sequentially the stimulus strength (Dixon, 1980). Behavioural assessment of mechanical allodynia has been performed at 0 (before surgery), 11, 16 and 21 dpl.

Ex vivo tissue processing

At 11, 16, and 21 dpl, rats were anesthetized with an i.p. injection of ketamine (10 mg/mL) and xylazine (1.17 mg/mL), and transcardially perfused with 0.5 M ethylenediaminetetraacetic acid (EDTA) in normal saline, followed by ice cold 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH = 7.4) (Sigma-Aldrich, St Louis, MO).

Spinal cords were isolated and post-fixed in 4% PFA in PBS at 4 °C overnight. Tissue samples were then washed in PBS and cryo-preserved in 30% sucrose in PBS at 4 °C for 3 days. Samples were embedded in optimum cutting temperature (OCT) medium and snap frozen in liquid nitrogen for cryosectioning using a cryostat (Reichert-Jung 2800). Twenty μm-thick axial section of spinal cords from the lumbar enlargement (L4-L5) were collected and stored at -80 °C until use.

RNA isolation and quantification

Total RNA was extracted from spinal cord slices (thickness of 20 μm) using the commercial RNeasy FFPE kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations, with different modifications carried out in order to improve the yield and purity of the extracted RNA. Briefly, the slides containing

spinal cord slices were taken out from the -80 °C and left to dry at room temperature (RT). Next, each slide was washed at least three times by using DEPC water and the spinal cord slides were harvested by employing a sterile scalpel and moved to microcentrifuge tubes. After the addition of Buffer PKD, each sample was vortexed, centrifuged, and mixed with proteinase K. Following three different incubation steps (56 °C, 80 °C, and 4 °C) and one centrifugation, the supernatant of each sample was transferred to a new microcentrifuge tube. Each tube was therefore added with a mixture of DNase Booster Buffer and DNase I stock and incubated at RT. At the end of the incubation, Buffer RBC and ethanol (100%) were added and the total solution content was moved to the RNeasy MinElute spin column. After two washing steps (Buffer RPE), the RNA was eluted by the addition to the columns of RNase-free water. The concentration of total RNA recovered from spinal cord slices was determined by measuring the fluorescence with Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and the absorbance at 260 nm with NanoDrop® ND-1000 (Thermo Fisher Scientific). The first allows more accurate measurements in terms of quantity compared with commonly used methods absorbance-based because the dyes part of the Qubit kit fluoresce only when bound to RNA. The additional measurement with NanoDrop® ND-1000 was carried out to verify the purity of the samples by analyzing the absorbance curves.

Gene Expression Analysis by Quantitative Real-Time PCR (qRT-PCR)

Gene expression analysis by qRT-PCR was performed as previously described (Caruso et al., 2019) with slight modifications. The reverse transcription of 20 ng of total RNA (for each sample) was accomplished by using the SuperScript III First-Strand Synthesis SuperMix kit (Thermo Fisher Scientific), while the quantification of each cDNA sample loaded in a 384-well plate was achieved by using a LightCycler® 480 System (Roche Molecular Systems, Inc., Pleasanton, CA, USA). The list containing the information of each primer used for this study is reported in

Table 1. The protocol employed for sample amplification, fluorescence data collection, and sample quantification is the same previously described elsewhere (Caruso et al., 2019).

Western Blot Analysis

To evaluate TGF-β1 and TGFβ-R2 expression levels, the slides containing eight spinal cord slices for each rat, obtained by the procedure described in the paragraph 2.3, were washed three times by using deionized water. Next the samples were pooled by employing a sterile scalpel and harvested into microcentrifuge tubes. The samples were resuspended in 3% sodium dodecyl sulfate (SDS) RIPA buffer supplemented by phosphatases and proteases inhibitors (1:100 dilution), incubated for 20 min at 100 °C, and for 20 min at 4 °C, then were sonicated and subsequently centrifuged for 15 min at 15.000 x g in refrigerate centrifuge to remove cellular debris.

Protein quantification was performed using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific), according to the manufacturer's specifications; subsequently, 30 µg of total proteins were denatured at 95 °C for 10 min, subjected to NuPage™ 10% bis–tris gel electrophoresis (Thermo Fisher Scientific) and transferred to nitrocellulose membranes.

The membranes were blotted with anti-TGF-β1 (1:500, Abcam ab92486, Cambridge, UK), anti-TGFβ-R2 (1:500, Cell signaling Technology Inc., Danvers, MA, USA; 79424), anti-GAPDH (1:2000, Millipore MAB 374, Burlington, MA, USA), and anti-β-actin (1:1000, Sigma Aldrich, St Louis, MO; A4700) primary antibodies in blocking buffer at 4 °C overnight. After washing in tris-buffered saline (TBS)/Tween 20X 0.1%, the membranes were incubated for 1 hour with IRDye® 800CW or 680LT secondary antibodies (1:15000) at RT in the dark. Bands were visualized using an Odyssey® infrared imaging system (LI-COR Biosciences,

Lincoln, NE, USA), while the densitometric analysis was carried out by using Image J software.

Immunofluorescence

Immunofluorescence was performed as previously described (Gulino et al., 2019; Vicario et al., 2021). Briefly, sections were washed in PBS and incubated with blocking buffer (10% normal goat serum (NGS) and 0.1% Triton X-100 in PBS) for 1 hour at RT. Sections were then incubated overnight at 4 °C with the following primary antibodies: mouse monoclonal (A60) anti-NeuN (Millipore Cat. No. MAB377, RRID: AB_2298772, 1:100), mouse monoclonal anti-GFAP (BD Biosciences Cat. No. 610566, RRID: AB_397916, 1:100), goat polyclonal anti-IBA1 (Novus Biologicals, Cat. No. NB100-1028, RRID: AB_521594, 1:500), and rabbit polyclonal anti-TGF-β1 (Abcam Cat. No. ab92486, RRID: AB_10562492, 1:100). The following day, sections were washed in 0.1% Triton X-100 in PBS 3 times at RT and then incubated 1 hour at RT with appropriate combination of secondary antibodies: goat polyclonal anti-mouse (Alexa Fluor 488, Thermo Fisher Scientific, Cat. No. A-11001, RRID: AB_2534069, 1:1.000), goat polyclonal anti-rabbit (Alexa Fluor 564, Molecular Probes, Cat. No. A-11010, RRID: AB_143156, 1:1.000) and donkey anti-goat (Alexa Fluor 647, Thermo Fisher Scientific, Cat. No. A-21447, RRID: AB_2535864, 1:1.000). Nuclei were counterstained with DAPI (1:10.000, Invitrogen, Waltham, MA, USA) for 5 min at RT and then mounted with BrightMount mounting medium (Abcam). Digital images were acquired using a Leica DM IRB fluorescence microscope and with Leica TCS SP8 confocal microscope and profile plots for immunofluorescence images were obtained as previously described (Vicario et al., 2019a; Spitale et al., 2020). Briefly, confocal images of ipsilateral dorsal horns were analyzed using ImageJ software. For each population marker (i.e. NeuN, Gfap, or Iba1) a profile plot was calculated and

superimposed to the corresponding TGF-β1 profile plot in order to highlight proximity and/or colocalization.

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism 9 (GraphPad Software, La Jolla, CA). Two-tailed unpaired Student's t-test was used for comparison of n = 2 groups. Comparisons of n > 2 groups were performed using a one-way analysis of variance (ANOVA). Statistical analyses of behavioral assessment of mechanical allodynia were performed using a two-way ANOVA repeated measure. One-way ANOVA, followed by Bonferroni's post hoc test, was used for multiple comparisons on molecular markers. Only two-tailed p-values of less than 0.05 were considered statistically significant. All data are represented as means ± SEM.

3. Results

3.1 The MOPr/DOPr agonist LP2 reduces the mechanical allodynia in CCI rats

We first evaluated the antinociceptive effect of LP2 through the behavioral assessment of mechanical allodynia following the experimental paradigm shown in **Figure 1A**. Neuropathic pain condition, produced by CCI model, decreased the withdrawal threshold, with a significant reduction at 11 dpl in CCI animals as compared to SHAM rats (**Figure 1B**). LP2 administration, started from 11 dpl (after withdrawal threshold measurements) recovered the withdrawal threshold at 16 and 21 dpl of CCI + LP2 treated rats as compared to CCI-vehicle rats.

3.2 Molecular mechanisms underlying the anti-allodynic effects of LP2: the key role of TGF-β1

In order to assess the impact of CCI on neuroinflammation in the spinal cord, the gene expression of TGF-β1 and its receptor (TGFβ-R2) along with two well-known

pro-inflammatory cytokines, IL-6 and IL-1β, was firstly investigated in the spinal cord at 11 dpl in SHAM and CCI animal groups (**Figure 2**). CCI represents a validated animal model of neuropathic pain where neuroinflammation in the spinal cord is known to play a key role in central sensitization (Nong et al. 2018). Interestingly we found that CCI significantly decreased the expression levels of TGF-β1 (**Figure 2A**) and of its receptor TGFβ-R2 (**Figure 2B**), while raised both IL-6 (**Figure 2C**) and IL-1β (**Figure 2D**) mRNA expression levels compared to the rats belonging to the SHAM group ($p < 0.05$ for all cytokines).

To understand the molecular mechanisms underlying the anti-allodynic effects of LP2 in CCI rats, we then assessed whether LP2 exerted its analgesic effects in our animal model of neuropathic pain by counteracting neuroinflammatory phenomena induced by CCI (SHAM *vs.* CCI *vs.* CCI + LP2) after 5 and 10 days of LP2 treatment, respectively (i.e. at 16 and 21 dpl) (**Figure 3-4**). **Figure 3A-B** clearly shows that the significant decrease in mRNA expression levels of TGF-β1 induced by CCI, still persisting after 16 and 21 days ($p < 0.05$ *vs.* SHAM). The same effect was observed for the TGFβ-R2 gene expression at 16 and 21 dpl (**Figure 4A-B**) ($p < 0.05$ vs. SHAM). Interestingly we found that the treatment with LP2 completely rescued both TGF-β1 (**Figure 3A-B**) and TGFβ-R2 (**Figure 4A-B**) mRNA levels in CCI rats ($p < 0.05$ *vs.* CCI) after 5 and 10 days of treatment. Unlike TGF-β1 and TGFβ-R2, the treatment with LP2 was not able to counteract the increased production of IL-6 and IL-1β CCI-induced (**data not shown**).

TGF-β1 activity in the CNS is regulated not only at a transcriptional level, but it shows a complex post-translational regulation through the conversion of latent TGF-β1 to active TGF-β1 by a variety of proteases, among which matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) play a central role in this conversion (Caraci et al., 2018).

To validate the role of TGF-β1 as a new pharmacological target in neuropathic pain, we therefore quantified the protein levels of active TGF-β1 and its receptor (TGFβ-

R2) in the spinal cord of CCI rats both in the absence and in the presence of LP2 treatment. Interestingly, we found that CCI procedure was able to induce a significant decrease of active TGF-β1 (**Figure 3C-D**) and TGFβ-R2 levels (**Figure 4C-D**) only 21 dpl ($p < 0.05$ *vs.* SHAM), but not at 16 dpl. Most importantly, LP2 treatment was able to rescue both TGF-β1 and TGFβ-R2 levels when compared with those in CCI rats ($p < 0.05$ *vs.* CCI; **Figure 3C-D; Figure 4C-D**), suggesting that only a long treatment with LP2 is able to rescue the TGF-β1 pathway in CCI rats.

3.3 The multimodal MOPr/DOPr agonist LP2 induces an increase of TGF-β1 which colocalizes with Iba1 in CCI rats

To evaluate the expression and localization of TGF-β1 in CCI model, we analyzed ipsilateral dorsal horns cell population (i.e., NeuN positive neurons, GFAP positive astrocytes, and Iba1 positive microglial cells) and their potential colocalization or proximity to TGF-β1 in the early phase of the neuropathy (**Figure 5A-C**). We observed that CCI induced an overall reduction of TGF-β1, which was found to be in close proximity to NeuN positive cells (**Figure 5A**) and to colocalize with Iba1 positive cells (**Figure 5C**). These data demonstrate a selective deficit of TGF-β1 in the spinal microglia in the CCI model. Such a deficit was also observed in the chronic stage of CCI (i.e., 21 dpl, **Figure 5D-F**). Indeed, profile plot analysis revealed that LP2 treatment was able to increase peri-neuronal TGF-β1 signals (**Figure 5D**), which weakly colocalize with NeuN (**Figure 5D**) and GFAP (**Figure 5E**) positive cells. Interestingly we found a strong colocalization between TGF-β1 and Iba1 positive cells (**Figure 5F**), thus indicating a critical role of microglial cells in the rescue of TGF-β1 mediated by LP2.

4. Discussion

Neuropathic pain represents a chronic pathological condition with a significant negative impact on a patient's quality of life with a prevalence among the general population ranging from 3% to 17% (Cavalli et al., 2019). Since the typical symptoms of this disease are often unresponsive to conventional therapy, new pharmacological targets have to be identified to improve current therapeutic approaches. In this scenario, the CCI model represents a validated animal model of neuropathic pain useful to identify the molecular mechanisms underlying chronic pain development as well as novel pharmacological targets for its management (Boccella et al. 2018; Coraggio et al. 2018; Caraci et al. 2019).

DOR represents a novel pharmacological target in the treatment of chronic neuropathic pain (Cahill et al., 2020; Kabli & Cahill, 2007). Recent studies have demonstrated that DOPr agonists counteract and prevent nociceptive behaviors in various chronic pain models, including neuropathic pain, while having minimal effect on sensory thresholds in the absence of injury (Turnaturi et al., 2019). We have previously demonstrated that the multimodal MOPr/DOPr agonist LP2 produced a significant anti-nociceptive and anti-inflammatory effect in tail-flick and formalin test, respectively (Pasquinucci et al., 2017, Pasquinucci et al., 2019). Moreover, the repeated administration of LP2 significantly inhibited the development of mechanical allodynia in neuropathic rats subjected to CCI and prevented CCI-induced Cx43 alterations and pro-apoptotic signalling in the CNS (Vicario, et al., 2019a; Vicario, et al., 2019b). Despite selective MOPr agonists are the cornerstones of moderate-to-severe acute pain treatment, their effectiveness and safety in chronic pain conditions is controversial. In contrast to MOPr, DOPr density and activity are up-regulated in chronic pain models and analgesic effects of DOPr selective agonists are enhanced during persistent inflammation (Turnaturi et al., 2019). Moreover, the activation of MOPr plays a crucial role in the regulation of the trafficking and membrane targeting of DOPr in conditions of persistent inflammation (Gendron et al., 2007). Direct coupling of MOPr-DOPrs in the form

of hetero-oligomers has been demonstrated in the spinal cord tissue and it was proposed to underlie the anti-nociceptive synergy between MOPr and DOPr agonists (Gomes et al., 2004). Thus, simultaneous activation of MOPr and DOPr with a dual target compound delivers potent analgesia in experimental models of chronic pain with strongly reduced dependence and tolerance development on the course of repeated administration (Starnowska-Sokół and Przewłocka, 2020). Nevertheless, the molecular mechanisms underlying the analgesic effects of DOPr agonists are still unclear as well as the different contribute of glial cells and neurons in the allodynic effects of these compounds. It is known that prolonged morphine treatment *in vivo* induces the translocation of DOPrs from intracellular compartments to neuronal plasma membranes (Hack et al., 2005). DORs are also strongly expressed both in astrocytes and microglial cells (Turchan-Cholewo et al., 2009), but it is presently unknown the contribute of glial DORs in the analgesic effects of DOPr agonists. Recent studies have demonstrated that the activation of glial cells, in particular microglia, toward a pro-inflammatory state plays a central role in the transition from acute to chronic pain (Caraci et al., 2019; Coraggio et al., 2018), also contributing to a state of opioid analgesic tolerance (Holdridge et al., 2007). In particular, peripheral damage and hyperactivity of primary sensory neurons promote neuroinflammation, in the spinal cord, through the release of several pro-inflammatory cytokines (e.g., TNF-α and IL-1β) by both activated astrocytes and microglial cells (Lees et al., 2015; Ji et al., 2014), finally leading to central sensitization and pain chronicization (Ji et al., 2014). Interestingly, the repeated activation of DOPr reduces the release of pro-inflammatory cytokines, such as TNF-α (Vicario et al, 2016), suggesting an impact of DOPr agonists on neuroinflammation in animal models of neuropathic pain. Along this line, an alternative approach that has been recently adopted in drug discovery in neuropathic pain is to develop new compounds able to promote the release of anti-inflammatory cytokines endowed with a neuroprotective and analgesic efficacy (Guo et al., 2021;

Piotrowska, et al., 2016). Among microglial anti-inflammatory cytokines, TGF-β1 has a protective role against the development of chronic neuropathic pain by inhibiting neuroinflammation and promoting the expression of endogenous opioids within the spinal cord (Lantero A. et al., 2012). TGF-β1 pathway has been proposed as a novel pharmacological target to increase the analgesic effects of opioids (Onichtchouk et al., 1999; Lantero et al., 2014, de la Puerta et al., 2019). In addition, a TGF-β1-opioid receptor signalling crosstalk results in improvement of endogenous and exogenous opioid analgesia in experimental models of neuropathic pain (Lantero et al., 2012; Lantero et al. 2014).

Starting from this evidence, we therefore examined the impact of CCI on mRNA levels of pro-inflammatory (IL-1β and IL-6) and anti-inflammatory (TGF-β1) cytokines in the spinal cord observing that CCI procedure increased IL-1β and IL-6 levels at 11days. These results are in accordance with previous findings showing a TNF-α induction in the same model in the sciatic nerve (Vicario et al, 2016). Previous works have also demonstrated an induction of IL-1β and IL-6 in affected nerves from CCI models (Liu et al., 2020; Khan et al., 2018). To the best of our knowledge our data demonstrate for the first time an induction of these cytokines in the spinal cord of CCI rats which co-occurs with a reduction of the expression levels of TGF-β1 and TGFβ-R2 mRNAs 11 dpl compared with SHAM control animals. Interestingly, in our model a significant decrease of both active TGF-β1 levels and TGFβ-R2 levels was detected only 21 dpl, suggesting a long-term and post-transcriptional regulation of TGF-β1 and its receptor in the CCI model or alternatively a selective deficit of them in sub-population of cells from spinal cord (microglial cells vs astrocytes/neurons; see below). TGF-β1 plays a protective role against the development of chronic neuropathic pain and the first demonstration of the anti-nociceptive effects of TGF-β1 has been obtained by Chen et al. (2013), with intrathecal administration of this peptide able to reduce both spinal neuroinflammation and excitoxicity (Chen et al., 2013). In the same animal model,

it has been demonstrated that the down-regulation of p38 and extracellular signal-regulated kinase (ERK) activity influences TGF-β1-induced analgesia during neuropathy (Chen et al., 2016). Different groups have analyzed the neurobiological links between TGF-β1 and opioid system in animal models of neuropathic pain (Tramullas et al. 2010; Lantero et al. 2014). Increased levels of a transmembrane pseudo-receptor structurally similar to TGF-β type I receptors, BAMBI (bone morphogenetic protein and activin membrane-bound inhibitor), and TGF-β receptors have been detected in areas with a high density of opioid receptors such as the cingulate cortex, periaqueductal grey matter, and the dorsal horns of the spinal cord, key areas of the inhibitory modulation of pain transmission (Tramullas et al., 2010). An enhancement of TGF-β1 signalling has been observed in mice lacking the TGF-β pseudo-receptor BAMBI, which leads to an increased synaptic release of opioid peptides and to a naloxone-reversible hypoalgesic/antiallodynic phenotype. In *in vivo* neuropathic pain models, the pleiotropic protective effects of TGF-β1 are achieved by the inhibition of neuroinflammatory phenomena (Chen et al., 2015; Echeverry et al., 2009), suppression of the neuronal hyperexcitability (Chen et al., 2013) and endogenous opioid system activation (Onichtchouk et al., 1999; Lantero et al., 2014; de la Puerta et al., 2019). Nevertheless, all these studied have not deeply investigated the possible presence of a TGF-β1 signalling deficit in the CCI model.

In the present work we demonstrated a selective deficit of TGF-β1 and its receptor in microglia cells from spinal cord of CCI rats detectable both at days 11 and 21 dpl as assessed by immunofluorescence analysis. Previous studies have found conflicting data on TGF-β1 levels in the CCI model, with reduced expression of this factor in dorsal root ganglion from CCI mice, followed by an increase at day 10 post-ligature (Xie et al., 2019) or reduced levels of TGF- β1 and its type I receptor in the red nucleus of rats two weeks after spared nerve injury (SNI) (Wang et al., 2015). Further studies are needed in the rat model of CCI to understand

whether the selective deficit of TGF-β1 and/or its receptor in microglia can be detected in an early phase of pain chronicization.

It is well-known that a 2-week subcutaneous infusion with recombinant TGF-β1 showed an anti-allodynic effect when compared with saline in mice subjected to sciatic nerve injury (Lantero et al., 2012), but alternative and more feasible approaches should be developed to rescue TGF-β1 levels in animal models of neuropathic pain. In the present work we demonstrated, for the first time, that a chronic treatment (10 days) with the multimodal MOPr/DOPr agonist LP2 was able to rescue the levels of active TGF-β1 and of TGFβ-R2 in the spinal cord from CCI rats and, most importantly, in microglial cells. Interestingly Reddy et al. found that activation of DOR in transplanted mesenchymal stem cells with the synthetic peptide [D-Ala^2, D-Leu^5]-enkephalin (DADLE) promotes the secretion of anti-inflammatory cytokines (IL-10/IL-4/TGF-β) (Reddy et al., 2017). Further studies are therefore needed, in primary cultures of microglial cells, to analyze the effects of LP2 as well as other DOPr agonists on TGF-β1 synthesis. A recent study, conducted in a neuropathic pain rat model generated through spinal nerve ligation (SNL), demonstrated that valproate mitigates SNL-induced allodynia by modulating microglial function, inhibiting neuroinflammatory response, finally promoting the expression of anti-inflammatory cytokines (TGF-β, IL-10 and IL-4) (Guo et al., 2021).

Our study, conducted in a validated animal model of neuropathic pain, is the first to demonstrate that the dual target MOPr/DOPr agonist LP2, when administered from the 11 dpl to 21 days, is able to reduce CCI-induced mechanical allodynia by rescue of TGF-β1 and TGFβ-R2 levels. Moreover, our results show the possible role played by microglial cells in modulating the rescue of TGF-β1 levels promoted by LP2 treatment. We believe that the rescue of TGF-β1 signalling by dual-target MOPr/DOPr agonist LP2 could be mediated by DOR activation in spinal microglia

and might represent a novel pharmacological approach to increase opioid analgesic efficacy of selective MOPr agonists.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICS STATEMENT

The study was authorized by the Institutional Animal Care and Use Committee (IACUC) of the University of Catania and by the Italian Ministry of Health (DDL 26/2014 and previous legislation; OPBA Project 946/2018-PR). Animal care followed Italian (D.M. 116192) and EEC (O.J. of E.C.L 358/1 12/18/1986) regulations on protection of animals used for experimental and scientific purposes.

AUTHOR CONTRIBUTIONS

CP, LP and FC gave substantial contributions to the conception and design of the work. AF, MG, RT, FS, NV, SS, NM and GC performed the experiments. FC, AF, MG, NV and GC analyzed the data. RP, SC, AM, LP participated in the design of the study. FC and CP drafted the work. All authors approved the version to be published.

FUNDING

This research was conducted with the support of the University of Catania, PIA.CE.RI. 2020-2022-Linea di intervento 2-Project DETTAGLI (UPB 57722172125). Is was also partially funded by grant from the Italian Ministero dell'Istruzione, dell'Università e della Ricerca, PRIN 2017, Grant no. 2017XKWWK9_004 to RP. This research was also funded by University of

Catania, Programma Ricerca di Ateneo unict 2020-2022- Linea 3; Project Asclepio (UPB 57722172132). NV was supported by the PON AIM R&I 2014-2020-E66C18001240007. FMS was supported by the International Ph.D. program in Neuroscience (Department of Biomedical and Biotechnological Sciences, University of Catania, Italy).

ACKNOWLEDGMENTS

The authors acknowledge the Center for Advanced Preclinical in vivo Research (CAPiR) and the confocal microscopy facility at the Bio-Nanotech Research and Innovation Tower (BRIT) of the University of Catania, for the technical contribution of the staff.

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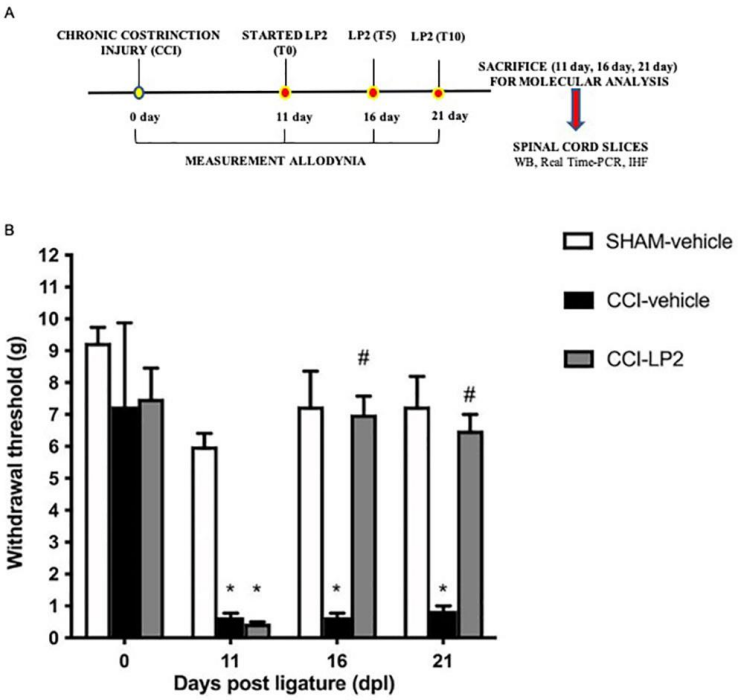

A
CHRONIC COSTRINCTION INJURY (CCI)
STARTED LP2 (T0)
LP2 (T5)
LP2 (T10)
SACRIFICE (11 day, 16 day, 21 day) FOR MOLECULAR ANALYSIS
0 day
11 day
16 day
21 day
MEASUREMENT ALLODYNIA
SPINAL CORD SLICES
WB, Real Time-PCR, IHF
B
SHAM-vehicle
CCI-vehicle
CCI-LP2
Withdrawal threshold (g)
Days post ligature (dpl)
0
11
16
21
#
#
*
*
*
*

Figure 1.

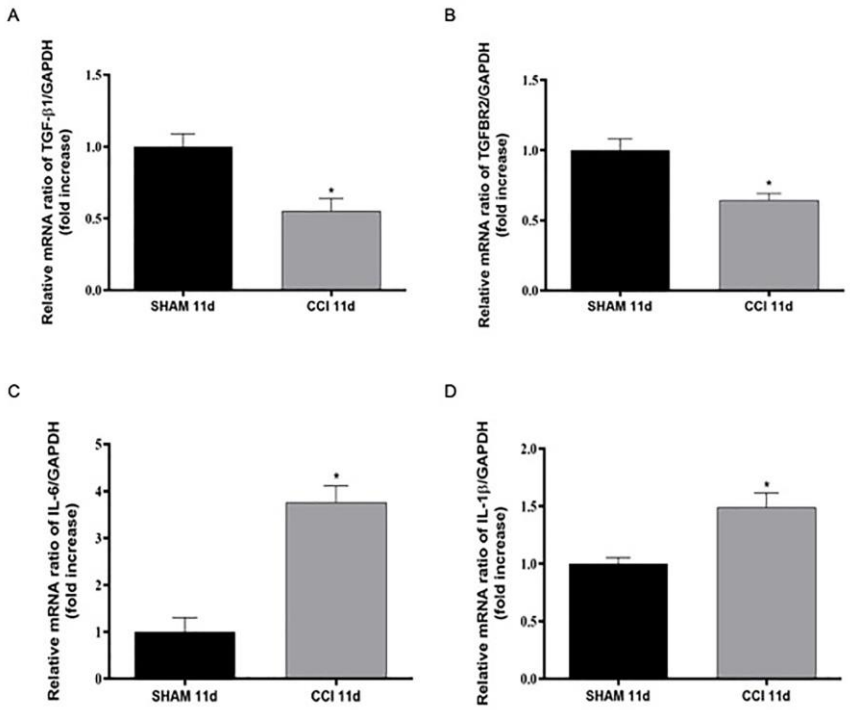

A
Relative mRNA ratio of TGF-β1/GAPDH (fold increase)
1.5
1.0
0.5
0.0
*
SHAM 11d
CCI 11d
B
Relative mRNA ratio of TGFBR2/GAPDH (fold increase)
1.5
1.0
0.5
0.0
*
SHAM 11d
CCI 11d
C
Relative mRNA ratio of IL-6/GAPDH (fold increase)
5
4
3
2
1
0
*
SHAM 11d
CCI 11d
D
Relative mRNA ratio of IL-1β/GAPDH (fold increase)
2.0
1.5
1.0
0.5
0.0
*
SHAM 11d
CCI 11d

Figure 2.

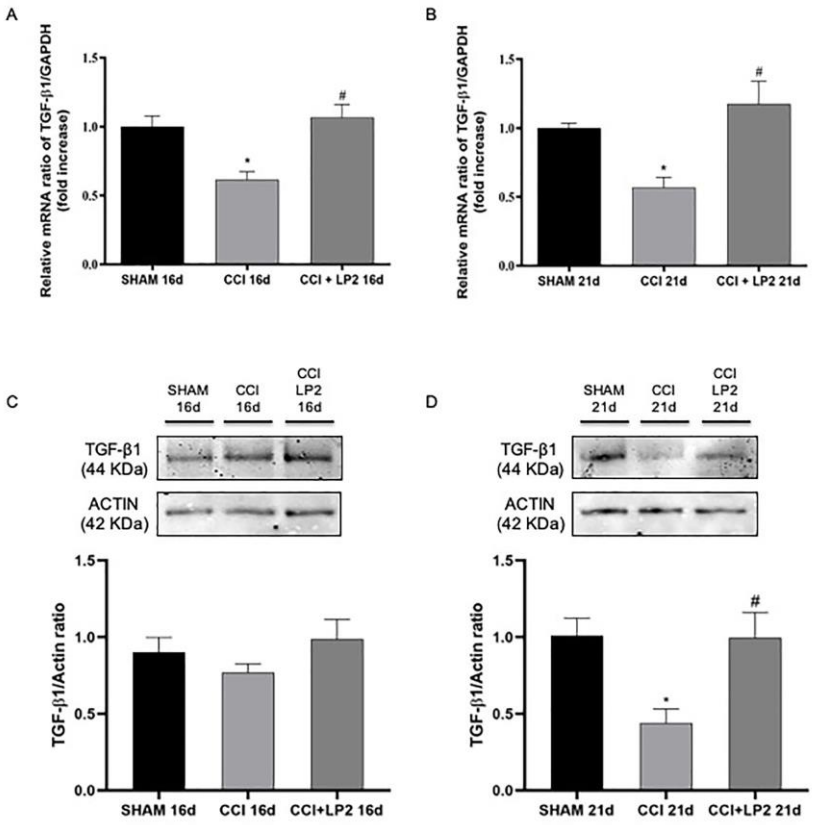
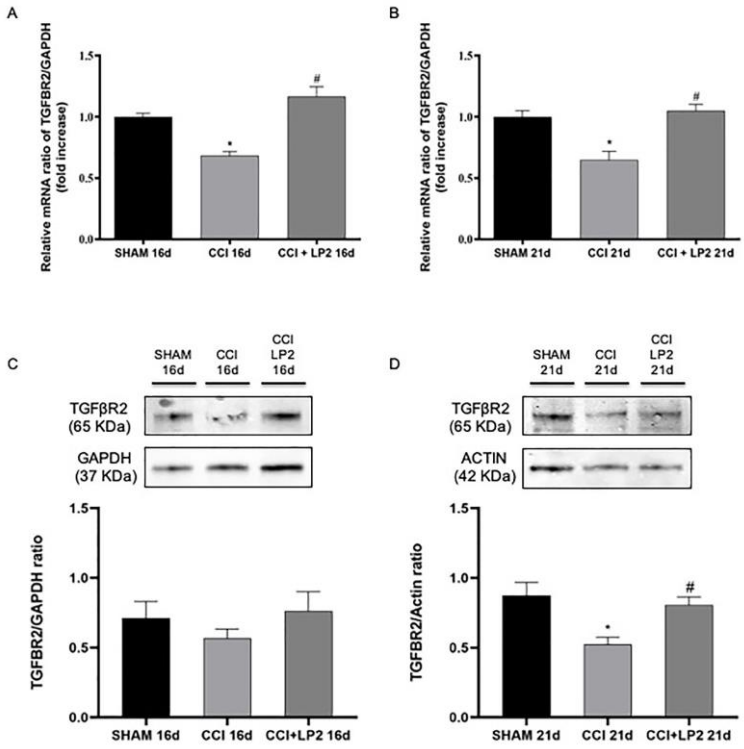

A
Relative mRNA ratio of TGF-β1/GAPDH (fold increase)
1.5
1.0
0.5
0.0
*
#
SHAM 16d
CCI 16d
CCI + LP2 16d
B
Relative mRNA ratio of TGF-β1/GAPDH (fold increase)
1.5
1.0
0.5
0.0
*
#
SHAM 21d
CCI 21d
CCI + LP2 21d
C
SHAM 16d
CCI 16d
CCI LP2 16d
TGF-β1 (44 KDa)
ACTIN (42 KDa)
TGF-β1/Actin ratio
1.5
1.0
0.5
0.0
SHAM 16d
CCI 16d
CCI+LP2 16d
D
SHAM 21d
CCI 21d
CCI LP2 21d
TGF-β1 (44 KDa)
ACTIN (42 KDa)
TGF-β1/Actin ratio
1.5
1.0
0.5
0.0
*
#
SHAM 21d
CCI 21d
CCI+LP2 21d

Figure 3.


A
Relative mRNA ratio of TGFBR2/GAPDH (fold increase)
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1.0
0.5
0.0
*
#
SHAM 16d
CCI 16d
CCI + LP2 16d
B
Relative mRNA ratio of TGFBR2/GAPDH (fold increase)
1.5
1.0
0.5
0.0
*
#
SHAM 21d
CCI 21d
CCI + LP2 21d
C
SHAM 16d
CCI 16d
CCI LP2 16d
TGFβR2 (65 KDa)
GAPDH (37 KDa)
TGFBR2/GAPDH ratio
1.5
1.0
0.5
0.0
SHAM 16d
CCI 16d
CCI+LP2 16d
D
SHAM 21d
CCI 21d
CCI LP2 21d
TGFβR2 (65 KDa)
ACTIN (42 KDa)
TGFBR2/Actin ratio
1.5
1.0
0.5
0.0
*
#
SHAM 21d
CCI 21d
CCI+LP2 21d

Figure 4.

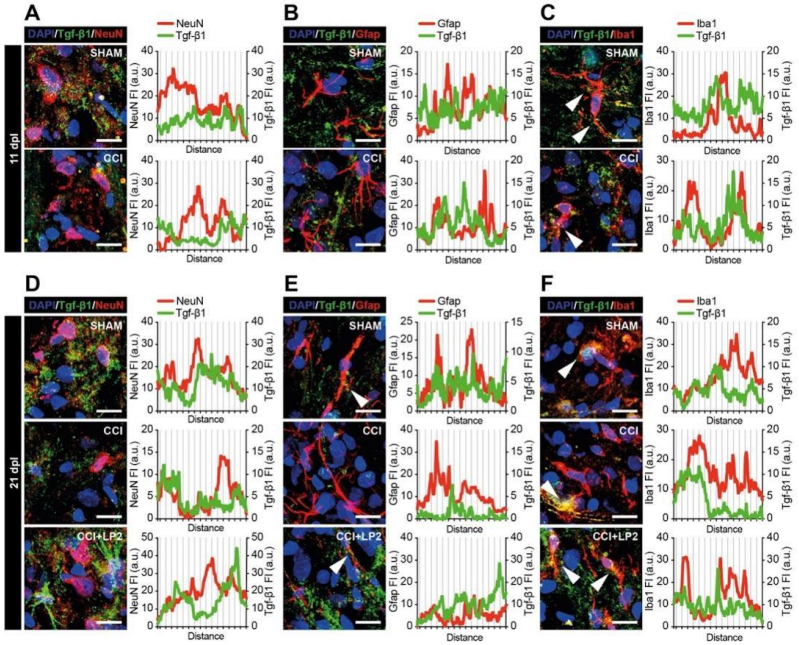

Figure 5.

Figure legends

Fig. 1 – **The MOPr/DOPr agonist LP2 shows an anti-allodynic effect in CCI animal model. (A)** Schematic representation of the experimental design. WB= Western Blot; Real Time-PCR= Real Time Polymerase Chain Reaction; IHF= Immunohistofluorescence analysis. **(B)** Withdrawal thresholds measured with von Frey's filaments on SHAM-vehicle, CCI-vehicle rats, and CCI + LP2-treated rats at 0, 11 (before the start of LP2 administration), 16, and 21 dpl. Data are shown as mean ± SEM of $n = 8$ rats per group. *$p < 0.001$ vs. SHAM-vehicle. #$p < 0.001$ vs. CCI-vehicle.

Fig. 2 – **CCI increases pro-inflammatory cytokines levels and reduces TGF-β1 levels 11 dpl.** Measurement of **(A)** TGF-β1 (t=3,593, df=4; F, DFn, Dfd= 1,031, 2, 2), **(B)** TGFβ-R2 (t=3,761, df=4; F, DFn, Dfd= 3,027, 2, 2), **(C)** IL-6 (t=5,955, df=6; F, DFn, Dfd= 1,332, 3, 3), and **(D)** IL-1β (t=3,206, df=5; F, DFn, Dfd=6,952, 3, 2) mRNA expression levels (RT-qPCR) in spinal cord of SHAM or CCI rats after 11 days. The abundance of each mRNA of interest was expressed relative to the abundance of GAPDH mRNA, as an internal control. Data are shown as mean ± SEM of $n = 3$-4 rats per group. Statistical analysis was performed using Student's t-test. *$p < 0.05$ *vs.* SHAM.

Fig. 3 – **LP2 treatment rescues TGF-β1 levels at 16 and 21 days after chronic constriction injury.** Measurement of TGF-β1 at 16 days and **(B-D)** TGF-β1 at 21 days mRNA and protein expression levels in spinal cord of SHAM, CCI, and CCI + LP2 rats. **(A-B)** The abundance of each mRNA of interest was expressed relative to the abundance of GAPDH mRNA, as an internal control. Data are shown as mean ± SEM of $n = 3$ rats per group. TGF-β1 16 days (F (2, 6)=10,02); TGF-β1 21 days (F (2, 7) = 9,961). **(C-D)** Representative immunoblots of TGF-β1 (44 kDa) in total protein extracts from spinal cord tissues at 16 (F (2, 15) = 1,222) and 21 (F (2, 9) = 6,638) dpl. Histograms refer to independent experiments means ± SEM of the TGF-β1 densitometric values normalized against actin used as internal control. Data are shown as mean ± SEM of $n = 4$-6 rats per group.

Statistical analysis was performed using one-way ANOVA with Bonferroni's *post-hoc* analysis. *p < 0.05 *vs.* SHAM; #p < 0.05 *vs.* CCI.

Fig. 4 – **CCI induces a reduction of TGFβ-R2 mRNA and protein expression levels reversed by a chronic treatment with LP2.** Effects induced by CCI procedure in the absence or presence of LP2 treatment at 16 and 21 dpl on TGFβ-R2 levels evaluated by RT-qPCR and western blot analysis. **(A-B)** The abundance of each mRNA of interest was expressed relative to the abundance of GAPDH mRNA, as an internal control. Data are shown as mean ± SEM of $n = 3$ rats per group. TGFβ-R2 16 days ($F(2, 7) = 22{,}66$); TGFβ-R2 21 days ($F(2, 5) = 13{,}51$). **(C-D)** Representative immunoblots of TGFβ-R2 (65 kDa) in total protein extracts from spinal cord tissues at 16 ($F(2, 12) = 0{,}8078$) and 21 ($F(2, 12) = 7{,}197$) dpl. Histograms refer to independent experiments means ± SEM of the TGFβ-R2 densitometric values normalized against GAPDH and actin used as internal controls by one-way ANOVA with Bonferroni's *post-hoc* for statistical analysis. Data are shown as mean ± SEM of $n = 4$-6 rats per group. *p < 0.05 *vs.* SHAM, # p < 0.05 *vs.* CCI.

Fig. 5 – **TGF-β1 profile in neurons, astrocytes and microglial cells in ipsilateral dorsal horns of CCI injured spinal cord.** Representative confocal images of TGF-β1 (green) and NeuN (red in **A** and **D**), GFAP (red in **B** and **E**) and Iba1 (red in **C** and **F**) immunohistofluorescence analysis in ipsilateral dorsal horns of SHAM and CCI rats at 11 dpl and of SHAM, CCI and CCI + LP2 treated rats at 21 dpl **(D-F).** Scale bars 10 μm. Profile plots of mean fluorescence intensity, expressed as arbitrary units (a.u.), are also shown.

Table 1. The list of primers used for quantitative real-time PCR (qRT-PCR).

Official name[#]	Official symbol	Alternative titles/symbols	Detected transcript	Amplicon Length	Cat. No.[§]
interleukin 1 beta	Il1b	Il-1b; IL-1beta; IL-1β	NM_008361 XM_006498795	150 bp 682 bp	QT01048355
interleukin 6	Il6	Il-6	NM_031168	128 bp	QT00098875
transforming growth factor, beta 1	Tgfb1	Tgfb; Tgfb-1; TGFbeta1; TGF-beta1	NM_011577	145 bp	QT00145250
transforming growth factor, beta receptor 2	Tgfbr2	Tgfbr2T; TGF-beta 2	NM_031132 XM_008766690	99 bp	QT00182315

[#]https://www.ncbi.nlm.nih.gov/gene/

[§]https://www.qiagen.com/it/shop/pcr/real-time-pcr-enzymes-and-kits/two-step-qrt-pcr/quantitect-primer-assays/

Chapter 6.

Review

Improving Cognition with Nutraceuticals Targeting TGF-β1 Signaling

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Abstract

Rescue of cognitive function represents an unmet need in the treatment of neurodegenerative disorders such as Alzheimer's disease (AD). Nutraceuticals deliver a concentrated form of a presumed bioactive(s) agent(s) that can improve cognitive function alone or in combination with current approved drugs for the treatment of cognitive disorders. Nutraceuticals include different natural compounds such as flavonoids and their subclasses (flavan-3-ols, catechins, anthocyanins, and flavonols), omega-3, and carnosine that can improve synaptic plasticity and rescue cognitive deficits through multiple molecular mechanisms. A

deficit of transforming growth factor-β1 (TGF-β1) pathway is an early event in the pathophysiology of cognitive impairment in different neuropsychiatric disorders, from depression to AD. In the present review, we provide evidence that different nutraceuticals, such as Hypericum perforatum (hypericin and hyperforin), flavonoids such as hesperidin, omega-3, and carnosine, can target TGF-β1 signaling and increase TGF-β1 production in the central nervous system as well as cognitive function. The bioavailability of these nutraceuticals, in particular carnosine, can be significantly improved with novel formulations (nanoparticulate systems, nanoliposomes) that increase the efficacy and stability of this peptide. Overall, these studies suggest that the synergism between nutraceuticals targeting the TGF-β1 pathway and current approved drugs might represent a novel pharmacological approach for reverting cognitive deficits in AD patients.

Keywords: Alzheimer's disease; cognition; transforming growth factor-β1; nutraceuticals; medicinal herbs; omega-3 and omega-6 fatty acids; carnosine

* *Published in Antioxidants 2021, 10, 1075.*

1. Nutraceuticals and Mental Health

Focus on Cognitive Function the term "nutraceutical" dates back to more than 30 years ago [1]. Among the numerous definitions, González-Sarrías et al. classified nutraceuticals as "a type of dietary supplement that delivers a concentrated form of a presumed bioactive(s) agent(s), nutrient or non-nutrient, but from food origin" [2]. Vegetables, containing a variety of bioactive compounds and micronutrients able to ameliorate the health status and/or decrease the risk of developing different diseases (e.g., omega-3 fatty acids), are also considered nutraceuticals [3]. According to World Health Organization , mental health, an integral and essential component of health, is "a state of well-being in which an individual realizes his or her own abilities, can cope with the normal stresses of life, can work productively

and is able to make a contribution to his or her community" (https://www.who.int (accessed on 23 June 2020)). In healthy people, cognition refers to the mental abilities allowing them to receive, acquire, and elaborate information from the surrounding environment and involving several brain areas [4]. Cognition is based on complex processes such as attention, perception, planning, learning, memory, and language [5], coordinated by executive control, emotional, and motivational components, that modulate the behavior of each individual person. An impairment of cognitive function represents a clinically relevant dimension in different neuropsychiatric disorders such as schizophrenia, depression, and Alzheimer's disease (AD) [6]. Among the natural compounds, flavonoids and their subclasses such as flavan-3-ols, catechins, anthocyanins, and flavonols have been associated with cognitive health, and can significantly improve cognitive function through multiple molecular mechanisms [7]. Flavonoids may promote synaptic plasticity changes, influencing memory and learning processes by acting on extracellular receptor kinase, Akt (also known as protein kinase B (PKB)) and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) [8]. Omega-3 fatty acids, essential polyunsaturated fatty acids also known as omega-3 oils, ω−3 fatty acids or n−3 fatty acids, and polyphenols are abundant micronutrients that are part of the human diet, and are known to exert protective effects on the central nervous system (CNS) thanks to their ability to modulate adult neurogenesis, synaptic and neuronal plasticity, promoting neuronal homeostasis and finally improving cognitive function [9,10]. Polyphenol-rich extract as an add-on to a healthy lifestyle may represent an additional pharmacological tool for the improvement of both working memory and attention [11]. Furthermore, an increased consumption of polyphenol-rich foods has been associated with better cognitive performance in elderly subjects with a high cardiovascular risk [12]. Plant-based foods, such as fruits, vegetables [13], wholegrains [14], nuts and legumes [15] are linked to many plausible effects toward human health, including

brain related disorders. These effects are mediated, at least partially, through the anti-oxidant and anti-inflammatory activity of vitamins and polyphenols [10]. The synergism between nutraceuticals and current approved drugs for the treatment of cognitive disorders can be used as a novel pharmacological approach to improve cognitive function in patients with neurodegenerative disorders. For example, the co-administration of vitamin D and memantine, an N-methyl-D-aspartate (NMDA) antagonist used for the treatment of moderate-to-severe AD, improved the cognitive performance in AD patients when compared with memantine alone [16]. Carnosine is a natural bioactive dipeptide synthesized starting from its two constituting amino acids, β-alanine and L-histidine, through the activity of carnosine synthase enzyme [17,18]. This nutraceutical dipeptide, widely distributed in the tissues and organs of vertebrates [19], has been shown to possess pro-cognitive effects under both physiological and pathological conditions. The dietary supplementation of carnosine in combination with its methylated analogue anserine for more than 12 weeks has been shown to improve cognitive function [20,21], also preserving verbal episodic memory and brain perfusion [20,22], and positively modulating network connectivity cognition-associated changes [20] in elderly subjects. With regard to the therapeutic effects in cognitive disorders, carnosine gave beneficial cognitive effects in Gulf War illness [23], mild cognitive impairment (MCI) [24], and AD [25,26] subjects. In a study carried out by Fonteh et al., a selective deficit of carnosine has been linked to cognitive decline in probable AD subjects [27]. In the present review, we will focus on selected nutraceuticals that are able to enhance cognitive function by targeting a specific pathway, the transforming growth factor-β1 (TGF-β1) pathway, which exerts a key role in the pathophysiology of cognitive disorders.

2. TGF-β1 in Cognitive Disorders

TGF-β1 is a well-known anti-inflammatory cytokine that can act as a neurotrophic factor exerting an essential role in the initiation and maintenance of neuronal differentiation and synaptic plasticity at CNS level. TGF-β1 is able to protect neurons against the damage induced by different stimuli such as excitotoxins, hypoxia/ischemia, and amyloid-β (Aβ) aggregates [28,29].

TGF-β1 signaling is initiated at the cell membrane surface through the binding of TGF-β to TGF-β type II receptor (TβRII) (homodimers) which recruits activin-like kinase 5 (ALK5)/TGF-β type I receptor (TβRI) (homodimers) forming a heterotetrameric complex with the ligand in which TβRII phosphorylates and activates TβRI [30,31] (**Figure 1a**).

Upon activation, TβRI phosphorylates Smad2 and Smad3 (R-Smads) that will form a complex with the Co-Smad protein Smad4 [32,33]. This complex will then translocate into the nucleus, regulating the transcription of genes involved in different cell functions such as proliferation, differentiation, and adhesion [34,35]. Not all Smad proteins are activators, in fact Smad6 and Smad7 inhibit activation of R-Smads, then inhibiting the genes' transcription taking place at nuclear level.

In addition to Smad-dependent pathways, TGF-β1 can also activate Smad-independent pathways, including the extracellular-regulated kinase pathway [35], the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [36], c-Jun amino terminal kinase (JNK) pathway [37], and the phosphatidylinositol-3-kinase (PI-3-K)/ AKT pathway [38], involved in several processes such as the inhibition of cell-cycle, suppression of immune response, and neuroprotection [39] (**Figure 1b**).

These receptor-activated, nonSmad transducers can mediate signaling responses either as stand-alone pathways or in combination with Smads, and they can also converge onto Smads to control Smad activities. TGF-β1 plays a key role in neuronal homeostasis function axon elongation, and synaptogenesis; TGF-β1 signaling through Smad2 and/or Smad3 is also essential for maintaining quiescent

microglia after injury [40]. Furthermore, this factor is able to inhibit free radical production and to induce apoptosis of stem/progenitor cells [40]. TGF-β1 can enhance synaptic plasticity by promoting the expression of brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (TrkB) [41,42]. In addition to the above described, TGF-β1 has been demonstrated to be essential for the transition from early (E-LTP) to late long-term potentiation (L-LTP), underlining its role in recognition memory formation [43]. TGF-β1 knockout (KO) mice present dendritic spine density reduction and hippocampal LTP impairment [44]. The dysfunction of TGF-β1 signaling has been associated with neurodegenerative disorders; an impairment of TGF-β1 signaling has been reported in AD pathogenesis, thereby contributing to Aβ accumulation, activation of microglia as well as to the progression of neurodegeneration [42,45]. In most cases AD patients exhibit decreased levels of nuclear Smad2, Smad3, and Smad4 in the temporal cortex [46], while TβRII expression is reduced at neuronal level in an early phase of cognitive decline [45]. The double action of TGF-β1 on Smad-dependent and Smad-independent signaling is relevant when considering the pathophysiology of cognitive decline in AD and the selective impairment of Smad signaling detected in AD brains [46]. According to this scenario, the identification of nutraceuticals able to activate these Smad-independent signaling pathways and counteract the deficit of Smad signaling might be relevant for preventing AD-related cognitive decline. Cognitive deficits are also clinically relevant in major depression, and common pathophysiological events have been identified in depression and AD, including neuroinflammation and an impairment of TGF-β1 signaling pathways [42]. Aβ injection into the dorsal hippocampus of rats has been connected to neurotoxic effects that were further amplified by intracerebroventricular (i.c.v.) injection of SB431542, a selective inhibitor of TβRI [38,47]. We have recently demonstrated a deficit of TGF-β1 signaling in a non-transgenic animal model of AD at hippocampal level [48], a brain area essential in

the storage and consolidation of short-term memory that is impaired in early stages of AD [49]. Different second-generation antidepressants, in particular selective reuptake inhibitors (SSRIs), such as fluoxetine, are able to increase TGF-β1 levels in depressed patients [50] and reverse memory impairment in AD animal models [51]. It has been demonstrated that fluoxetine exerts neuroprotective effects in an in vitro experimental model of Aβ-induced neurodegeneration via a TGF-β1-mediated mechanism [52]. Furthermore, a chronic treatment with fluoxetine, or the new multimodal antidepressant vortioxetine, has been shown to completely reverse depressive-like phenotype and memory deficits in Aβ-injected mice by the rescue of hippocampal TGF-β1 levels [48]. Along this line, we can hypothesize that nutraceuticals targeting TGF-β1 signaling pathways can synergize with antidepressants to rescue cognitive function both in depression and AD. Astrocytes represent the main source of TGF-β1 in the CNS and in the absence of pathological conditions this cell type synthesizes and releases this neurotrophic factor in different brain regions [53]. However, a TGF-β1 neuronal expression has also been reported [54,55]. In addition, TGF-β1 can be synthesized and secreted from microglia in response to inflammatory cytokines [40,56]. An in vivo study conducted by Yap et al. using an animal model of ischemic stroke [57] demonstrated that astrocytes are able to secrete Interleukin 6 (IL-6) with consequent inhibition of T helper 1 cell differentiation and the promotion of regulatory T cells (Tregs) with an increase in TGF-β1 levels contributing to the effect of hyperforin on neuroangiogenesis and functional recovery. TGF-β1 expression and activity are primarily regulated through the conversion of latent TGF-β1 to active TGF-β1 by a variety of proteases, among which matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) play a central role in this conversion [58]. Psychotropic drugs, such as fluoxetine, promote the release of active TGF-β1 by favoring the activation of MMP-2 in astrocytes, and the ensuing maturation of latent TGF-β1 [52]. We can speculate that an increased conversion of latent TGF-β1 to

active TGF-β1 might underlie the increased secretion of TGF-β1 by cortical astrocytes induced by some nutraceuticals such as hesperidin [59] and therefore hypothesize that cortical astrocytes might represent an ideal cellular target for natural compounds (e.g., flavonoids) [60] able to promote the secretion of TGF-β1 and then improve cognitive function. It is known that physical activity promotes cognitive and memory functions by modulating the signaling pathway of neurotrophic factors [61] and, in turn, physical activity can exert "neuroprotective effects" after brain injury [62]. Indeed, physical activity from one hand promotes neurogenesis via synthesis and release of BDNF, one of the key neurotrophic factors involved in brain plasticity [63], and on the other hand it increases TGF-β1 plasma levels [64], thus suggesting that physical activity can be considered as an add-on strategy to the conventional drug treatment. According to this hypothesis, a very recent study carried out by Szymura and collaborators showed that the concentrations of TGF-β1 and BDNF increased in the blood samples obtained from healthy older adults as well as in subjects suffering from Parkinson's disease (PD) after 12 weeks of regular balance training of moderate intensity [65]. An open question remains regarding how and when a rescue of TGF-β1 levels can affect global cognitive function in these patients and what impact might be of nutraceutical targeting the TGF-β1 pathway.

3. Nutraceuticals Targeting TGF-β1 Pathway: Evidence from Preclinical Studies

Medicinal plants are used in traditional medical practice to alleviate or, in the best scenario, cure human suffering and illness. Medicinal plants represent a wide source of bioactive phytochemicals that play a key role in preventing chronic diseases such as cancer and diabetes [66]. In recent years, herbs have been considered an alternative approach for the treatment of neuropsychiatric disorders, and in particular of anxious and cognitive disorders, due to their good safety profile

compared to current approved drugs [67]. Each class of these phytochemical compounds contains a wide range of active compounds characterized by different potencies, with some of them presenting multifunctional activity [68]. Different plants or natural compounds extracted from medicinal herbs with the potential ability to improve cognitive functions have been identified during the last decades. Among them, polyphenols, aromatic compounds isolated from fruits, vegetables, and grains have shown the ability to suppress neuroinflammation and improve memory and cognitive impairment [69]. In particular, among the subclasses of flavonoids associated with the improvement of cognitive status [7], flavonols and flavanones are able to increase the levels of TGF-β1 [70,71].

3.1. Medicinal Herbs

John's wort, known botanically as Hypericum perforatum, is a natural agent with antidepressant activity [72], which has also recently been considered as an enhancer on cognitive function [73]. The extract of H. perforatum has proved to be neuroprotective in animal models of AD [74] and the hypericin, one of the most effective active compounds, is able to prevent stress-induced memory deficits and improve recognition memory induced by chronic stress in rats [75]. Hypericin promotes wound-healing phenomena [76] by inducing vascular-endothelial growth factor (VEGF) and TGF-β1 production in the burn wound area [77]. Furthermore, it has been demonstrated by Yechiam et al. that the acute administration of a low dose of H. perforatum (500 or 250 mg of H. perforatum quantified to either 1 or 0.5 mg of hypericin) has a positive effect on short-term verbal memory in healthy subjects [78]. In a different study employing an animal models of stroke, hyperforin, the main active ingredient derived from H. perforatum, showed the ability to promote neuroangiogenesis and functional recovery by stimulating the production of IL-4, IL-6, and TGF-β1 [57]. Both IL-4 and TGF-β1 exerted a key role in promoting the protective activity of hyperforin in post-stroke angiogenesis

and recovery. In a recent preclinical study, the intranasal administration of hyperforin was able to improve post-stroke social isolationinduced exaggeration of post-stroke depression and anxiety and promoted hippocampal neurogenesis and cognitive function by rescuing TGF-β1 levels [79]. Flavonoids, a large family of polyphenolic secondary metabolites found in plants, prevent the cognitive deficits associated with chronic inflammation in vivo [80–82]. Flavonoid induced activation of neuronal and glial signaling has been linked to the regulation of mammalian target of rapamycin (mTOR), vascular endothelial growth factor B (VEGF-B), and TGF-β1, promoting changes in synaptic plasticity and neurogenesis, which ultimately positively influence memory, learning and cognition [8,83]. Among flavonoids, hesperidin, a naturally flavanone glycoside present in Citrus sinensis [84], has been shown to improve memory performance in adult mice through increased secretion of TGF-β1 by cortical astrocytes [59]. This natural compound is also able to improve post-stroke depressive and anxiety behavior promoting neurogenesis at hippocampal level and memory function by TGF-β signaling [79]. In a different study, conducted by Li et al., a treatment for 10 days with hesperidin ameliorated behavioral impairments and neuropathology of transgenic amyloid precursor protein/presenilin 1 (APP/PS1) mice, also reducing microglial activation and TGF-β1 type 1 receptor in both the cortical cortex and hippocampus [85]. Icariin, the major constituent of flavonoids from Epimedium brevicornum, has demonstrated a relevant neuroprotective activity in animal models of AD as well as the ability to ameliorate the cognitive deficits induced by permanent occlusion of bilateral common carotid arteries (BCCAO) by reducing the BCCAO-induced TGF-β1 over-expression and Smad2/3 phosphorylation [86]. Icariin exerts a protective role in AD counteracting oxidative stress phenomena [87] and, most importantly, prevents memory deficits in Aβ-injected rats [88] by rescuing the BDNF signaling pathway and reverting decreases in postsynaptic density protein (PSD-95) and the phosphorylated form of cAMP response element-

binding protein (p-CREB) levels. We have recently found a similar deficit of PSD-95 paralleled by a deficit of hippocampal TGF-β1 in the same animal model of AD [48]. It is also known that TGF-β1 signaling and the BDNF pathway are strictly interconnected, and that TGF-β1 enhances the expression of both BDNF and TrkB [41]. We can hypothesize that the neuroprotective effects of icariin can be mediated by an increased TGF-β1 production and the following release of BDNF, but new preclinical studies in the same animal model of AD should be conducted to validate this hypothesis.

3.2. Omega-3 and Omega-6 Fatty Acids

Omega-3 and omega-6 fatty acids represent two main families of fatty acids that cannot be synthesized by the human body, are therefore "essential", and need to be introduced by the diet. Several studies have reported a positive correlation between omega3 supplementation and a reduced risk of developing cognitive decline and dementia [89–91]. In addition to this evidence, it has been shown that omega-3 fatty acids are able to influence brain development and improve reference memory and mood [89,92,93]. Along this line, the reduction in omega-3 and/or omega-6 intake by the diet contributes to cognitive decline [94]. In vivo studies have demonstrated that the deficiency in omega-3 intake could also be associated with reduced biosynthesis of noradrenaline and dopamine in rat brains and then linked to a decreased learning ability [95], whereas omega-3 chronic administration improves reference memory and learning [96], and increases neuroplasticity of nerve membranes [97]. The benefit of omega-3 supplementation on cognition has also been observed in different clinical trials; in this regard, Fontani et al. have shown a positive effect of omega-3 polyunsaturated fatty acids on cognitive domains in healthy subjects, in particular, an improvement in attentional and physiological functions, particularly those involving complex cortical processing [98]. Based on the above-mentioned information, omega-3 levels and/or omega-6/omega-3 ratio could represent novel pharmacological tools for the prevention of cognitive

impairment during aging and in the prodromal phase of AD [99]. Omega-3 fatty acids are characterized by an anti-inflammatory activity, as demonstrated in different studies on diseases including diabetes, arthritis, cancer, depression, and AD [100–102]. Lower arachidonic and docosahexaenoic acids (DHA) levels were associated with higher pro-inflammatory (e.g., IL-6) and lower anti-inflammatory (e.g., TGF-β1 and IL-10) cytokines concentrations [103]. Sharma et al. demonstrated that omega3 fatty acids possess an inhibitory activity in ovarian cancer cells in which TGF-β1, Smad-3, and p21 levels were increased [104]. The DHA showed immunomodulatory and antiinflammatory activities in an animal model of atopic dermatitis by increasing TGF-β1 expression and suppressing the secretion of pro-inflammatory cytokines by CD4+ T cells and macrophages [105]. Recently, Xu and colleagues showed a positive effect of omega-3 supplementation in a chronic renal failure animal model by regulating the nuclear factor erythroid 2–related factor 2 (Nrf2) and TGF-β/SMAD pathway [106]. The ability of omega-3 fatty acids to increase the synthesis of TGF-β1 has been shown both in vitro and in vivo [107,108]; in particular, a multicenter, randomized, double-blind, placebocontrolled trial conducted by Krauss-Etschmann and co-workers employing 311 pregnant women, long-term fish oil supplementation—containing a high concentration of omega-3 and omega-6—was associated with decreased mRNA levels of T(H)2-related molecules in the fetus and decreased maternal inflammatory cytokines, combined with an increased production of TGF-β1 acting as a master regulator in decreasing maternal inflammation [109]. Preclinical studies show that omega-3 intake is associated with an improvement in cognitive deficits paralleled by an antioxidant effect in animal models of AD, and also that the chronic administration of docosahexaenoic acid improves learning ability in aged rats [110,111]. Furthermore, a reduction in neuroinflammatory phenomena and Aβ-amyloid accumulation have been observed following the administration in vivo of omega-3 fatty acids [112,113]. Unfortunately in these studies the authors did not

explore the impact of omega-3 fatty acids on TGF-β1 signaling, but recent studies in animal models of depression, a well-known risk factor for AD, support the hypothesis that omega-3 fatty acids can stimulate in vivo the secretion of TGF-β1 from microglial cells [114]. Gu et al. demonstrated that the endogenous omega-3 polyunsaturated fatty acid (PUFA) administration is able to counteract depressive-like behavior lipopolysaccharide (LPS)-induced by rescuing TGFβ1 levels and by balancing microglial M1 and M2 phenotypes [114]. Interestingly, lower concentrations of omega-3 fatty acids (in particular, eicosapentaenoic acid (EPA)) have been detected in humans in fasting plasma associated with lower TGF-β1 levels [103], and the supplementation with high dose of omega-3 fatty acids is able to reduce depressive symptoms in adolescent depressed patients [115]; despite this evidence, new clinical studies both in depressed and AD patients are needed in order to understand whether the cognitiveenhancing activity of omega-3 PUFA can be mediated by a rescue of TGF-β1.

3.3. Multifunctional Nutraceuticals Able to Target TGF-β1 Signaling: Focus on Carnosine and Its Therapeutic Potential in Cognitive Disorders

Among the multitude of nutraceuticals to be considered as novel therapeutic tools in improving cognition and/or counteracting cognitive disorders such as AD, recent evidence suggests a relevant therapeutic potential for the naturally occurring dipeptide carnosine (β-alanyl-L-histidine), a nutraceutical characterized by a multimodal and neuroprotective activity that includes the scavenging of reactive species [116], the negative regulation of pro-inflammatory markers [117], and the modulation of immune cells (e.g., macrophages and microglia [18,47,118–120]), and could thus play an important role in preventing and/or counteracting cognitive disorders often characterized by high levels of oxidative stress and neuroinflammation [121].

The ability of carnosine to modulate the activity of the above-mentioned immune cells is clinically relevant, since it has been shown that the dysfunction of both macrophages and microglia, the resident innate immune cells in the CNS, strongly contribute to cognitive decline detected in different neurodegenerative disorders such as Down syndrome, MCI, and AD [122–124]. With specific regard to TGF-β1, there are several studies showing the ability of carnosine to positively modulate the synthesis and the release of this pleiotropic cytokine [18,47]. In particular, in a study carried out by Fresta et al., carnosine, used at a physiological concentration (5–20 mM), was able to increase the mRNA expression levels of TGF-β1 in LPS-stimulated macrophages; this activity was also accompanied by the amelioration of the macrophage energy status, the down-regulation of the expressions of pro-inflammatory molecules and pro-oxidant enzymes, as well as the positive modulation of the expression levels of different members of the antioxidant machinery [18]. All these modulatory activities are of interest when considering different CNS disorders characterized by cognitive decline deriving from increased oxidative stress combined with neuroinflammation. Still considering the immune cells, in a model of an Aβ-induced neuroinflammation, carnosine was able to increase the gene expression levels as well as the protein secretion of TGF-β1, simultaneously preventing microglial cell death and lowering oxidative stress [47], all factors that are strictly connected to the risk of developing dementia and, more in general, to the aging-related cognitive decline [125]. In the same study, the key role played by TGF-β1 in mediating the beneficial effects of carnosine was validated by using a selective inhibitor of the type-1 TGF-β receptor (SB431542). In addition to the above-mentioned in vitro studies, the ability of carnosine to increase the production of TGF-β1, also playing a key role in hippocampal synaptic plasticity and memory [43], has also been observed in vivo in a mouse model of type 2 diabetes [126], a known risk factor for the development of AD [127,128]. When considering the therapeutic potential of carnosine, it should be also taken into

account that the strong preclinical evidence is also strengthened by human studies showing an enhancement of cognition in elderly people as well as in subjects suffering of brain-related disorders [129]. All these data suggest a multimodal mechanism of action of carnosine underlying its therapeutic potential for the treatment of cognitive disorders, especially through the positive modulation of TGF-β1 production. Nevertheless, a major unmet need in this field remains that of increasing the bioavailability of carnosine both in rodents and humans after its systemic administration [125]. Carnosine administration in humans only leads to a small increase in circulating carnosine, because of its fast degradation by serum carnosine dipeptidase 1 (CNDP1) [130]. In light of the above metabolism, during the last decades lots of efforts have been made in order to develop new approaches or new formulations of carnosine able to improve its bioavailability and target delivery. A first approach might be the use of potent and selective inhibitors of CNDP1, such as carnostatine, in combination with carnosine, to increase carnosine's bioavailability [131]. Alternatively, intranasal administration of carnosine has been proposed to bypass the blood-brain barrier (BBB) and first-pass metabolism [132,133]. Recent studies suggest that novel formulations can be developed to increase the therapeutic potential of carnosine.

3.4 Increasing Carnosine Delivery and Its Bioavailability: Focus on Vesicular, Nanoparticulates Systems and Derivatives

In recent decades, the delivery of carnosine into innovative formulations (drug delivery systems) has attracted a lot of interest (**Figure 2**). In particular, vesicular, nanoparticulate systems and carnosine derivatives have been investigated (**Table 1**).

From 2001 to 2007, the first strategy investigated was the derivatization of carnosine to increase its stability to carnosinases, representing an important limit for the therapeutic use of this molecule due to the reduction in its bioavailability

[145]. Carnosine was derivatized with β-cyclodextrin and trehalose; both formulations demonstrated an antioxidant efficacy at concentrations 10–20 times lower than that reported for other synthetic derivatives [134,136]. Derivatization was also studied to facilitate the site-specific transport to different tissues. Bellia et al. investigated a new carnosine derivative (BioCar) with biotin [136]. They demonstrated an increase in the stability of this derivative towards the hydrolytic action of serum CNDP1. Moreover, the binding affinity of BioCar to avidin and streptavidin were studied with the aim to exploit the potential functionalization of gold nanoparticles.

Among the few published studies, great attention has been focused on magnetic nanoparticles coated with L-carnosine [137]. This strategy can be advantageous for its dual effect: achieving nanoparticle stability and enhancing the carnosine therapeutic effect. Peptide and/or proteins are some of the most promising materials serving as protective layers on superparamagnetic iron oxide nanoparticles (SPION) [138]. Carnosine-coated iron oxide nanoparticles have been prepared via co-precipitation of iron oxide in the presence of carnosine. The synthesized carnosine-coated magnetic nanoparticles might be applied to diagnosis and targeted drug delivery for cancer therapy [139]. Farid et al. developed stimuli-responsive magnetic nanoparticles coated with carnosine as promising nanoplatforms for breast cancer therapy. Surface grafting of magnetic nanoparticles with the dipeptide carnosine maintained nanoparticles' colloidal stability, preventing their agglomeration. In vitro cytotoxicity results revealed superior cytotoxic effects of carnosine-coated magnetic nanoparticles on human breast cancer cell lines compared with a carnosine solution. In vivo chemotherapeutic activity on Ehrlich Ascites tumor Bagg Albino (Balb)-C mice model showed that carnosine-coated magnetic nanoparticles exhibited a significant reduction in tumor size with no observed systemic toxicity. In addition, carnosine-coated magnetic nanoparticles

displayed superior anti-angiogenic effects compared with a carnosine solution [137].

Another interesting application can be found in the study of Lu et al., in which Fe3O4 nanoparticles/poly(lactic-co-glycolic acid) (PLGA) polymer-loaded dexamethasone functionalized with carnosine were prepared and investigated as a drug delivery platform for simultaneous BBB crossing and treatment of ischemic stroke. The incorporation of this dipeptide has also played an efficient role in BBB crossing transcytosis under lipoprotein receptor-related protein (LRP) receptors to access the brain tissues [140].

Among different encapsulation strategies, vesicular systems have been investigated using phospholipids, surfactants or polymers, thus obtaining liposomes, niosomes or polymerosomes, respectively [146]. In order to improve the efficacy and stability of carnosine, nanoliposomes were prepared by the thin film hydration method comparing the effects of three different lipids on the vesicles' features (size, zeta potential, phase transition temperature and fluidity) [141]. Authors were able to demonstrate that 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as well as 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were able to provide the ideal nanoliposomes with the smallest size and highest encapsulation efficiency, probably due to the higher saturation degree compared to 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC).

Maestrelli et al. condensed carnosine with lipoic acid, obtaining a lipoic acid-based transient receptor potential akyrin type-1 antagonist, which was successfully encapsulated into niosomes for brain targeting [142]. Free carnosine and carnosine-loaded niosomes were investigated by in silico and in vitro studies to evaluate their effects on modifications of bovine serum albumin (BSA) and their interactions with specific amino acids [143]. Moulahoum et al. demonstrated the occurrence of a dose-dependent inhibition of advanced glycation end-products (AGE), advanced oxidation protein products (AOPP), and BSA aggregation, thus demonstrating the

potential of carnosine-loaded niosomes as a valid strategy in the treatment of age-related protein modification. Recently, a novel strategy based on carnosine encapsulation in lipoprotein receptor-related protein-1 (LRP-1)-targeted functionalized polymersomes for the treatment of ischemic stroke was developed [144]. This formulation showed neuroprotective effects at a dose of carnosine three orders of magnitude lower than that of free carnosine. The LRP-1-targeted functionalization was relevant for brain targeting, allowing a time-dependent polymerosome accumulation in the brain.

4. Conclusions and Perspectives

Nutraceuticals deliver a concentrated form of a presumed bioactive(s) agent(s) from food/vegetables that can improve cognitive function alone or in combination with current approved drugs for the treatment of cognitive disorders. Drug discovery in the field of cognitive disorders is currently focused on the identification of active principles, with strong neuroprotective activity and high therapeutic potential. Nutraceuticals include different natural compounds such as flavonoids and their subclasses (flavan-3-ols, catechins, anthocyanins, and flavonols), omega-3, and carnosine that can improve synaptic plasticity and increase cognitive function through multiple molecular mechanisms. Rescue of cognitive function still represents an unmet need in the treatment of neurodegenerative disorders such as AD. A deficit of TGF-β1 pathway is an early event in the pathophysiology of cognitive impairment in different CNS disorders, from depression to AD.

In the present review, we provided evidence that different nutraceuticals such as *H. perforatum* (hypericin and hyperforin), flavonoids such as hesperidin, omega-3, and carnosine can target TGF-β1 signaling, increase TGF-β1 production in the CNS and finally enhance cognitive function both in rodent models of cognitive disorders and in humans. The bioavailability of these nutraceuticals, in particular carnosine, can be significantly improved with novel formulations (nanoparticulate systems,

nanoliposomes, niosomes or polymerosomes) that increase the efficacy and stability of carnosine finally increasing its therapeutic potential in humans. The studies examined in the present review also suggest that the synergism between nutraceuticals targeting the TGF-β1 pathway and the drugs currently approved for the treatment of cognitive disorders might represent a novel pharmacological approach for rescuing cognitive function in patients with AD.

Author Contributions

All authors listed have made a substantial, direct, and intellectual contribution to the review. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by University of Catania, Programma Ricerca di Ateneo unict 2020–2022- Linea 2; Project 3N-ORACLE.

Conflicts of Interest

The authors declare no conflict of interest.

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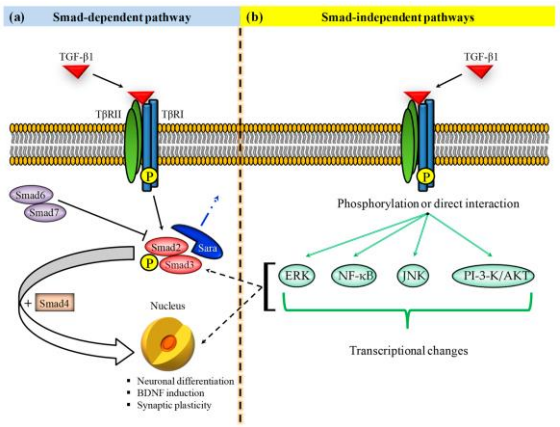

Figure 1.

(a) Smad-dependent and (b) Smad-independent TGF-β1 signaling pathways are involved in the regulation of various cellular processes, including cell growth/proliferation, differentiation, cell migration, invasion, and extracellular matrix remodeling. (**a**) TGF-β1 binds to the TβRII homodimers allowing the dimerization with TβRI homodimers, the activation of the kinase domain of TGF-βRI kinase domain, and the phosphorylation of both SMAD2 and SMAD3.

These phosphorylated proteins interact with SMAD4 leading to the formation of a heterotrimeric complex able to translocate into the nucleus with the subsequent activation or repression of different genes involved in neuronal homeostasis. (**b**) TGF-β1 can also recruit Smad-independent signaling pathways such as ERK, NF-κB, JNK, and PI-3-K/AKT. These non-Smad transducers can mediate signaling responses alone or in combination with Smads, also converging onto Smads to control Smad activities. P indicates phosphorylation. TβRII = TGF-β type II receptor; TβRI = TGF-β type I receptor; BDNF = brain-derived neurotrophic factor; Sara = Smad anchor for receptor activation; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells; ERK = extracellular signal-regulated kinase; PI-3-K = phosphatidylinositol-3-kinase; JNK = c-Jun amino terminal kinase.

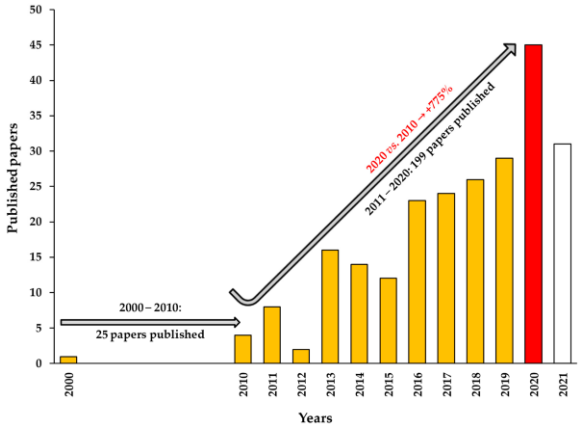

Figure 2.

Number of published papers from January 2000 to 2021 in which the words "carnosine and nanoparticles" were used in Science Direct (last update 1 May 2021). The production of papers from 2000 to 2010 is discontinued (one in 2000, two in 2004, four in 2005, four in 2007, three in 2008, and nine in 2009). The bar representing 2020, currently the most productive year, is highlighted in red. As clearly depicted, during the last decade (2011–2020) the number of publications has increased significantly compared to the previous ten years (2000–2010), 25 vs. 199 published papers (almost 8 times more). The current number of published papers in 2021, still in progress and indicated with a white bar, suggests that this year could represent the most productive of the last two decades.

Table 1. List of drug delivery strategies for carnosine.

	Delivery System	References
Carnosine derivatives	▪ Derivatized with β-cyclodextrins ▪ Derivatized with threalose ▪ Derivatized with biotin	(Lanza, Bellia et al. 2011) (Bellia, Oliveri et al. 2013)
Nanoparticulate systems	▪ Magnetic nanoparticles coated with L-carnosine	(Durmus, Kavas et al. 2011, Khramtsov,

	▪ Fe_3O_4 nanoparticles/poly(lactic-co-glycolic acid) (PLGA) polymer loaded dexamethasone functionalized with carnosine	Barkina et al. 2019, Farid, Gaafar et al. 2020) (Lu, Zhang et al. 2021)
Vesicular systems	▪ Nanoliposomes ▪ Niosomes ▪ Polymerosomes	(Maherani, Arab-Tehrany et al. 2012) (Maestrelli, Landucci et al. 2019, Moulahoum, Sanli et al. 2019) (Kim, Kim et al. 2020)

General discussion

In the present thesis we have conducted different studies to validate the role of TGF-β1 pathway as a new pharmacological target for the treatment of CNS disorders with a special focus on AD. At the same time, we have also identified different drugs able to rescue of TGF-β1 pathway and then prevent Aβ-induced toxicity. The evidence discussed in the present thesis suggest that a deficit of TGF-β1 is present not only in experimental models of Alzheimer's disease and depression, but also in neuropathic pain, a disease that often co-occurs with depression. In this enlarged scenario TGF-β1 can act with its neuroprotective action directly on neurons as well as preventing and reducing microglia activation.

It is known that TGF-β1 secreted both by astrocytes and neurons is able to regulate microglia activation and to reduce the release of inflammatory cytokines and reactive species. Endogenous TGF-β1 is implicated in several processes of brain development, such as astrocyte differentiation, and neuronal migration and survival. In physiological conditions, TGF-β1 production and release from astrocytes is increased and directed to neurons which in turn are protected by astrocytes against excitotoxic death (Gomes, Sousa Vde et al. 2005). In addition, TGF-β1 expressed by neurons can protect this cell type from CNS inflammation

and injury playing also a key role in the regulation of neuronal development and survival.

It has been demonstrated that TGF-β1 is neuroprotective against amyloid-related neurotoxicity by increasing Aβ clearance through BV-2 microglia cells but the role of this pleiotropic factor on neurons is not completely understood. Fang et al., by using primary hippocampal neuron cultures treated with $A\beta_{1-42}$ oligomers, showed that TGF-β1 pre-treatment exerts a neuroprotective effect against amyloid-induced neuronal degeneration and apoptosis. In addition, silencing type I TGF-β1 receptor gene in neurons, the anti-inflammatory and anti-apoptotic activity of TGF-β1 is abolished suggesting that the neuroprotective effect of this factor is mediated by its type I receptor (Fang, Sun et al. 2018).

TGF-β1 also synergize with other neurotrophins such as NGF and BDNF to protect neurons against toxic insults and to maintain neuronal health and exerting neurotrophic effects by the action of a high-affinity transmembrane receptor complex consisting of ALK5 and TGF-βR2.

In this context, in human AD brain it has been showed an impairment of TGF-β1 signaling with a particular deficit of its type 2 receptor (TGF-βR2) in patients with mild dementia (MMSE score, 21–25). A specific neuronal TGF-βR2 deficit may be specific to AD given that its levels were decreased in AD brain patients, but not in the brain tissue of patients with other type of neurodegenerative diseases such as frontotemporal dementia, Lewy body's disease, and PD (Tesseur, Zou et al. 2006). In addition, a reduced neuronal TGF-β1 signaling increased amyloid deposition and neurodegeneration in transgenic AD mice. In cultured neurons, estrogen-stimulated production of TGF-β1 by glial cells (Sortino, Chisari et al. 2004) or exogenous application of TGF-β1 in vivo (Prehn, Bindokas et al. 1996) have been shown to reduce Aβ-induced neurotoxicity.

A study conducted by Diniz et al., demonstrated that Aβ oligomers reduce the levels of astrocytic TGF-β1 both in vitro and in vivo paralleled to a decreased levels of

synaptic proteins in hippocampal neurons suggesting that a deficit of TGF-β1 signaling in astrocytes might be one of the mechanisms of Aβ-induced synaptotoxicity (Diniz, Tortelli et al. 2017).

However, the role of TGF-β1 in AD pathology is unambiguous because in transgenic AD animal models the co-expression of TGF-β1 accelerates the deposition of Aβ (Wyss-Coray, Masliah et al. 1997) and AD mice that overexpress TGF-β1 develop AD-like vascular and meningeal alterations (Gaertner, Wyss-Coray et al. 2005). In addition, vessel-derived TGF-β1 contributes to inflammatory processes in AD brain (Grammas and Ovase 2002). Furthermore, no change in the expression of TGF-β1 and TGF-β3 were observed in aging astrocytes isolated from different cortex areas and at hypothalamic level, except for TGF-β2 in the aged cerebellum suggesting that the differences observed across the studies in TGF-β1 expression may depend from brain region and cell type analyzed playing thus distinct roles in the synaptic dysfunction observed in AD (Boisvert, Erikson et al. 2018). These data suggested that TGF-β1 could promote the accumulation of Aβ in cerebral blood vessels, but on the other hand, TGF-β1 is able to reduce Aβ deposition at brain parenchyma level (Caraci, Battaglia et al. 2011).

In addition, increased levels of TGF-β1 were found in CSF of AD patients, but Mocali et al., observed a reduction of TGF-β1 total and cleaved (active) forms at plasma level of AD subjects (Tarkowski, Issa et al. 2002, Mocali, Cedrola et al. 2004). Consider the disagreement over these differences observed across the studies, the answer could be due to the evaluation of TGF-β1 levels in distinct stages of AD and therefore the increased TGF-β1 levels might have a protective role against neuronal death observed in the AD later stages.

It is known that $A\beta_{1-42}$ oligomers, the more toxic species in AD brain, when injected i.c.v. in mice, can induce both cognitive and depressive-like symptoms paralleled to a neuroinflammatory phenomena (Ledo, Azevedo et al. 2013).

We identified in our non Tg-animal model of AD, obtained by i.c.v. injection of Aβ oligomers, a selective impairment of hippocampal TGF-β1 signaling paralleling with a depressive-like phenotype that persists until 19 days after i.c.v. injection and memory deficits induced by Aβ oligomers.

Interestingly, we found that the chronic treatment with fluoxetine and vortioxetine was able to reverse this phenotype as well as restores recognition and reference memory impairment in Aβ-injected mice by completely rescuing TGF-β1 levels. Moreover, these antidepressant drugs showed a protective role against the synaptotoxic effects of Aβ oligomers by rescuing hippocampal synaptophysin and PSD-95 levels in Aβ-injected mice. Taking into consideration these evidences, we can say that the rescue of TGF-β1 signaling with second-generation antidepressants might represent a new pharmacological strategy to prevent both amyloid-induced depression and cognitive decline in AD.

An open question in the field of translational neuropharmacology remains to establish whether i.c.v. $A\beta_{1-42}$ oligomers can induce transient or long-term memory deficits in mice, although, different researcher groups demonstrated that this pre-clinical animal model is useful to investigate the molecular mechanisms by which Aβ oligomers interfere with cognitive processes (Balducci and Forloni 2014). In our amyloid-related depression animal model, we evaluated the cognitive deficit Aβ-induced by employing a well-known pre-clinical behavioral test, the ORT or also known as the novel object recognition test (NOR), a test based on the function of the hippocampus, the perirhinal and the medial temporal lobe cortices, widely used to measure memory functions in rodents as well as it is used to study the effects of different pharmacological treatments. It is known that the perirhinal cortex plays an important role in object recognition memory and the integrity of the medial temporal lobe is fundamental for recognition memory formation, and when some cortex damage occurs, the performance in recognition memory tasks is impaired (Antunes and Biala 2012). Taking in mind these informations, we can assume that

the cognitive deficits Aβ-induced in our non-Tg animal model are related to hippocampus functions impairment. In addition, we found that i.c.v. injection induces a contemporaneous deficit of reference and recognition memory, assessed 14 days after Aβ i.c.v. injection. We adopted this non-Tg AD animal model because we know from our previous work that the amount of Aβ oligomers injected reaches a cerebral concentration comparable with the concentration of soluble Aβ observed in AD brains, e.g., close to 1 μg/g (Leggio, Catania et al. 2016).

To further validate the involvement of TGF-β1 signaling pathway in memory deficits and depressive-like phenotype induced by Aβ oligomers, could be crucial in future studies the use of SB431542, a selective inhibitor of TGF-β1 pathway and therefore a validated tool to evaluate cellular and behavioural actions mediated by endogenous TGF-β1 in our amyloid-related depression animal model.

However, the results obtained from the present study and discussed in the present thesis together with the previous finding of other research groups that show how TGF-β1 can modulate amyloid deposition, suggest that the rescue of TGF-β1 signaling may be a novel therapeutic approach in AD, simultaneously targeting a neurodegenerative pathway and preventing Aβ deposition.

Starting from this evidence, we focused our attention on oxidative stress phenomena that increases the production and aggregation of Aβ, which in turn exacerbates neuronal oxidative damage contributing to the pathological development of AD. In addition, oxidative and nitrosative stress pathways are involved in the pathogenesis of depression by interacting with neurogenesis and neuroplasticity, neuroinflammation and monoamine reuptake process. We hypothesized the possible activation of oxidative system induced by Aβ and that fluoxetine and/or vortioxetine chronic treatment enhances the antioxidant machinery counteracting the possible pro-oxidant effect induced by Aβ in our amyloid-related depression model by also analyzing the possible correlation between the rescue of TGF-β1 pathway and the antioxidant effects of antidepressant

drugs. Our data showed that the i.c.v. injection of Aβ oligomers induced a significant increase in iNOS and NOX2 enzymes in the hippocampus, a brain area strongly affected in both depression and AD, and more important that the antidepressant chronic treatment was able to abolish the pro-oxidant enzymes over-expression Aβ-induced. We showed that the chronic treatment with fluoxetine and vortioxetine enhances the levels of antioxidant enzymes responsible for inhibition of ROS formation or removal of free radicals. In particular, a deficit of GPX1 has been related to increased Aβ-mediated neurotoxicity and our data show that the treatment with second-generation antidepressants is needed to rescue the expression of GPX1 at gene, but not at protein level. In fact, while the expression levels of GPX1 mRNA was significantly decreased in the hippocampus of Aβ-injected mice and rescued by both antidepressants, no differences were observed at protein level. Then we cannot exclude a post-trascriptional regulation of GPX1 gene and in our experimental conditions, the disagreement between gene and protein expression levels might be due to a compensatory response of the antioxidant system. Specifically, the antidepressant treatment starting 7 days before Aβ injection could prevent the decreased GPX1 gene expression Aβ-induced making the hippocampus less susceptible to oxidative stress (Alasmari, Bell et al. 2018).

These results confirmed the ability of antidepressant drugs to exert immune-regulatory effects by reducing the levels of pro-inflammatory cytokines and stimulating the synthesis of anti-inflammatory cytokines such as TGF-β1, also decreasing the production of reactive species and/or enhancing key elements of the antioxidant machinery.

It has previously demonstrated that fluoxetine is neuroprotective against Aβ toxicity via a paracrine signaling mediated by TGF-β1, which does not result from a simplistic SERT blockade, in fact at therapeutic concentrations (100 nM-1 μM), fluoxetine significantly prevented Aβ-induced toxicity in mixed glia-neuronal cultures, a well-established in vitro model of Aβ-induced neurodegeneration

(Caraci, Tascedda et al. 2016). By using mixed cultures of cortical cells treated with Aβ oligomers (2 μM for 48 h), we were able to compare, for the first time, the well-known neuroprotective activity of fluoxetine 1 μM, with the protective effects of the new multimodal antidepressant drug vortioxetine. The vortioxetine pre-treatment in Aβ-treated mixed cultures significantly prevent, even though not completely, Aβ toxicity at 250 nM with the maximal neuroprotective effect at the dose of 1 μM. It cannot be excluded that both antidepressants could exert their therapeutic potential by enhancing the release of TGF-β1 from microglial cells, as observed in our in vivo experiments, then rescuing the antioxidant system in neurons through the activation of TGF-β1 signaling.

All together, the data obtained by using a non-Tg model of AD, demonstrated that oxidative stress, taking place as a consequence of pro-oxidant enzymes activation, along with the previously showed deficit of TGF-β1, represents one of the neurobiological links between depression and AD. This neurobiological link could be fundamental because other multimodal compuonds that counteract oxidative stress can also rescue TGF-β1 levels. In particular, we used an experimental model of Aβ-induced neuroinflammation where BV-2 microglial cells were challenged with a μM concentration of Aβ oligomers known to strictly correlate with oxidative stress and ROS formation. We demonstrated that carnosine, an endogenous dipeptide with antioxidant activity widely distributed in excitable tissues like muscles and the brain, was able to decrease the secretion of pro-inflammatory cytokines, simultaneously rescuing IL-10 levels and increasing the synthesis and the release of TGF-β1 in an established in vitro model of Aβ-induced neuroinflammation. Interestingly, we found that carnosine decreased Aβ toxicity in a dose-dependent manner in mixed glia-neuronal cultures treated with Aβ oligomers 2μM for 48h, with a stronger protective effect at the concentration of 10 mM. The ability of SB431542, a selective inhibitor of the type-1 TGF-β1 receptor, to completely prevent the effects of carnosine suggests that TGF-β1 release and

activation of Smad-dependent signaling is essential in mediating the neuroprotective efficacy of carnosine against Aβ toxicity.

Future studies should be conducted in our non-Tg animal model of AD to assess whether carnosine can prevent amyloid-related cognitive deficits and depressive-like behavior by the rescue of TGF-β1 signaling, as observed in our in vitro experimental model of Aβ-induced neurodegeration and neuroinflammation, exerting an effect comparable with antidepressant drugs endowed with neuroprotective activity such as fluoxetine.

It is known that depression often co-exists and exacerbates neuropathic pain condition decreasing the individuals' quality of life. Depression can induce neural plasticity changes in areas involved in the control of the nociceptive system and may "predispose" the brain to persistent pain sensitivity. In addition, strong evidence showed that neuroinflammation plays an important role in the induction and maintenance of depression-pain syndrome as well as in pain chronicization (Campos, Antunes et al. 2020). Preclinical studies demonstrated that TGF-β1 plays a key role in the inhibition of neuropathic pain condition by suppressing neuroimmune responses of neurons and glia as well as promoting the endogenous opioids expression at spinal cord level (Lantero, Tramullas et al. 2012, Lees, Fivelman et al. 2015). A study conducted by Chen et al., demonstrated that the intrathecal (i.t.) administration of TGF-β1 is able to attenuate the spinal neuroinflammation and microglia activation in CCI-induced neuropathic pain model (Chen, Huang et al. 2013). In this context, in vivo experimental models represent a useful tool to screening the effects of candidate pharmacological treatments and can provide the evidence of possible neurobiologic effects of a treatment in the drug development process. We adopted a validated animal model of neuropathic pain, the CCI model, developed by Bennett and Xie (1988) which is able to determine the induction of pronounced mechanical allodynia lasting up to 2 months, to examine the molecular mechanisms underlying the analgesic effects of

the MOPr/DOPr agonist LP2. We found that this dual-target benzomorphan-based compound, when administered from the 11 days post-ligature to 21 days, is able to reduce CCI-induced mechanical allodynia by rescue of TGF-β1 and its type 2 receptor at spinal cord level. Moreover, a selective deficit of TGF-β1 in the spinal microglia of CCI rats was detectable both in the early and in the chronic phase of neuropathic pain. Since the deficit of TGF-β1 as well as of its receptor was observed in the spinal cord from CCI rats, our aim was to specifically identify the cell type characterized by this deficit. Interestingly we found that LP2 induces an increase of TGF-β1 which colocalizes with Iba1 in CCI rats suggesting the possible role played by microglial cells in modulating the rescue of TGF-β1 levels promoted by LP2 treatment.

Available therapies with MOPr agonists often provide incomplete pain relief and common side effects are reported. For this reason, a novel drug discovery strategy in chronic pain is the development of MOPr/DOPr dual-target compounds that are able to activate both types of receptors given that the co-expression of both receptors in areas involved in pain modulation and the role played by MOPr activation in the regulation of DOPr trafficking. DOR are strongly expressed both in astrocytes and microglial cells, but it is presently unknown the contribute of glial DOR in the analgesic effects of DOPr agonists which counteract and prevent nociceptive behaviors in various chronic neuropathic pain models. Presently, it is not known whether MOPr or DOPr agonists can positively modulate TGF-β1 pathway and whether rescue of TGF-β1 signalling can contribute to increase the analgesic effects of MOPr/DOPr compounds. Taking into consideration the results obtained in our study, we believe that the rescue of TGF-β1 signalling by dual-target MOPr/DOPr agonist LP2 could be mediated by DOR activation in spinal microglia and might represent a novel pharmacological approach to increase opioid analgesic efficacy of selective MOPr agonists.

Finally, we moved from rodent to humans, to validate the role of TGF-β1 in cognitive decline exploring the hypothesis that a deficit of TGF-β1 as a biomarker of cognitive decline in neuropsychiatric disorders characterized by an increase risk to develop AD such as DS patients.

The need of biomarkers for cognitive decline is particularly important among aging DS individuals. It is known that DS subjects present an elevated risk for AD development due to increased Aβ resulting from the APP over-expression on chromosome 21. Neuropathological similarities exist between DS and AD, but Aβ deposition occurs very early in DS and exponentially increases after 40 years. Therefore, a long asymptomatic period probably precedes the onset of dementia and in order to provide appropriate care to DS patients, it is of critical importance to found new approaches that comprehensively assess health status and cognitive function, allowing to follow the AD progression in DS patients. In this context, blood-based biomarkers offer the advantage of being very accessible, less expensive and better tolerated than neuroimaging and CSF biomarker approaches. Moreover, these biomarkers could have relevance for diagnosis and precision medicine for AD in DS patients. In order to characterize new blood-based biomarkers of AD in DS, we analyzed TGF-β1 plasma levels by ELISA assay according to manufacturer's instructions (R&D Systems, cod. DB100B) in a cohort including: young and adult DS without AD [young DS (<35 years) without AD n=19 and old DS (>35 years) without AD n=10)] as well as old DS with AD (DSAD; >35 years n=8) recruited at IRCCS-Oasi Maria S.S., Troina, Italy and compared with healthy age- and sex- matched controls (n=36) (project GR-2019). DSAD patients were categorized in Early Onset, Middle Stage, Late Stage dementia according to the Dementia Scale for Down syndrome (DSDS). To assess cognitive decline, the Test of Severe Impairment (TSI) was administered at baseline (T0) (range 0-24, lower scores indicate greater impairment). Premorbid severity of intellectual disability (ID) in DS patients was classified into 2 groups, using the

International Classification of Diseases (ICD)-10: mild/moderate (IQ 35–70) and severe/profound (IQ <35). Participants or their legal representative gave written informed consent, as approved by the Ethics Committee of Oasi Research Institute. By performing ELISA assay in DS cohort, we observed a significant decrease in TGF-β1 plasma levels from DS patients compared to age- and sex- matched healthy subjects (CTRL) and this deficit was correlated in the first cohort of DS patients with global cognitive function deterioration evaluated by TSI score.

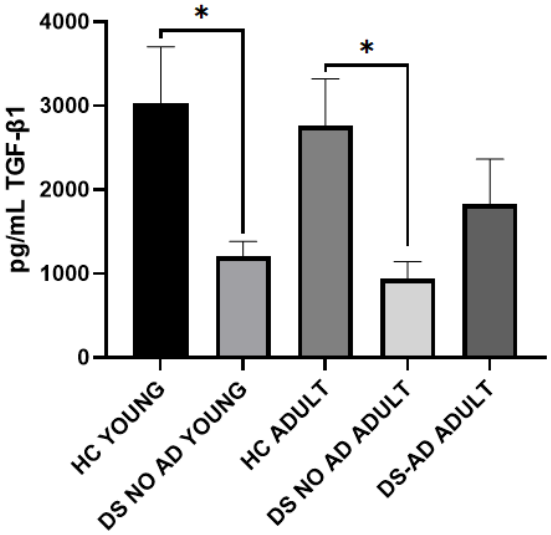

Figure 6. TGF-β1 plasma levels are reduced in DS patients.

Interestingly, the levels of TGF-β1 are significantly reduced also in young DS patients without AD compared to healthy age- and sex- matched controls suggesting that the deficit of TGF-β1 is an early and long-lasting event in the pathophysiology of cognitive decline in DS.

The impairment of TGF-β1 signaling in young and adult DS without AD clinical signatures could contribute to the development and/or worsening of AD pathology in these patients potentiating neuroinflammatory phenomena in an early preclinical phase of AD. These results support preliminary evidence obtained in the first cohort of DS patients where Caraci, Iulita, and Cuello showed that baseline plasmatic levels of TGF-β1 are significantly reduced in older DS patients without AD. On the

other hand, the increased TGF-β1 plasma levels detected in adult DS-AD patients, compared with DS subjects without dementia, might be reconsidered as a compensatory mechanism in response to advanced neuroinflammatory phenomena in a late phase of the disease where TGF-β1 is over secreted to limit, without success, the temporal and spatial extent of neuroinflammation and neurotoxicity.

The results discussed in the present PhD thesis could support our hypothesis that a deficit of TGF-β1 can occur in periphery in an early preclinical phase of AD in DS patients, even before the onset of cognitive decline and could be considered as a potential blood-based biomarker for early diagnosis of AD in DS subjects.

A second cohort of DS patients, will be recruited for a long-term (24 months) prospective longitudinal study at Oasi research Institute in order to validate the role of plasma TGF-β1 as a novel biomarker able to predict the rate of cognitive decline in DS patients.

Of note, higher levels of Nf-L plasma, total-tau, and age were all shown to be associated with increased risk for AD in DS subjects (Petersen, Rafii et al. 2021). The evaluation of plasma Nf-L increased levels or brain hypometabolism, both markers of neurodegenerative changes, may occur in DS patients even before the deposition of Aβ detectable by PET. Interestingly, in a recent study conducted by Fortea et al., plasma Nf-L concentrations were the first biomarkers to change by age 28–30 years, more than 20 years before prodromal AD diagnosis (Fortea, Vilaplana et al. 2020). In addition, Peterson et al. showed that plasma total-tau and Nf-L (alone or in combination) will be easily accessible biomarkers of AD-related neurodegeneration (Petersen, Rafii et al. 2021) in DS subject.

In our second cohort of DS patients we are planning to perform the measureament of Nf-L plasmatic levels in order to correlate the reduced plasma levels of TGF-β1 with the rate of neurodegeneration and confirming the role of TGF-β1 as a new blood-based biomarker to predicte the rate of cognitive decline in DS subjects.

Finally, in this context PBMCs can be used as a simple, non-invasive, and inexpensive peripheral experimental model to identify the biochemical and molecular signatures of AD in DS given that they could reflect in the periphery the biological alterations found at CNS level as well as could represent a useful tool for the identification of new biomarkers for the diagnostic and/or therapeutic approach of AD in DS patients (Esteras, Alquézar et al. 2016).

In our preliminary data, we mantained PBMCs obtained from DS and healthy control subjects in fresh culture medium and after 48 h of incubation at 37°C PBMC cultures were treated with fluoxetine 1µM (24 hours), while the other untreated cultures were kept as control. Conditioned-culture media were collected for ELISA assay while the PBMCs pellet culture (1 x 10^6 cells) will be used to RNA extraction. We found that TGF-β1 levels are reduced in PBMC medium from DS patients compared to PBMCs controls treated or not with fluoxetine. TGF-β1 are reduced both in plasma and PBMCs in the same DS subjects and interestingly, we found that fluoxetine treatment was able to increase TGF-β1 levels in the culture media of PBMCs from DS patients with values comparable to control.

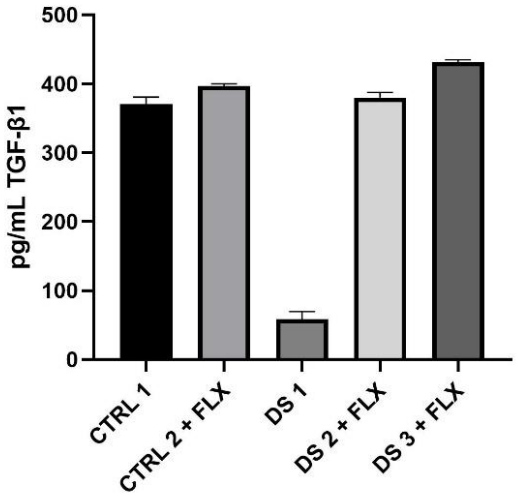

Figure 7. Fluoxetine is able to increase TGF-β1 release in the culture media of PBMCs from DS patients.

A second step will be to evaluate the TGF-β1 baseline levels before fluoxetine treatment in the same patients in order to confirm that fluoxetine, an SSRI endowed with neuroprotective activity, is able to rescue TGF-β1 levels in DS subjects at higher risk to AD development.

An increase in pro-inflammatory cytokines levels in DS patients with AD pathology has been recently suggested (Petersen and O'Bryant 2019) and a strong neurobiological link exists in AD brain between an early pro-inflammatory process and a deficit of TGF-β1 (Caraci, Spampinato et al. 2012). We hypothesize that Aβ accumulation process sustains a pro-inflammatory status and a deficit in TGF-β1 at peripheral level.

However, future long-term studies are needed to validate TGF-β1 as a new blood-based biomarker in epidemiological community-based populations.

The diagnosis of AD in DS individuals is challenging due to the underlying intellectual disability and cognitive decline associated with aging of these subject. For this reason, the identification of novel biomarkers could have a significant clinical impact for the menagement of AD pathology in DS patients. Then, the combination of biological and neuropsychological markers will increase the diagnostic accuracy of dementia in DS subjects and this strategy will be essential to identify DS patients at high risk of AD developmemt, in order to to establish which patients should be included in future secondary prevention trials to study potential disease-modifying drugs.

In conclusion, overall the data presented in this PhD thesis suggest that TGF-β1 signaling pathway offer potential opportunities for being a new pharmacological target in neurodegerative diseases including AD.

A number of agents have been studied that have targeted various components of TGF-β1 pathway. In fact, different drugs classes including SSRIs such as fluoxetine and the new multimodal antidepressant vortioxetine are able to increase TGF-β1 levels and different studies showed that antidepressant drugs enhance the plasma levels of TGF-β1 as well as BDNF levels in vivo and are able to increase neurogenesis. Interestingly, acetyl salicylic acid accelerates the antidepressant effect of fluoxetine in animal model of depression (Brunello, Alboni et al. 2006) and at the same time increases TGF-β1 synthesis and release in vivo studies. Among psychotropic drugs, lithium is able to induce TGF-β1 from glial cells and this could be the molecular mechanism underlying its neuroprotective activity in AD (Caraci, Copani et al. 2010).

Taking into account the results discussed in this thesis we have also hypothesized that natural molecules such as carnosine or nutraceutical products targeting TGF-β1 signaling pathways can synergize with antidepressant drugs in order to rescue cognitive impairment in depression and AD (Grasso, Caruso et al. 2021). In particular, hyperforin, the *H. perforatum* main active ingredient, showed the ability to promote hippocampal neurogenesis by stimulating the production of TGF-β1 as well as rescues the neurotrophin's levels promoting thus an improvement in cognitive function. Hesperidin, a flavanone glycoside found primarily in Citrus fruit, has been shown to improve memory performance in adult mice by increasing TGF-β1 secretion from cortical astrocytes. Among the subclasses of flavonoids associated with better cognitive health, flavonols and flavanones are able to increase TGF-β1 (Panek, Kawalec et al. 2020). Recently, in animal models of depression, it has been shown that omega-3 fatty acids stimulate the secretion of TGF-β1 from microglial cells (Gu, Li et al. 2018). In addition, the bioavailability of these nutraceuticals can be significantly improved by drug delivery systems as well as by employing an intranasal drug delivery in order to bypass the BBB, avoid

some systemic side effects and overcome the problem with first-pass metabolism (Panek, Kawalec et al. 2020).

Targeting cognitive deficits in depression, by possibly rescuing TGF-β1 signaling, could improve the efficacy of the pharmacological treatment, because a high rate of patients are considered treatment-resistant and also considering that no new drugs have been approved by FDA for the treatment of cognitive impairment in depression. We believe that TGF-β1 signaling might represent a new pharmacological target for the treatment of cognitive impairment observed in different neuropsychiatric disorders from depression to AD.

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