Beta-Amyloid oligomeric and monomeric states: implications for Alzheimer's Disease

Doctorate Thesis

Marianna Flora Tomasello

UNIVERSITY OF CATANIA

International Ph.D. program in Neuropharmacology

XXIIV cycle

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Coordinator: Prof. Filippo Drago

Tutor: Prof. Agata Copani

Co-Tutor: Dr. Giuseppe Pappalardo (CNR-IBB)

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ABBREVIATIONS

Aß, beta-amyloid;

ADDL, Aβ-Derived-Diffusible-Ligands

α2M, alfa2-macroglobulin;

ApoE, apolipoprotein E;

APP, amyloid precursor protein;

BACE; beta-site APP cleaving enzyme;

BBB, blood brain barrier;

CNS, central nervous system;

CSF, cerebro spinal fluid;

ECE, endothelin converting enzyme;

EGR1, early growth response factor;

ERK, extracellular regulated kinase;

Foxo1, forkhead box protein O1;

IDE, insulin degrading enzyme;

IGF-1R, insulin-like growth factor-1 receptor;

IR, insulin receptor;

IRS, insulin receptor substrate;

LRP, LDL receptor related protein;

MAPK, mitogen activated protein kinase;

NMDA, N-methyl-D-aspartate receptors

p110/ p85, catalytic/ regulatory subunit of PI3K;

PDK, phosphatidylinositide- dependent kinase;

PI3K, PI3 kinase;

PI3,4P, phosphatidylinositide 3,4-diphosphate;

PI3,4,5P, phosphatidylinositide 3,4,5-triphosphate;

PPP, Picropodophyllin

PS, presenilin;

RAF, RAF proto-oncogene serine/threonine protein kinase;

RAGE, receptor for advanced glycation end-product;

RAS, RAS small GTPase;

Stat, Signal transducer and activator of transcription protein;

SOS, son of sevenless;

SP1, specificity protein-1;

ABSTRACT

Alzheimer's Disease (AD) is by far the most common cause of dementia affecting more than 35 million people worldwide. Despite considerable research, there is still no cure for this neurodegenerative disease and available treatments are only symptomatic. In the controversial literature about AD, a predominant idea refers to the crucial role of amyloid-β protein (Aβ) in the pathogenesis of the disease; in fact, the feature in the brain of AD patients is the presence of extracellular plaques mainly composed of Aβ. Nevertheless, Aβ is physiologically produced in healthy individuals. Due to its biophysical properties, under certain conditions, Aβ may self-aggregate into multiple forms, ranging from 4 kDa monomers and including higher-order oligomers, protofibrils, and mature fibrils. For many years, the fibrillar Aβ assemblies, similar to what seen in amyloid plaques, have been considered mainly responsible for neurodegenation associated with AD. However, the quantity and temporal progression of amyloid plaques do not correlate well with the clinical evolution of the disease. There is now extensive evidence that soluble Aß oligomers disrupt synaptic transmission and plasticity in AD. Moreover, new studies strengthen evidence that people with sporadic AD make normal amounts of Aß, and that the toxic buildup is due to altered peptide disposal. These data are in line with the finding previously reported by our group that Aß in its non-toxic monomeric state has a broad neuroprotective effect *in vitro*. This effect depends on the stimulation of type-1 insulin-like growth factor (IGF-I) receptors and/or other receptors of the insulin superfamily.

The aim of this PhD thesis was to decipher Aβ activities, focusing on the relationship between the structure/aggregation state and the neurotoxic/biological activity. In **paper I** we have addressed the issue of Aβ toxicity, whereas the properties of the non-toxic form of Aβ, the monomer, have been considered in **paper II** and **III**. In **paper I**, the neurotoxic activity of Aβ was investigated in a particular model in which anabolic-androgenic steroid (AAS) sensitize neurons to the toxicity of Aβ oligomers. We found that, concentrations of the AAS that were not neurotoxic by themselves were able to increase neuronal susceptibility to the apoptotic stimulus provided by Aβ. **In paper II** and **III,** we have demonstrated that the Aβ, in its non-toxic monomeric state, activates type I IGF receptors and mimics the metabolic actions of IGFs in neurons and peripheral cells. In neurons, endogenous Aß release was required to uphold glucose uptake during activation, and exogenously added Aß monomers caused the translocation of type-3 glucose transporters to the plasma membrane with ensuing glucose uptake. We suggest that pathological aggregation of Aβ monomers, as occurring in AD, might impair neuronal ability to cope with transient needs in energy provision.

INTRODUCTION

1. ALZHEIMER DISEASE

Dementia is increasingly being recognized as one of the most important medical problems in the elderly with a prevalence rising from 3% at the age of 60 to at least 60% at the age of 90 years (Ferri et al., 2005). Within the spectrum of dementias, the most common form was first described by the German psychiatrist and neuropathologist Alois Alzheimer in 1906 and was named after him Alzheimer's dementia or Alzheimer's disease (AD) (Berchtold et al 1998). Most often, AD is diagnosed in people over 65 years of age (Brookmeyer et al., 1998) although the less-prevalent early-onset Alzheimer's can occur much earlier. In 2006, there were 26.6 million sufferers worldwide. Alzheimer's is predicted to affect 1 in 85 people globally by 2050 (Brookmeyer et al., 2007). In addition to the tragedy of affected pepople, AD places psychological and economical burdens on caregivers, and represents a major public health problem being among the most costly diseases for the society in Europe and in the United States. Despite all scientific efforts, at the moment, effective pharmacotherapeutic options for the prevention and the treatment of AD are lacking.

1.1 General characteristics

Although AD develops differently for each individual, there are many common symptoms. Early symptoms are often mistakenly thought to be 'age-related' concerns, or manifestations of stress. In the

early stages, the most common symptom is difficulty in remembering recent events. When AD is suspected, the diagnosis is usually confirmed with tests that evaluate behaviour and thinking abilities, eventually followed by a brain scan. As the disease advances, symptoms may include confusion, irritability and aggression, mood swings, trouble with language, and long-term memory loss. As sufferers decline, they often withdraw from family and society (Tabert et al., 2005). Gradually, bodily functions are lost, ultimately leading to death. Since the disease is different for each individual, predicting how it will affect the person is difficult. AD develops for an unknown and variable amount of time before becoming fully apparent, and it can progress undiagnosed for years. On average, the life expectancy following diagnosis is approximately seven years (Mölsä et al., 1986.) Fewer than three percent of individuals live more than fourteen years after diagnosis (Mölsä et al., 1995).

1.2 History

AD was initially observed in a 51-year-old woman named Auguste D. Her family brought her to Dr. Alzheimer in 1901 after noticing changes in her personality and behavior. The family reported problems with memory, difficulty speaking, and impaired comprehension. Dr. Alzheimer later described Auguste as having an aggressive form of dementia, manifesting in memory, language and behavioral deficits. Dr. Alzheimer noted many abnormal symptoms, including difficulty with speech, agitation, and confusion. He followed her care for five years, until her death in 1906. Following her death, Dr. Alzheimer performed an autopsy, during which he found dramatic shrinkage of the cerebral cortex, fatty deposits in blood vessels, and

atrophic brain cells. He also noticed two major histopatological lesions, neurofibrillary tangles and senile plaques, which have become pathological hallmarkers of AD (**Fig a**).

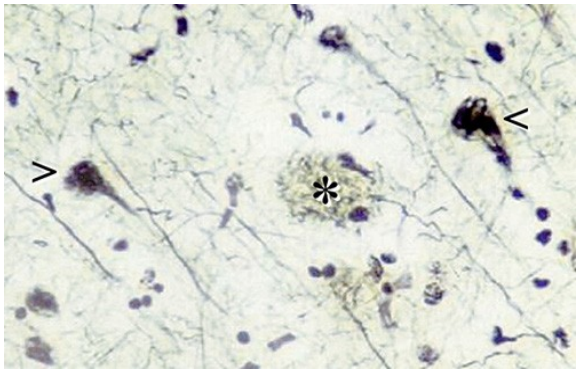

Fig a: *Microscopic alterations of brain tissue affected by AD. A plaque is shown in the center of the field (asterisk) as a round mass formed by heterogeneous material. Neurons with fibrillary tangles in their cell bodies appear as dense, inverted triangles on the left and right sides of the picture (arrowheads).*

Senile plaques and neurofibrillary tangles are located predominantly in areas displaying neuronal loss, i.e the neocortex, hippocampus, amygdale and nucleus basalis. Subsequent researchers have identified the biochemical component of the extracellular and intracellular deposit observed in AD brain. The major constituent of the extracellular plaques is a 40-42 aminoacid peptide termed amyloid-beta (Aß). Neurofibrillary tangles are composed of bundles of highly phosphorilated Tau proteins paired into helical or coiled frilaments (Deshpande et al., 2008).

1.3 Causes

The cause for most Alzheimer's cases is still essentially unknown (except for 1% to 5% of cases where genetic differences have been identified). It is widely recognized that the disease is associated with plaques and tangles in the brain, but competing hypotheses exist trying to explain the cause of the disease:

Cholinergic hypothesis: the oldest, on which most currently available drug therapies are based, is the *cholinergic hypothesis* (Francis et al., 1999), which proposes that AD is caused by reduced synthesis of the neurotransmitter acetylcholine. The cholinergic hypothesis has not maintained widespread support, largely because medications intended to treat acetylcholine deficiency have not been very effective.

Amyloid hypothesis: in 1991, the *amyloid hypothesis* postulated that beta-amyloid (Aβ) deposits are the fundamental cause of the disease (Hardy et al., 1991; Mudher et al., 2002). Support for this postulate comes from the location of the gene for the amyloid precursor protein (APP) on chromosome 21, together with the fact that people with trisomy 21 (Down Syndrome) who have an extra gene copy almost universally exhibit AD by 40 years of age (Nistor, 2007, Lott et al., 2005). Also APOE4, the major genetic risk factor for sporadic AD, leads to excess amyloid buildup in the brain (Polvikoski, 1995). Further evidence comes from the finding that transgenic mice that express a mutant form of the human APP gene develop fibrillar amyloid plaques and Alzheimer's-like brain pathology with spatial learning deficits (Games, 1995). An experimental vaccine was found to clear the amyloid plaques in early human trials, but it did not have

any significant effect on dementia (Holmes, 2008). However, as the deposition of amyloid plaques does not correlate well with neuron loss (Schmitz, 2004), researchers have been led to suspect the non-plaque Aβ oligomers (aggregates of many monomers) as the primary pathogenic form of βA. A more detailed description about the more recent formulation of the *amyloid hypothesis* will be given in paragraph 3.

Tau hypothesis: the *tau hypothesis* is the idea that tau protein abnormalities initiate the disease cascade (Mudher et al., 2002). In this model, hyperphosphorylated tau begins to pair with other threads of tau. Eventually, they form neurofibrillary tangles inside nerve cell bodies (Goedert et al., 1991). When this occurs, the microtubules disintegrate, collapsing the neuron's transport system (Iqbal, 2005). This may result first in malfunctions of biochemical communication between neurons and later in the death of the cells (Chun et al., 2007).

Other hypotheses: Herpes simplex virus type 1 has also been proposed to play a causative role in people carrying the susceptible versions of the apoE gene (Itzhaki et al., 2008). Another hypothesis asserts that the disease may be caused by age-related myelin breakdown in the brain. Iron released during myelin breakdown is hypothesised to cause further damage. Homeostatic myelin repair processes contribute to the development of proteinaceous deposits such as beta-amyloid and tau (Bartzokis et al., 2004 and 2007). Oxidative stress and dys-homeostasis of biometal metabolism may be significant in the formation of the pathology (Su et al., 2008; Kastenholz et al., 2009). AD individuals show 70% loss of locus coeruleus cells that provide norepinephrine

that locally diffuses from "varicosities" as an endogenous antiinflammatory agent in the microenvironment around the neurons, glial cells, and blood vessels in the neocortex and hippocampus. It has been shown that norepinephrine stimulates mouse microglia to suppress Aβ-induced production of cytokines and their phagocytosis of Aβ. This suggests that degeneration of the locus ceruleus might be responsible for increased Aβ deposition in AD brains (Heneka et al., 2010).

1.4 Pathophysiology

1.4.1 Neuropathology:

Alzheimer's disease is characterised by loss of neurons and synapses in the cerebral cortex and certain subcortical regions. This loss results in gross atrophy of the affected regions, including degeneration in the temporal lobe and parietal lobe, and parts of the frontal cortex and cingulate gyrus (Wenk, 2003). Studies using MRI and PET have documented reductions in the size of specific brain regions in people with AD as they progress from mild cognitive impairment to Alzheimer's disease (Fig.b), and in comparison with similar images from healthy older adults (Desikan et al., 2009; Moan, 2009).

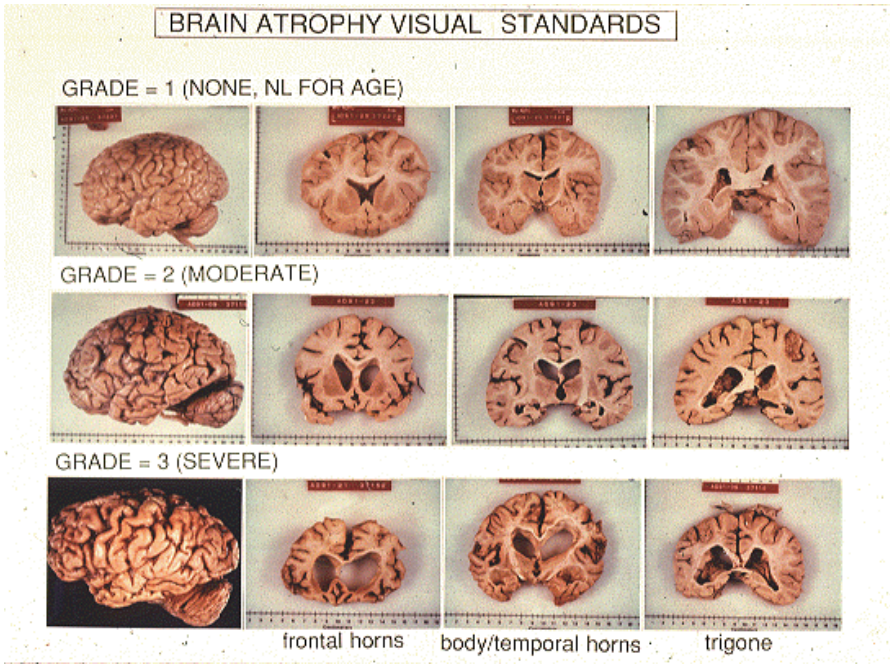

***Fig b**: brain atrophy in moderate and in severe AD. From the ADRC Neuropathology Core of the Washington University School of Medicine in St Louis*

1.4.2 Biochemistry:

Alzheimer's disease has been identified as a *protein misfolding disease* (proteopathy), caused by accumulation of abnormally folded amyloid beta and amyloid tau proteins in the brain (Hashimoto et al., 2003). These misfolded peptides accumulate either intra- or intercellularly in a variety of organs, including the liver, spleen, and, most importantly in the brain, causing severe neurological disorders. The aggregates are often in the form of regular amyloid fibrils. Amyloids are rich in beta-sheet structure, with hydrogen bonding between monomers parallel to the fibril axis, leading to fibrils of indefinite length. Both amyloid

plaques and neurofibrillary tangles are clearly visible by microscopy in brains of those afflicted by AD (Tiraboschi et al., 2004). Although many older individuals develop some plaques and tangles as a consequence of ageing, the brains of people with AD have a greater number of them in specific brain regions such as the temporal lobe (Bouras et al., 2004). Plaques are made up of aggregates of a small peptide, Aβ which is produced from a larger protein called *amyloid precursor protein (APP)* through the sequential action of processing enzymes called *secretases.* AD is also considered a tauopathy due to abnormal aggregation of the tau protein. *Tau* stabilises microtubules when phosphorylated, and is therefore called a microtubule-associated protein. In AD, tau undergoes chemical changes, becoming hyperphosphorylated; it then begins to pair with other threads, creating neurofibrillary tangles and disintegrating the neuron's transport system (Hernández et al., 2007).

1.4.3 Genetics and other risk's factors

Genes have a varied influence on developing AD, ranging from the autosomal dominant inheritance in the familial forms (1-5% of cases) or FAD, to the polygenic background in late-onset (>65 years of age) sporadic AD (≥95% of cases). Patients with either sporadic AD or FAD share common clinical and neuropathological features, including synaptic and neuritic loss and the two major histopathological hallmarks, extracellular plaques and intracellular neurofibrillary tangles (NFT), and finally profound neurodegeneration in many brain regions. In addition to the genetic component, the risk for developing AD, the age at onset and the course of the disease are influenced by several other factors including sociodemographic, life style,

environment and comorbid medical conditions (Papassotiropoulos et al., 2006). Age and female sex represent risk for developing AD. Poor education, low mental ability, traumatic brain injury, stroke and history of depression can also predispose to AD. The genetic component however, seems to be of major importance, since according to twin studies, a major part of the risk for sporadic AD is genetically determined (Gatz et al., 1997). Autosomal dominant forms of early-onset FAD are in many cases determined by specific mutations of the gene located on chromosome 21 encoding for the APP, or of the genes mapped on chromosomes 14 and 1, encoding presenilin-1 (PS-1) and presenilin-2 (PS-2), identified as putative gamma-secretases (Cruts et al., 1998). All of these genes are involved in the processing of Aβ. Identification and characterization of dominant mutations of these genes was instrumental for the understanding of the biological mechanisms leading to enhanced Aβ accumulation and senile plaques generation. The familial forms usually have an early onset and greater severity than sporadic cases and they are also associated with a greater load of protein aggregates (Hardy and Gwinn-Hardy, 1998). In contrast with the familial AD, the causing factors of the Aβ accumulations and other pathological mechanisms remain mostly unclear in the sporadic form. The first specific genetic cause of AD to be identified was the occurrence of missense mutations in APP (Goate, 1998). Mutations within APP are clustered around the N and C termini of the Aβ domain, and the usual phenotype involves increased generation of Aβ species that end(s) at residue 42. The APP gene maps to the long arm of chromosome 21. According to this finding, patients with Down's syndrome develop Aβ deposits and neuronal degeneration (Head et al., 2001). In 1990, it was discovered that a

substitution of glutamine for glutamic acid at codon 693 of APP causes the rare condition of cerebral hemorrhagic amyloidosis of the Dutch type. Subsequentely more than 20 different families have been discovered to have APP gene mutations as a cause of FAD. As with most APP mutations, the mutations in PS appear to act by increasing the generation of the Aβ species (Levy-Lahad et al., 1995; Sherrington et al., 1995). The highly homologous presenilin-1 (PS1) and -2 (PS2) genes encode two highly conserved 43-50 kD multipass transmembrane proteins that are involved in Notch 1signaling pathway critical for cell fate decision (Selkoe and Kopan, 2003). PSs are endoproteolitically cleaved by a presenilinase to form the N-terminal ~28 kD fragment and the C-terminal ~18 kD fragment (Thinakaran et al., 1997). Both fragments are critical components of the γ-secretase complex (Selkoe and Kopan 2003). Nearly 50% of cases of early onset familial Alzheimer's disease are linked to the PS1 gene. Genetic surveys have identified as many as 75 missense mutations in presenilin 1 and three in presenilin 2 as molecular causes of early-onset AD in several hundred families worldwide. Some of the mutations merely alter the ratio between Aβ42 and the other major forms—e.g., Aβ40—without increasing Aβ42 levels (Shioi, 2007). This suggests that presenilin mutations can cause disease even if they lower the total amount of Aβ produced and may point to other roles of presenilin or a role for alterations in the function of APP and/or its fragments other than Aβ. The complex genetic model of sporadic AD suggests that several heterogeneous susceptibility sets of genes may converge on the pathological processes that underlie the disease. However, so far only the apolipoprotein E (APOE) gene has been definitively associated with the risk for AD (Brouwers et al., 2008). APOE is involved in lipid

transport and metabolism. Furthermore, it plays a specific role in the central nervous system, including neuronal development, regeneration and certain neurodegenerative processes. The polymorphism of the APOE gene determines three isoforms of APOE protein (ε2, ε3, ε4) with different conformation and lipid binding properties (Cedazo-Mínguez and Cowburn, 2001). The APOE ε4 isoform prefers very low density lipoprotein and it is less effective in cholesterol transport as compared to the other APOE isoforms (Cedazo-Mínguez and Cowburn, 2001). Membrane cholesterol modulates the cleavage of the APP protein and in the presence of the ε4 isoform the balance is shifted to the production of Aβ (Stefani and Liguri, 2009).The APOEε4 allele increases the risk of the disease by three times in heterozygotes and by 15 times in homozygotes (Blennow et al., 2006). However, this "genetic" effect is not necessarily purely genetic. For example, certain Nigerian populations have no relationship between presence and dose of APOEε4 and incidence or age-of-onset for Alzheimer's disease (Hall et al., 2006; Gureje et al., 2006). Geneticists agree that numerous other genes also act as risk factors or have protective effects that influence the development of late onset Alzheimer's disease (Waring et al., 2008), but results such as the Nigerian studies and the incomplete penetrance for all genetic risk factors associated with sporadic Alzheimers indicate a strong role for environmental effects. Over 400 genes have been tested for association with late-onset sporadic AD, most with null results (Blennow et al., 2006).

2. THE AMYLOID BETA PROTEIN : FROM PRODUCTION TO CLEARANCE

The main component found in amyloid deposits is the 4 kDa amyloid-β-protein produced by the proteolytic cleavage of the amyloid precursor protein (APP) (Verdile et al., 2004). The amyloid precursor protein is a 100-130 kDa integral membrane protein, widely expressed in cells, with a single membrane-spanning domain, a large extracellular glycosylated N-terminus and a shorter cytoplasmic C terminus. The proteolitic processing pathway leading to the formation of Aβ from the amyloid precursor protein (APP) has been well characterized in a number of cell lines (Selkoe, 2000). Ten isoforms of human APP have been identified (Octave, 1995) among which eight contain the Aβ sequence. The isoform mainly expressed in the human brain is a 695-aa protein named APP695 (Kang et al., 1987). The physiological function of APP is not fully understood, but increasing evidences suggest its involvement in the regulation of neuronal survival, neurite outgrowth, synaptic plasticity and cell adhesion (Mattson, 1997). Several identified mutations in the APP sequence are linked with inherited forms of AD, known as early onset familial FAD (Tanzi, 1999). Most of these mutations are located within or near the Aβ sequence and result in increased Aβ production. APP is delivered to the surface membrane where it is subject to proteolitic processing mediated by three different secretase: α-secretase, β-secretase (called BACE-1*: beta-site APP cleaving enzyme-1)* and γ-secretase. The enzymatic γ-secretase cleavage is carried out by a protein complex consisting of four proteins: presenilin, nicastrin, Aph-1 and Pen-2. APP can undergo cleavage down one of at least two pathways. In the

first pathways, cleavage by the enzyme α-secretase prevents Aβ formation and instead produces the neuroprotective sAPP α-fragment from the cell surface and leaves an 83-amino-acid carboxy-terminal APP fragment (C83). However if sequential cleavage by β and then γ-secretase predominates, Aβ is formed. Two main forms of Aβ can be identified, the shorter, 40 amino acid Aβ (1-40) species, and the longer, 42 amino acid Aβ (1-42) species (**Fig. c**).

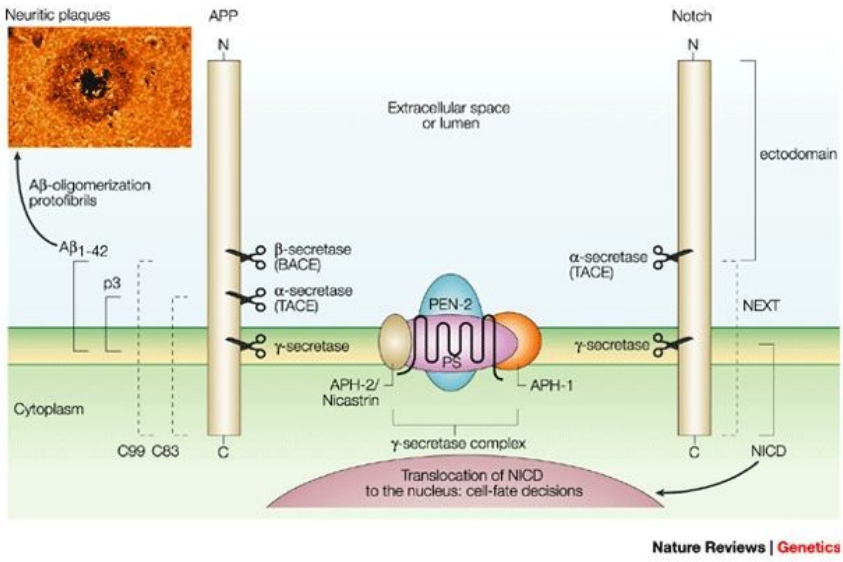

***Fig. c**: APP processing. From Monica Driscoll & Beate Gerstbrein. Nature Reviews Genetics 4, 181-194 (March 2003).*

The Aβ (1-40) form accounts for 90% of all Aβ normally released from cells and it appears to contribute only to later phases of the disease pathology (Asami-Odaka et al., 1995). The Aβ (1-42) form only accounts for 10% of secreted Aβ, however, it is the predominant form found in the amyloid plaques of AD (Lippa et al., 1998). The Aβ

(1-42) peptide aggregates and polymerises into amyloid fibrils more readily than the Aβ (1-40) species, and these properties are thought to confer the peptide's pathogenecity (Hensley et al., 1994). Under physiological condition Aβ level are tightly regulated by the combined activity of production/degradation. For Aß clearance several mechanisms are known: i) enzymatic degradation by activated microglia or by insulin degrading enzyme (IDE), neprilysin, endothelin converting enzyme (ECE), and angiotensin converting enzyme (ACE); ii) receptor-mediated transport across the blood brain barrier (BBB) by binding to the low-density lipoprotein receptor related protein (LRP) either directly, or after binding to apolipoproteinE (ApoE) and/or α2-macroglobulin (α2M) to be delivered to peripheral sites of degradation, e.g., liver and kidney. (reviewed in Tanzi et al., 2004). Most enzymes are produced by neurons or glia, but some are expressed in the cerebral vasculature, where reduced Aβ-degrading activity may contribute to the development of cerebral amyloid angiopathy (CAA). Neprilysin and IDE are expressed both neuronally and within the vasculature (Turner et al 2004). The production and degradation of Aβ has given many insights into potential target process for therapeutic intervention aimed at preventing Aβ formation or accelerating its degradation. The secretases enzymes have received attention as attractive drug target, as they are directly involved in Aβ biogenesis. It has been shown that BACE-1 levels are increased in post-mortem brain sections from AD patients (Holsinger et al., 2002) as well as in brains from Tg2576 mice (Harada et al., 2006) expressing the Swedish mutation of APP (APPSW), a well established mouse model for AD. Interestingly, in these rodent brains mRNA levels of BACE-1 were not elevated,

suggesting that elevation of BACE-1 activity is a posttranscriptional mechanism. Enhanced activity in regions with high plaque load leads to the assumption that the plaques themselves influence BACE-1 activity.

During the last years, the γ-secretase protease, despite the complexity of its multi-subunit, has been target of small-molecule compounds developed to inhibit or modulate activity of this enzyme and some of these have already entered clinical trials.

3. THE NEW AMYLOID HYPOTHESIS: SOLUBLE Aβ OLIGOMERS AS INITIATING FACTORS IN AD

Since the first description of presenile dementia by Alois Alzheimer in 1907, senile plaques and neurofibrillary tangles (NFTs) are considered the key pathological hallmarks of AD. The identification of A*β* in plaques and genetic studies that identified mutations in the *APP*, *PS1*, and *PS2* genes, leading to the accumulation of A*β* in the early-onset familial dementia, resulted in the formulation of the "Amyloid Cascade Hypothesis" (ACH; Fig. **d**). Since FAD has a similar phenotype to late-onset AD, it was assumed that this amyloid deposition could explain the pathogenesis of all types of AD. According to the ACH, the deposition of A*β* is the initial pathological trigger in the disease, which subsequently leads to the formation of NFTs, neuronal cell death and dementia. While there is considerable evidence supporting this hypothesis, there are observations that seem to be inconsistent.

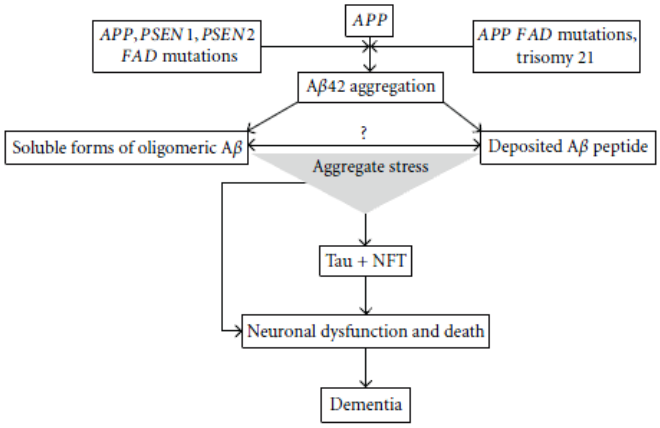

Fig d: *The Amyloid cascade hypothesis. From Reitz et al., 2011*

In fact, it has been reported that the quantity and temporal progression of amyloid plaques do not correlate well with the clinical evolution of the disease (Katzman, 1986; Dickson et al., 1995). Moreover, plaques have been reported also in non-demented individuals. Alterations in synaptic connectivity and/or strength have been observed in transgenic mouse models of AD before the appearance of senile plaques (Jacobsen et al., 2006; Lanz et al., 2003). These observation have led to a critical reexamination of some assumption of this hypothesis, with increased recognition that soluble non-fibrillar Aβ assemblies are the major neurotoxins which cause synaptotoxicity in the early phases of the pathology (Shankar and Walsh, 2009). The gradual accumulation and aggregation of this small hydrophobic peptide initiates a slow but deadly cascade that leads to synaptic alteration, microglial and astrocytic activation, modification of tau protein and progressive neuronal loss associated with multiple neurotransmitter deficiencies and cognitive failure (Hardy and Selkoe, 2002).

The neurotoxicity of Aβ oligomers has been confirmed by distinct experimental approaches, including the use of synthetic or native Aβ peptides, cell culture systems over-expressing the amyloid precursor protein (APP) from which Aβ is derived, and APP transgenic mice (Lesne et al, 2006; Walsh et al., 2002). Today, diffusible oligomers of Aβ are considered metastable neurotoxic molecules that exist for prolonged periods without conversion to fibrillar structures. Therefore, attention has been focused on Aβ oligomeric species to elucidate the underlying mechanism of neuronal degeneration. Many different types of soluble assembly of synthetic Aβ, including protofibrils (PFs), annular structures, paranuclei, Aβ-derived diffusible ligands (ADDL), and globulomers have been described over the last two decades (table a) (Teplow et al., 1998). In particular, ADDLs have been shown to inhibit hippocampal long-term potentiation (Wang et al., 2009) and to cause death in different culture systems (Kim et al., 2003).

Recently, an apparent dodecamer of natural Aβ, named Aβ*56, has been detected in the brains of an APP transgenic mouse line (Lesne et al., 2006). It has been suggested that Aβ*56 might represent an *in vivo* analogue of synthetic ADDLs. In order to obtain an antibody that specifically recognize the oligomeric state of Aβ, Kayed and coworkers developed a conformation-dependent antibody named A-11 (Kayed et al., 2003). Surprisingly, it was able to bind soluble oligomers among all other all other types of amyloidogenic proteins and peptides examined, indicating that they have a common structure and may share a common mechanism of toxicity. In the absence of high resolution oligomer's structures, the conformation dependent antibody A-11 has been used to provide a more rational mean of

classifying amyloid oligomers (Glabe, 2008). Moreover, the oligomer-specific antibody A-11 has been reported to inhibit the amyloidogenic mediated toxicity of all proteins and peptides able to bind it. The pathogenic relevance of natural Aβ oligomers is supported by the finding that their formation is increased by expressing AD-causing mutations within APP or presenilin genes in recombinant cells (Xia et al., 1997). Moreover, putative ADDL-like oligomeric assemblies have been isolated from post-mortem AD brains and their presence correlates with memory loss (Gong et al., 2003). When micro-injected in living rats (Walsh et al., 2002) or added *in vitro* to hippocampal slices (Townsend et al., 2003), natural oligomers of human Aβ are acutely toxic on synaptic functions. In rats, Aβ oligomers have also been shown to interfere rapidly and reversibly with the memory of a learned behaviour (Klyubin et al., 2005). Noteworthy, the evidence that Aβ immunotherapy neutralizes the synaptotoxic effects of soluble oligomers has led to the notion that antibody-mediated inactivation of Aβ oligomers might be a therapeutic strategy for early AD (Rowan et al., 2005).

Even thought the involvement of Aβ remains central in AD pathology, many other structural and functional features of AD should be taken in consideration. These include inflammatory response, oxidative stress and other pathological changes linked to aging. Through this point of view, AD is due to the combined consequences of all the pathological changes, including among many others the effects of the Aβ and tau protein (Mattson, 2004).

Oligomeric assembly	Characteristics
Protofibril (PF)	Intermediates of synthetic Aβ fibrillization; up to 150 nm in length and ~5 nm in width; β-sheet structure: bind Congo red and Thioflavin T
Annular assemblies	Doughnut-like structures of synthetic Aβ; outer diameter of ~8–12 nm; inner diameter of ~2.0–2.5 nm
Aβ-derived diffusible ligands (ADDLs)	Synthetic Aβ oligomers smaller than annuli; might affect neural signal-transduction pathways
Aβ*56	Apparent dodecamer of endogenous brain Aβ; detected in the brains of an APP transgenic mouse line and might correlate with memory loss
Secreted soluble Aβ dimers and trimers	Produced by cultured cells; resistant to SDS; resistant to the Aβ-degrading protease IDE; alter synaptic structure and function

Tab a: *Aβ oligomeric assemblies*

3. Aß AGGREGATION

Aggregation is one of the common consequences of a polypeptide chain failing to reach or maintain its functional three-dimensional structure. Such events can be associated with specific mutations, misprocessing phenomena, aberrant interactions with metal ions, changes in environmental conditions, such as pH or temperature, or chemical modification (oxidation, proteolysis). Perturbations in the conformational properties of the polypeptide chain may increase the population of partially unfolded, or misfolded, species that are much more aggregation-prone than the native state.

Amyloid fibrils characterized as highly intractable thread-like species are associated with many neurodegenerative diseases. Although neither the mechanism of amyloid formation nor the origin of amyloid toxicity is currently completely understood, during the last decade determination using using x-ray diffraction, cryoelectron, microscopy and solid-state NMR has provided important informations towards the comprehension of the aggregation mechanisms of amyloidogenic

proteins. In particular X-ray diffraction analyses of isolated amyloid protein fibrils (Bonar et al., 1969; Sunde and Blake, 1998) revealed that all proteinaceous amyloid fibrils were ordered in secondary structure, with the polypeptide backbone assuming the beta pleated sheet conformation and oriented perpendicular to the fibril axis. Aß fibrillogenesis *in vitro* was firstly described as a nucleation-dependent polymerization process (Jarrett and Lansbury, 1993). The kinetic of this type of process is controlled by two key parameters, nucleation rate and elongation rate. Monomeric Aß has been shown to associate non covalently to form nuclei or "seeds" from which soluble protofibrils and then full-length insoluble fibrils arise (**Fig e**). Along this pathway, small oligomeric intermediates and short fibrillar structures (protofibrils) have been observed. The transient appearance of protofibrils precedes that of fibrils during Aß aggregation in vitro, and this has suggested that protofibrils are precursors to fibrils (Esler et al., 1996). Typically, Aß fibrils are straight, unbranched, 7-12 nm wide, formed by 5-6 elementary filaments (protofilaments) around 1.5-2 nm in diameter, that may be wrapped or twisted around each other with regular helicity in a rope-like structure. Aß fibrils are ~10 nm in diameter and can reach lengths of 1 μm or more (Sunde et al., 1997). Each protofilament appears to have a highly ordered inner core that X-ray fibre diffraction data suggest as a cross-ß structure. In this structural organization, the ß-strand runs perpendicular to the protofilaments' axis, resulting in a series of ß-sheets that propagate along the direction of the fibril (Stefani and Dobson, 2003). Aß monomers possess regions of ß-strand and turn that appear to be

retained in the fibrillar structure as shown with solution NMR by Hou *et al* (Hou et al., 2004). Aß oligomers are globular aggregates that generally lack a well-defined secondary structure. Aß was observed to form both small spherical oligomers of about 5 nm in diameter with molar masses in the 20-50 kDa range (Lambert et al., 1998) as well as large spherical oligomers with diameters around 15 nm and molar masses approaching 1 million Da (Huang et al., 2000). Aß protofibrils are linear aggregates that appear early in the amyloid pathway. They share structural epitopes with oligomers and like them lack the well-defined secondary structure of filaments and fibrils. These structures are generally considered to be intermediates in the amyloid formation pathway (Harper et al., 1997; Teplow, 1998).

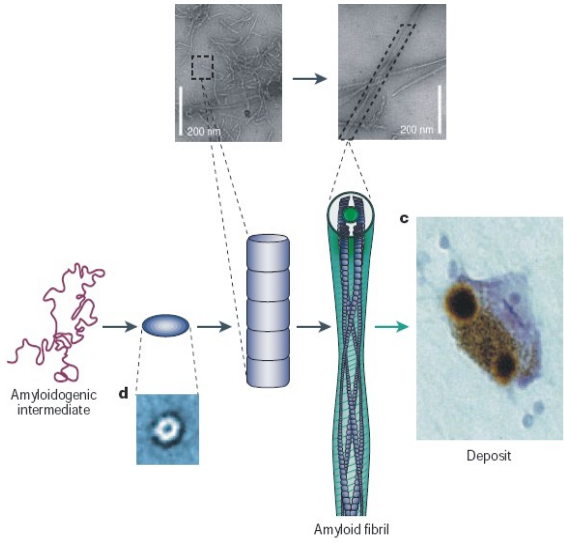

Fig e: *schematic rappresentation of the general mechanism of aggregation to form amyloid fibrils. Unfolded or partially unfolded proteins associate with each other to form small, soluble aggregates that undergo further assembly into proto fibrils or protofilaments and then mature fibrils. Top electron microscope images*

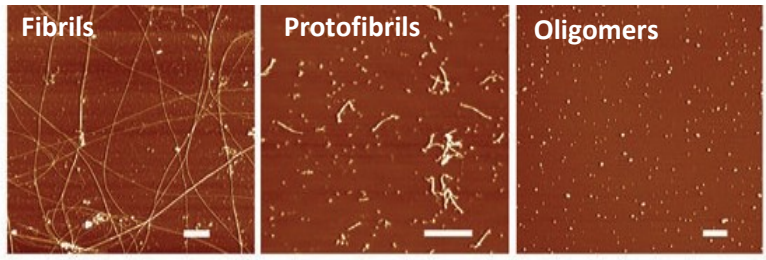

Fig f: *different (biophysical) assembly states of Aβ. The assembled forms obtained form incubation of synthetic Aβ are highly sensitive to preparation and incubation. Widely differing proportions of insoluble fibrils, soluble PFs and oligomers also known as ADDLs .*

3.1 Factors affecting Aß aggregation

Beyond external factors, intrinsic properties of Aβ sequence itself can be considered one of the factors involved in the process of aggregation. Aβ monomer is unstructured, unfolded 4 kDa protein, rich in hydrophobic residues. These hydrophobic amino acids cluster at the C-terminus of the peptide located in the transmembrane part of APP and in position 17-21, referred to as the central hydrophobic cluster. Residues 10-24 have been reported to be α-helical, whereas the N-terminal portion of the peptide consists of an extended strand. It has been suggested that Aβ can form a partly unfolded helix containing intermediate, which accelerates fibril formation. However, the completely unfolded peptide also appear to be able to form fibrils, but at a slower rate (Fezoui and Teplow, 2002). Pathogenic mutations within the Aβ peptide are associated with AD. Five pathogenic intra-

Aβ mutations have been identified and named as the ethnic origin of the affected families. All five intra Aβ mutations, with the exception of Flemish's (A21G) mutation, increased the aggregation rate of Aβ (Walsh et al., 2001; Murakami et al., 2002). Moreover, the Arctic (E22G) and Dutch (E22Q) mutations are associated with accelerated Aβ protofibril formation (Yamamoto et al., 2004). *In vitro* studies have also demonstrated that Aβ assembly can be accelerated by several extrinsic factors such as ionic strength, temperature, acidic pH, protein concentration (Barrow and Zagorski, 1991; McLaurin et al., 2000). It is likely that the development of amyloid aggregates *in vivo* depends on the combined action of the above mentioned biophysical factors and many different biomolecules (including proteins, proteoglycans, lipids, metals and other small molecules) that have been reported to be associated with amyloid plaques in AD brains.

4. PHYSIOLOGICAL ROLE(S) OF Aß PEPTIDE

In the controversial literature about AD, a predominant idea refers to the crucial role of Aβ in the pathogenesis of the disease. However the production of Aβ through the endoproteolytic cleavage of APP is a physiological process that occurs normally in neuronal cells (Cirrito et al., 2003). The nucleotide sequence of Aβ has been well conserved through evolution and is present in virtually all vertebrates that have been investigated (Coulson et al., 2000). After production, Aβ is exported outside the brain by the low density lipoprotein receptor related protein-1 (LRP-1). The amount of Aβ synthesized outside of the brain is instead transported inside *via* the receptor for advanced glycation end-products (RAGE) (Mackic et al., 1999). In human CSF, Aβ (1–40) is present a concentration of approximately 2–

3 ng/mL, while the concentration of Aβ (1–42) is roughly an order of magnitude lower (Ida et al., 1996). The concentrations of Aβ (1–40) and Aβ (1–42) in the CSF are relatively high early in life, decrease slightly in middle age and then increase again in old age (Shoji et al., 1998). It is interesting to note that in AD the levels of soluble Aβ (1–40) in the CSF are not different from those in age-matched cognitively normal people, while the levels of Aβ (1–42) are 37–75% lower in AD. Aβ is present in the sera of most people at concentrations of around 185 ± 86 pg/mL for Aβ (1–40), and 52 ± 23 pg/mL for Aβ (1–42) (Ida et al., 1996). The tightly regulated bidirectional trafficking of Aβ across the blood brain barrier suggests a biological role of the protein for which the production and removal of the peptide must be maintained into a specific range of concentrations. Accordingly, several metalloproteases, including neprilysin, insulin-degrading enzyme (IDE) and endothelin converting enzymes, have been reported to act in Aβ clearance (Qiu et al., 2006). During the past decade, few physiological activities have been proposed for the peptide. Essentially, based on findings from differents grups, two main aspects have emerged: the modulation of synaptic activity, and the neurotrophic function. We will discuss separately these two aspects.

4.1 Aß as modulator of synaptic activity

The use of transgenic mice has recently provided a strong hint toward this concept. BACE 1 knock-out mice, which lack Aβ formation, have behavioral deficits (Harrison et al., 2003) and synaptic dysfunctions (Ohno et al., 2004; Wang et al., 2008), including a reduced activity-dependent strengthening of presynaptic release at mossy fiber synapses. Similar to BACE 1 knock-out, APP null mutant mice show

an impaired formation of LTP and, as a consequence of this synaptic impairment, they have reduced learning and memory (Muller et al., 1994; Dawson et al., 1999; Senechal et al., 2008). These finding might well be related to the loss of function of either BACE 1 or APP, rather than to the missing production of Aβ. Interestingly enough, however, the transgenic approach strengths the older finding that physiological Aβ production sustains survival in cultured neurons (Plant et al., 2003). Along this line is the demonstration that picomolar concentrations of synthetic Aβ, which are likely to approximate the endogenous level of the peptide, enhance synaptic plasticity and memory in the hippocampus (Puzzo et al., 2008). In the same system, high nanomolar concentrations of Aβ led to the well known impairment of synaptic functions, suggesting that the concentration level of the peptide is crucial for its physiological activity.

Several lines of evidence converge to indicate that Aβ is released in normal brains during synaptic activity. Kamenetz and colleagues (Kamenetz et al., 2003) first reported that Aβ is secreted from healthy neurons in response to neuronal activity, and in turn down-regulate excitatory synaptic transmission. This negative feedback loop, in which neuronal activity promotes Aβ production and Aβ decreases synaptic activity, would provide a physiological homeostatic mechanism to maintain the levels of neuronal activity. Recently, the regulation of the endogenous synaptic release of Aβ has been addressed in rodent hippocampal cells and slices (Abramov et al., 2009). This study shows that acute increases in Aβ levels expand, reversibly, the number of active synapses and the amount of neurotransmitter released at each synapse, whereas enduring inhibition of Aβ clearance results in a reduction in the number of synapses. Thus,

Aβ appears to be a modulator of synaptic activity requiring a fine balance between production and removal. Accordingly, sequestration of endogenous Aβ by the monoclonal antibody 4G8 disrupts memory in adult rats, whereas hippocampal injection of physiological concentrations of Aβ rescues the amnesia produced by the anti-Aβ antibody (Garcia-Osta et al., 2009). Different from normal Aβ concentrations, the high levels of peptide present in transgenic mice over-expressing human APP are *per se* sufficient to elicit epileptiform activity and seizure, even at an early stage of the pathology and in the absence of neuronal loss (Palop et al., 2007). This Aβ-induced aberrant neuronal activity has been suggested to trigger compensatory inhibitory responses causally linked to cognitive decline. In AD patients, 7% to 21% of individuals with sporadic AD are estimated to have at least one unprovoked clinically apparent seizure during the illness. The relationship between this phenomenon and AD is even stronger in the case of autosomal dominant early onset AD (Palop and Mucke 2009). Once again, the levels of soluble Aβ may be critical for the dual effect of Aβ at the synapse.

The discovery that Aβ binds to the α7 subunit of nicotinic acetylcholine receptors (nAChRs) with high affinity has provided a strong support to the old hypothesis of a cholinergic deficit responsible for the cognitive dysfunction in AD. Nevertheless, whereas higher concentrations of Aβ desensitize α7-containing nAChRs (Pettit et al., 2001; Lee et al., 2003), low concentrations of the peptide appear to activate pre-synaptic nAChRs (Dineley et al., 2002; Lee et al., 2003), which are responsible for glutamate release during LTP.

Additional effects of Aβ at the synapse have been reported as being solely disruptive and linked to an impairment of AMPA and NMDA

receptor trafficking (Gu et al., 20009; Schenk et al 1999) or the disassembly of the post-synaptic density (Roselli et al., 2005); studies refer to synthetic preparations of Aβ oligomers, and clearly suggest that the concentration of Aβ in the synaptic cleft affects its aggregation state, and that differently assembled Aβ aggregates have different effects on synaptic activity. Nevertheless, the true identity of the Aβ species that act as modulators of synaptic activity, especially in the case of endogenous released Aβ (Abramov et al., 2009), remains unclear.

4.2 Aβ monomers : the neuroprotective species

Based on the notion that synaptic activity regulates the expression of gene products that are important for neuronal survival (West et al., 2002), the evidence that Aβ can act as a synaptic modulator is *per se* suggestive of a pro survival role of the peptide. One of the first pieces of evidence supporting a physiological role for Aβ dates back to 1989, when the 1-28 fragment of the peptide was shown to have neurotrophic activity (Whitson et al., 1989). The following years have been characterized, instead, by an extensive amount of research on the toxic effects of aggregated forms of Aβ. Indirect evidence for the implication of Aβ in the normal neuronal metabolism can be found in several papers published in this field. The *in vitro* inhibition of either β- or γ-secretase seems to affect the viability of cortical neurons, which are rescued by adding picomolar concentrations of Aβ1-40/42 (Pettit et al., 2001).

The contribution of Aβ to physiological neuronal activity is strengthened by the observation that the addition of Aβ (1-42) to cultured neurons enhances glucose uptake and metabolism via the

induction of hypoxia-inducible factor-1 (Souceket al., 2003). More important, indirect evidence for a neuroprotective activity of Aβ has been recently obtained in patients who underwent invasive intracranial monitoring after acute brain injury. Results provided by the authors show a strong correlation between the Aβ levels in the CSF and the patients' neurological status, with Aβ concentrations increasing when the neurological status improves and falling when the neurological status declines (Brody et al., 2008).

Our group recently identified the nature of the neuroprotective effect of Aβ1-40/42, demonstrating that the protective activity of Aβ is confined to the monomeric, low concentrated form of the peptide. In neurons undergoing death by trophic deprivation, synthetic Aβ (1-42) monomers had a rescuing effect mediated by the activation of the phosphatidyl-inositol-3-kinase (PI-3-K) pathway. The activation of the PI-3-K pathway, which is a main surviving path in neurons (Franke et al., 1997), could be reconducted to the stimulation of IGF-1 receptors and/or other receptors of the insulin superfamily (Giuffrida et al., 2009). Interestingly, Aβ1-40/42 monomers had a broad rescuing effect that included neuroprotection against excitotoxic cell death, a process that contributes to several neurodegenerative diseases (Doble et al., 1999). We should highlight that monomers of the artic-mutant Aβ (1-42) do not share the same neuroprotective properties of the Aβ 40/42 peptides (Giuffrida et al., 2009). Conformational studies of the different Aβ monomers indicate that the neuroprotective Aβ 40/42 species share similar folding properties and have similar conformational features, thus suggesting that they might bind to specific recognition sites on the neuronal surface.

A dysregulation of Insulin/IGF-1 signaling is thought to sustain a crucial role in the pathogenesis of AD. Some evidence indicates that insulin/IGF-1 resistance, as occurs in type 2 diabetes, is linked to the development of late-onset forms of AD (Li et al., 2007; Sabayan et al., 2008), and alterations of both insulin receptors and IGF-1 receptors have been reported in the AD brain (Moloney et al., 2008).

5. ROLE OF IGF-1-RECEPTOR AND INS-RECEPTOR SIGNALLING IN THE PATHOGENESIS OF AD

In different clinical studies, an association between type 2 diabetes and neurodegenerative diseases, as well as a decline in memory, have been described (Janson et al., 2004; Ott et al., 1999; Stewart and Liolitsa, 1999; Lovestone, 1999). Recent longitudinal studies have shown that glucose intolerance and impairment of insulin secretion are associated with a higher risk to develop dementia or AD (Ott et al., 1996; Luchsinger et al., 2004; Ronnemaa et al., 2008). Moreover, patients with AD frequently present impaired glucose metabolism or type 2 diabetes (Janson et al., 2004). In the last years there has been growing evidence for an influence of insulin and insulin-like growth factor (IGF-1) signalling in the pathogenesis of neurodegenerative diseases. To some extent vascular complications of type 2 diabetes or glucose intolerance might be leading to neurodegeneration due to insufficient neuronal nutrient supply as well as impaired Aß clearance from the brain (Pluta et al., 2003). Furthermore, disturbed cerebral perfusion due to endothelial dysfunction, and alteration of the blood brain barrier, results into an upregulation of APP expression and Aß deposition (Sadowski et al.,

2004). Also, hyperinsulinemia as it is present in type 2 diabetes may play an important role in formation of senile plaques (de la Monte et al., 2005). Alternative mechanisms might be directly related to insulin/ IGF-1 signaling, suggesting a common pathogenetical cerebral signaling pathway in type 2 diabetes and AD. Postmortem analyses of brains from AD patients revealed a markedly downregulated expression of the insulin receptor (IR) and its downstream signaling targets (Talbot et al., 2012), suggesting AD to be a neuroendocrinological disease which some authors refer to as "brain-type diabetes" or "type 3 diabetes" (Steen et al., 2005). Moreover, disturbances of the IR/IGF-I receptor (IGF-IR) signalling cascade progresse with severity of neurodegeneration (de la Monte et al., 2005). These findings raise the question whether the changes in the neuronal insulin/IGF-1 signaling cascade are cause or consequence of neurodegeneration in AD.

In the last years a role of IR/IGF-IR signaling in different aspects of the AD pathogenesis has been emphasized using different model organisms and human studies. IR/IGF-IR mediated signals might be involved in regulation of tau phosphorylation, APP cleavage, Aß transport, and degradation as well as memory formation, aging and longevity. Here follows a review of the possible role of IR/IGF-IR signaling in these processes.

5.1 Insulin, Insulin-like growth factor and their receptors

Both insulin and IGF-I belong to the same protein family and are filogenetically very ancient (Mattson, 2002). While insulin is best known as a glucoregulatory signal and IGF-I as a potent growth factor and a mediator of growth hormone actions on somatic growth, both are

also important modulators of brain function (de Pablo and de la Rosa, 1995). For instance, it is accepted that brain energy balance is regulated by the two hormones (Bondy and Cheng, 2002; Schwartz et al., 1992), but many other non-metabolic actions of insulin/IGF-I on the brain are gradually being unveiled. For example, it is very likely that the remarkable ability of insulin/IGF-I to modulate neuronal excitability and synaptic plasticity (Blair and Marshall, 1997; Carro et al., 2000; Castro-Alamancos and Torres-Aleman, 1993; Fadool et al., 2000; Gonzalez de la Vega et al., 2001; Gutierrez- Ospina et al., 1997; Kanzaki et al., 1999; Wan et al., 1997; Man et al., 2000) underlies the modulatory effects of these hormones on cognitive processes (Aleman et al., 1999; Craft et al., 2000). Therefore, it is not coincidental that insulin/IGF-I functions appear dysregulated in widely different neurodegenerative diseases as evidenced by impaired cellular responses to these hormones and/or changes in their circulating levels (Blum-Degen et al., 1995; Busiguina et al., 2000; Craft et al., 1998, 1999). This is indeed the case for AD.

As with other hormone/growth factor receptor families, insulin and IGF-I receptors show relative promiscuity and can bind both IGF-I and insulin with a near 100-fold difference in their binding affinity. Although this may appear to be a sufficiently large difference in receptor affinity, pericellular concentrations of insulin and IGF-I may vary widely. Very high levels of either peptide can be reached in specific locations such as for example in insulin-producing pancreatic islets (beta cells are rich in IGF-I receptors) or in tissue areas with high levels of IGF-binding proteins (IGFBPs) that will result in local accumulation of IGFs. To make the situation even more complex,

hybrid insulin/IGF-I receptors with the ability to bind both ligands with similar affinity have been described (Federici et al., 1999). In fact, the IR and the IGF-1R are heterotetrameric receptor tyrosine kinases consisting of two α- and ß-subunits linked by disulfide bonds (Fig. **g**). The α-subunits are located exclusively extracellularly (Van Obberghen et al., 1981; Kasuga et al., 1983; Ullrich et al., 1986), the transmembrane and intracellular parts of the ß-subunits contain an insulin regulated tyrosine-specific protein kinase (Chou et al., 1987; Ebina et al., 1987). Alternative splicing of exon-11 leads to synthesis of two insulin receptor isoforms (IRa and IRb). IRb binds insulin with high affinity, whereas IRa binds insulin or IGF-2 with comparable affinity. Hybrid receptors composed of αß-heterodimers from the IGF-IR and the IRb selectively bind IGF-1, whereas hybrid receptors composed of IGF-IR and IRa bind IGFs and insulin with similar affinities (White, 2006). IR and IGF-1R expression overlaps in many brain regions (Bondy et al., 2004), and also hybrid insulin/IGF-1 receptors, with an unclear physiological role, are highly present in the brain (Bailyes et al., 1997). Noteworthy, insulin has a low affinity for both IGF-IR and hybrid receptors that, instead, are bound by IGF-1 with higher affinity than insulin (reviewed in Belfiore et al., 2009). Specifically, at least in purified receptors from human placenta, the concentration of unlabeled IGF-I for half-maximal inhibition of $_{125}$I-IGF-I binding appears to be 0.1–0.2 nM for hybrids and 0.05–0.01 nM for IGF-1R. By contrast, unlabeled insulin required for half-maximal inhibition of $_{125}$I- insulin binding is 3–5 nM for hybrids and 0.3–0.5 nM for IRs, confirming the relatively low affinity of hybrids for insulin (Soos et al., 1993). The evidence that IGF-1 inhibits $_{125}$I-insulin binding to hybrid receptors or IGF-1R more effectively than insulin (1

and 0.04 nM (IGF-I) vs. 4 and 4 nM (insulin) for hybrid receptors and IGF-IR, respectively) (Soos et al., 1993) and also stimulates the kinase activity of hybrid receptors more significantly than insulin (Kasuya et al., 1993) suggests that hybrid insulin/ IGF-1 receptors might have the functional properties of an IGF-IR.

5.2 The Insulin receptor and the IGF-1 receptor signalling systems

The signaling mechanisms and the biological effects of insulin and IGF-1 have been studied mainly in classical insulin target tissues, such as skeletal muscle, fat and liver, with respect to glucose uptake, regulation of cell proliferation, gene expression and the suppression of hepatic glucose production. Over the past few years, it has become clear that insulin and IGF-1 also have profound effects in the central nervous system (CNS), regulating key processes such as energy homeostasis, neuronal survival, and longevity as well as learning and memory.

The binding of insulin or IGF-1 to their receptors induces conformational changes stimulating the autophosphorylation of the intracellular ß-subunit (Kahn et al., 1978). Tyrosine-phosphorylated IR/IGF-1R ß-subunits recruit and subsequently phosphorylate tyrosine residues of the intracellular insulin receptor substrates (IRS). The IRS protein family has at least four members, IRS-1 to -4 (Sun et al., 1991; Lavanet al., 1997). These proteins are homolog in structure and function but show distinct tissue distribution. IRS-1 and IRS-2 are widely distributed throughout different tissues and the brain, whereas IRS-3 is only expressed in rodent adipose tissue, and IRS-4 is predominantly localized in hypothalamus, thymus, skeletal muscle, heart, kidney, and liver (Giovannone et al., 2000; Schubert et al.,

2003). Upon its activation, the IRS proteins bind several Src homology (SH-2) domain containing cellular signaling proteins, such as p85, the regulatory subunit of phosphatidylinositide(PI)3-kinase, growth factor receptor binding protein(GRB-2) and SH2-Phosphatase (SHP-2). Activation of the catalytic subunit of the PI3-kinase results in phosphorylation of phosphatidylinositide-diphosphate (PI4,5P) to generate phosphatidylinositide-triphosphate (PI3,4,5P). This leads to activation of several downstream targets, such as phosphoinositide-dependent protein kinase (PDK)-1, protein kinase B (PKB, AKT), p70S6kinase, glycogen synthase kinase (GSK)-3ß and BAD a proapoptotic member of the Bcl-2 family. Phosphorylation of GSK-3ß and BAD inactivates these proteins and thereby inhibits further signalling leading to apoptosis. Activated AKT phosphorylates the forkhead transcription factor Foxo, which triggers its nuclear exclusion and thereby regulates transcription of genes involved in development, growth, stress resistance, apoptosis, metabolism and aging (Partridge et al 2008; Naimi et al., 2007). Several Foxo target genes might play an important role for the pathogenetics of AD (review in Calnan et al., 2008): catalase and MnSOD (manganese superoxid dismutase) are crucial in promoting longevity by possibly acting as antioxidants due to reduced insulin-like signalling in various species such as *drosophila* (Curtis et al., 2007) and *C. elegans* (Chavez et al., 2007). However, data from mammals are inconsistant and more research is needed concerning Foxo signaling in mice and men. The regulation of Fas ligand through Foxo is at least partially responsible for IGF-1 and insulin mediated anti-apoptotic effects. A phathological role of Foxo has been recently described in *C. elegans*. Cohen and coworkers (Cohen et al., 2006) showed that DAF-16-mediated signaling, the

ortholog of Foxo in mammals, might be important in promoting amyloidogenesis.

Furthermore, AKT phosphorylates tuberin (TSC2), which inhibits its GAP activity (guanosine triphosphataseassociated protein) towards the small G protein RHEB (RAS homolog enriched in brain), which causes an accumulation of the RHEB-GTP complex that activates mammalian target of rapamycin (mTOR) (Astrinidis and Henske, 2005). Another major physiological role of insulin and IGF-1 is the regulation of gene transcription via the MAP kinase cascade. Following insulin and IGF-1 stimulation, IRS proteins and GAB (GRB-2 associated binder)-1 bind to the SH2 domains of several small adaptor proteins such as GRB-2. These proteins then interact with the GDP/GTP exchange factor SOS (son of sevenless) leading to activation of the small G-protein RAS and subsequently to the recruitment of c-RAF to the membrane. Activated c-RAF activates MEK, which then activates extracellular signal-regulated kinase (ERK)-1/-2 [37]. ERK-1/-2 mediated signals are involved in long-lasting neuronal plasticity, including long-term potentiation and memory consolidation (review in Sweatt, 2001). In contrast, overactivation of ERK-1/-2 also leads to apoptotic cell death in case of oxidative stress or growth factor deprivation (review in Zhuang and Schnellmann, 2006). However, ERKs seems to be an essential gene since animals lacking ERK-2 die in early embryonic development (Saba-El-Leil et al., 2003).

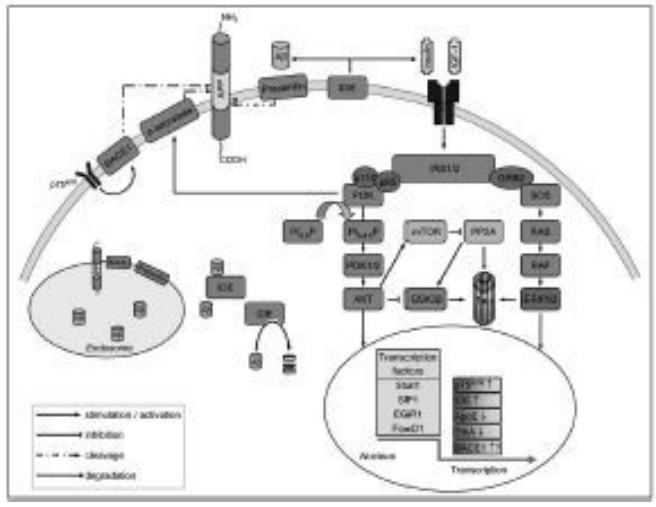

Fig. g. ***Cerebral IR/IGF-1 signaling in APP metabolism.*** *Binding of insulin and IGF-1 to their receptors leads to autophosphorylation of the ß-subunit of the IR/IGF-1R and recruitment of IRS-1/-2. IRS proteins activate mainly two pathways, the PI3 kinase pathway, and the RAS-RAF-MAP kinase cascade. Tau phosphorylation via IR/IGF-1 signaling is influenced by GSK-3ß and the tau phosphatase PP2A. Interestingly, mTOR signaling couples the activity of PP2A and GSK-3ß in a way that the activities of both enzymes change always in the same direction, suggesting that only a dysregulation of either PP2A or GSK-3ßinduces tau hyperphosphorylation. The PI3K pathway as well as the RAS-RAF-MAPK cascade regulate different transcription factors involved in transcriptional regulation of metabolism and clearance of ß- amyloid. APP is cleaved by α, ß-secretase (BACE-1) and γ-secretase (presenilin). ß - and subsequent γ-cleavage of APP leads to generation of ß -amyloid1-40/1-42. IGF-1 signaling promotes a switch from TrkA to p75NTR expression leading to increased ß-secretase activity due to an upregulation of BACE-1 expression. Recent data suggest that not only BACE-1 is influenced by IR/IGF-1R mediated signals but also α-secretase activity is stimulated by the PI3K pathway. From Freude et al., 2009.*

5.3 The Insulin and IGF-1 system in the brain

In 1978 the localization of insulin receptors in the CNS was demonstrated for the first time (Havrankova et al., 1978). According to numerous studies, IRs and IGF-1Rs as well as IRS-1 and IRS-2 are widely distributed throughout the developing and mature brain mediating the intracellular effects of mainly insulin and IGF-1 (Hill et al., 1986; van Houten and Posner 1979; Baskin et al., 1986). The insulin and IGF-1 receptor have distinct expression patterns in the mammalian brain. The highest IR density is found in olfactory bulb, hippocampal formation, hypothalamus, and cerebral cortex. The IGF-1R is expressed throughout the rodent CNS with high levels of expression detected in the developing cerebellum, midbrain, olfactory bulb, and in the ventral floor plate of the hindbrain (Garcia-Segura et al., 1997; Rotwein et al., 1988; Lesniak et al., 1988; Bondy et al 1990). Postmortem studies in adult humans showed the highest IGF-1R density in hippocampus, amygdala and parahippocampal gyrus (Schulingkamp et al., 2000). Whereas the density of brain IRs decreases during age, IGF-1Rs do not fade or even increase suggesting that particularly insulin mediated signals are involved in aging and possibly cause age associated cognitive decline (Chung et al., 2002). Even when IGF-1R expression is not altered during aging, serum IGF-1 levels decrease (Carro et al., 2002), leading to a reduction at least in peripheral IGF-1 action and possibly central IGF-1 effects, which also might alter cognitive function. However, the specific role of insulin and/or IGF-1 in aging, and which hormone is the most important player, in the development of neurodegenerative diseases, is not clear yet. Since insulin is not or very little produced in the CNS, the question raises how it enters the CNS. Insulin has been shown to cross

the blood brain barrier (BBB) by different mechanisms: extracellular pathways, non-saturable transmembrane diffusion or saturable active transport (Banks et al., 1985¸ Baura et al., 1993). Currently, the majority of studies suggest that the largest proportion of insulin crosses the BBB by receptor-mediated transport (King et al., 1985; Schwartz et al., 1990). The ability of endothelial cells to bind and internalize insulin might largely contribute to insulin transport across the BBB (Jialal et al., 1986; Pardridge et al 1984). An alternative transport mechanism is provided by the circumventricular organs and the choroid plexus. The circumventricular organs are known to be supplied by a vascular system with a "leaky" BBB, so that plasma solubles can diffuse freely into these areas and hereby promote insulin transport to brain and CSF (Porte et al., 1998). Peripherally injected insulin enters the CSF rapidly, and leads to activation of neuronal IR signalling within 10 min (Freude et al., 2005). Although this may vary among other species, less than 1% of the applied insulin reaches the CSF (Banks et al., 1998). Interestingly, there is a considerable variation in the speed of insulin transport to the brain. The transport rate into the olfactory bulb is up to eight times higher than into the whole brain suggesting that the olfactory system has a specific role in delivering peptides to the brain (Banks et al., 2004). Accordingly, an insulin formulation which is applied intranasally has been developed. Intranasally administered insulin is rapidly transported to the CSF without altering plasma glucose levels giving the opportunity to study insulin action in the CNS independent of the peripheral glucose lowering effect (Kern et al., 1999).The transport of insulin into the brain is influenced by various conditions. A decreased transport has been described in fasting, obesity, dexamethasone treatment, aging and

in AD (Baskin et al., 1985; Baura et al 1996). In AD patients insulin levels in the CSF are decreased, whereas pheripheral plasma insulin is elevated, and the ratio of CSF insulin to plasma insulin levels declines even further with the severity of dementia (Craft et al., 1998). In contrast to insulin, there is a considerable amount of IGF-1 produced within the CNS during development and to a lesser extent in the mature brain (Rotwein et al., 1988). However, besides the local expression of IGF-1, recent studies suggest that IGF-1 might be crossing the BBB via an insulin-like mechanism (Pan and Kastin, 200). Using $_{125}$I-labeled IGF-1 in rats it has been shown that a high amount of [$_{125}$I]-IGF-1 was transported into the brain, especially in paraventricular regions (Reinhardt and Bondy, 1994). Yu *et al.* (Yu et al., 2006) reported the existence of different transporter systems for IGF-1 and insulin in mice overlapping in their affinities, and thereby potentially inhibiting the transport of the other hormone. Taken together, the BBB is an important interface between the blood and the CNS compartment regulating uptake of insulin and IGF-1 into the brain. However, the molecular mechanisms by which different conditions like aging or AD decrease insulin transport to the brain are not known yet. Whether these mechanisms contribute to the pathogenesis of AD and cognitive decline is still unclear.

5.4 The IR/IGF-1R signalling is disturbed in AD

IR and IGF-1R signaling is markedly disturbed in the CNS of AD patients (Frolich et al., 1999; Moloney et al., 2008). Postmortem studies have shown that mRNA levels of insulin and its receptor decrease, with an almost 80% reduced IR expression in severe AD (Steen et al., 2005; Rivera et al., 2005). Accordingly, Frolich *et al*.

found that neuronal tyrosine kinase activity is decreased in AD patients compared to age-matched controls. The overall expression of IGF-IR is reduced in AD brains dependent on the severity of the disease. However, in some cases IGF-1R density was found to be increased in neurons next to amyloid plaques (Moloney et al., 2008; Rivera et al., 2005). Brain IGF-1 mRNA levels diminish in severe AD, whereas IGF-1 serum levels are increased in early stages of disease, suggesting that IGF-1 resistance plays a role in the pathogenesis of AD (Rivera et al., 2005; Vardy et al., 2007). IRS-1/-2 protein expression is reduced in AD brains and inactivating serine-phosphorylation of IRS-1 at Ser312 and Ser616 is increased leading to impaired IR and IGF-1R signaling (Moloney et al., 2008; Talbot et al., 2012). Thus, IR/IGF-1R downstream signal transduction is impaired in AD brains, leading to the hypothesis that cerebral insulin/IGF-1 resistance might be involved in the pathogenesis of AD. At present the leading pathological finding in AD is the accumulation of toxic Aß peptides. Townsend *et al*. (Townsend et al., 2007) demonstrated, in primary hippocampal neurons from mice, that soluble human Aß compromises the activation of the IR and IGF-IR downstream kinases, if administered to hippocampal cells. Furthermore, *in vitro* models revealed that soluble Aß peptides bind to the IR and interfere with its insulin-induced autophosphorylation (Townsend et al., 2007; Xie et al., 2002; Zhao et al., 2008). A further non insulin-, non amyloid-dependent hypothesis for late-ons*et A*D suggests that the IR may be desensitized for its natural ligands due to inhibition of receptor function by noradrenalin and/or cortisol, the levels of which both increase with age (Townsend et al., 2007; Hoyer, 2004).

Thus, insulin and IGF-1 signaling is impaired in brains of AD patients raising the question whether cerebral insulin resistance contributes to the pathogenesis of AD or rather might be a compensatory phenomenon.

5.5 IR/IGF-1R signalling enhances learning and memory in humans

The role of insulin signaling in the regulation of learning and memory formation in human and rodent brain is discussed. The conflicting information on insulin action in the CNS arises from difficulties in dissecting the direct actions of insulin in the brain from the effects resulting from hypoglycemia after peripheral administration of insulin (reviewed in Watson et al., 2004). Given that IRs are widely expressed in the hippocampus, the most important brain region for learning and memory, it seems to be plausible that decline of IR signaling leads to cognitive impairment (Squire, 1986; Craft and Watson, 2004). Interestingly, trials using the Morris water maze task, evaluating spatial learning and memory, revealed an upregulation of IR mRNA in cornu ammonis (CA1) and dentate gyrus as well as increased IR/IGF-1R signaling during training, emphasizing the role of insulin in learning and memory (Zhao et al., 1999). Experiments with adult mice lacking liver IGF-1 production (LID mice) with an up to 85% reduction in circulating IGF-1 showed impaired spatial memory in the Morris water maze task compared to wild type littermates (Svensson et al., 2006). These findings might possibly explain the reduction of cognitive functions during aging, since IGF-1 serum levels diminish with increasing age, even under physiological conditions (Dik et al., 2003). Unexpectedly, studies with neuronal/brain IR knockout mice

(NIRKO) could not provide any evidence for impairment in learning and memory, proposing that insulin resistance alone is not a key feature in dementia and neurodegeneration (Schubert et al., 2004). Contemplating these results one should consider that the NIRKO model was generated using Cre recombinase under control of the Nestin-promoter deleting the IR at early stages in embryogenesis. Thus, a lack of the IR might be compensated over time by other mechanisms, which could explain the unaltered learning and memory formation in these mice. Clinical trials in healthy humans under hyperinsulinemic euglycemic clamp conditions showed a negative shift in transcortical direct current potentials, indicating that circulating insulin can rapidly act on brain activity independent from its systemic effects (Hallschmid et al., 2004). Longitudinal studies revealed that insulin resistance with persisting hyperinsulinemia comes along with an elevated risk for disturbed cognition, impaired memory and AD (Young et al., 2006; Peila et al., 2004). In AD patients as well as in healthy subjects, hyperinsulinemic euglycemic clamp studies revealed an improving effect of insulin on cognitive functions (Craft et al., 1996; Kern et al., 2001). Intranasal application of insulin in healthy humans directly entered the CSF and improved memory function and cognitive capacity especially in women without influencing peripheral blood glucose levels (Benedict et al., 2007 and 2008). These gender specific findings suggest an influence of sex hormones. Accordingly, Clegg *et al*. demonstrated that insulin sensitivity in rat brains differ, depending on estrogen levels (Clegg et al., 2006). Craft *et al*. found that AD patients have higher plasma insulin but lower CSF insulin levels (Craft et al 1998). A possible explanation could be that central hypoinsulinemia is caused by reduced transport via the BBB or that

peripheral hyperinsulinemia might be reactive to central hypoinsulinemia, mediated by so far non distinctive pathways. Very recent data suggest that intranasal insulin administration in AD patients improves memory as well, providing a possible therapeutic option (Reger et al., 2008). Tschritter *et al*. (Tschritter et al., 2006) used a magnetoencephalography approach during a two-step hyperinsulinemic euglycemic clamp to assess cerebrocortical insulin effects in humans. In lean humans, stimulated cortical activity increased with insulin infusion relative to saline. In obese humans, these effects were suppressed, suggesting cerebral insulin resistance in these patients. Moreover, cerebrocortical insulin resistance was found in individuals carrying the Gly972Arg polymorphism of IRS-1, which is considered to elevate the risk to develop type 2 diabetes (Tschritter et al., 2006). Thus, cerebral insulin resistance might lead to impaired learning and memory, whereas administration of insulin in non-insulin resistant subjects promotes cognitive function. However, the molecular mechanism by which insulin enhances cognitive abilities and whether central hypoinsulinemia observed in AD contributes to the cognitive decline in AD currently remains unclear.

5.6 IR/IGF-1R signalling alters Tau phosphorylation

As discussed above, Tau is a microtubule-binding protein that ligates tubulin and accounts for the stability of microtubules. If hyperphosphorylated, tau aggregates and interferes with intraneuronal metabolism and transport leading to neurodegeneration (Avila and Diaz-Nido, 2004). Tau phosphorylation state is regulated by site-specific dephosphorylation through certain phosphatases and by kinases phosphorylating tau protein at specific sites. Protein

phosphatase 2A (PP2A) is the major phosphatase with 70% tau phosphatase activity in human brains (Avila, 2008). This implies a protective role of PP2A in neurodegeneration which is consistent with the finding that PP2A activity is reduced in AD brains (Liu et al., 2005 and 2008). The phosphorylation of tau is mainly promoted by GSK-3ß and cyclin-dependent kinase 5 (Cdk5). Besides these kinases, activated c-Jun N-terminal kinases (JNK) and ERK-1/-2 signaling lead to an increase in tau phosphorylation and therefore might be of importance in AD pathogenesis. GSK-3ß is a serine/threonine kinase, modulated by insulin/IGF-1 signaling. When the IR/IGF-1R pathway is activated, GSK-3ß is phosphorylated by AKT at Ser9 leading to its inactivation (Flaherty et al., 2000; Cho and Johnson, 2004; Jamsa et al., 2004; Stoothoff and Johnson 2005). However, PP2A dephosphorylates GSK-3ß (review in Millward et al., 1999), which then phosphorylates tau at several sites leading to the phosphorylation/dephosphorylation equilibrium of tau (Overview: see Fig. **1**). Thus, impaired IR/IGF-1R signaling might lead to hyperphosphorylation of tau protein and an increased formation of neurofibrillary tangles. In SHSY5Y cells, a human neuroblastoma cell line, as well as in primary cultures of rat cortical neurons, insulin administration leads to tau hyperphosphorylation (de la Monte et al., 2003; Lesort and Johnson, 2002; Lesort et al., 1999). In contrast, insulin and IGF-1 administration in NT2N cells, cultured human neurons, decreases tau phosphorylation (Hong and Lee, 1997). In primary cortical neuron cultures, Meske *et al*. (Meske et al., 2008) found that insulin treatment causes a regulatory interaction between PP2A and GSK-3ß. Inhibition of PI3-kinase leads to activation of GSK-3ß and PP2A. Enzyme activity of both enzymes always changed in the same direction. This balanced

response seemed to induce a steady state in tau phosphorylation at GSK-3ß/PP2A-dependent sites (Meske et al., 2008). Thus, only a dysbalance of insulin/IGF-1 regulated tau kinases and phosphatases might lead to tau hyperphosphorylation, partially explaining the different results obtained under different conditions. Besides *in vitro* studies, different *in vivo* models were used to investigate tau phosphorylation. In IGF-1 knockout mice a substantial increase of site-specific tau phosphorylation at Ser396 and Ser202 could be demonstrated, while tau mRNA as well as tau protein levels remained unchanged (Cheng et al., 2005). This suggests a protective role of IGF-1 to prevent tau hyperphosphorylation. Hyperphosphorylation of tau at Thr231 was found in NIRKO mice (Schubert et al., 2004). Furthermore, in IRS-2 knockout mice hyperphosphorylation of tau at Ser202 was demonstrated (Schubert et al., 2003). The pattern of tau phosphorylation in NIRKO mice is different from IRS-2 deficient mice, suggesting that not only insulin resistance, occurring in both mouse models, but also other factors might be involved, e.g. hyperinsulinemia to overcome insulin resistance. In type 2 diabetes hyperinsulinemia is a common feature to compensate for the underlying insulin resistance. In wild type mice, peripheral administration of supraphysiological insulin doses causes hyperinsulinemia and induces increased site specific tau phosphorylation at Ser202 within 10 minutes (Freude et al., 2005). However, in NIRKO mice peripheral hyperinsulinemia following insulin stimulation did not increase tau phosphorylation (Freude et al., 2005), suggesting that the cerebral effect of peripherally administered insulin is mediated via the IR and not by the IGF-IR. Streptozotocin (STZ) is specifically toxic for pancreatic beta cells. Chronic treatment

causes impairment of insulin secretion, and STZ mice serve as model of type 1 diabetes. In STZ mice, increased tau phosphorylation at multiple phosphorylation sites has be shown, which was reversible after peripheral insulin treatment (Clodfelder et al., 2006; Planel et al., 2007). The increased phosphorylation was most likely due to decreased activity of PP2A. These results show that insulin deficiency causes an increase of tau phosphorylation. Thus, hyperinsulinemia as well as complete lack of insulin result in increased tau phosphorylation, leading to the hypothesis that hyperphosphorylation of tau follows from an imbalance of insulin-regulated tau kinases and phosphatases. Currently the majority of *in vivo* studies suggest that severely impaired IR/IGF-IR signalling leads to tau hyperphosphorylation.

5.7 APP metabolism and aging is targeted by the IR/IGF-1R signalling

The APP processing by secretases has been widely discussed in paragraph 2. During aging changes in the cerebral expression levels of the neurotrophin receptors, TrkA (tyrosine kinase receptor A) and p75NTR (p75 neurotrophin receptor) have been described. In the human neuroblastoma cell line SHSY5Y, as well as in primary cultured neurons, chronic treatment with IGF-1 leads to a switch from TrkA to p75NTR expression as seen in aging brains (Costantini et al., 2006). This switch causes increased ß-secretase activity indirectly by activation of neuronal sphingomyelinase which is responsible for hydrolysis of sphingomyelin and the active liberation of the second messenger ceramide (reviewed in Puglielli, 2008). Ceramide is responsible for the molecular stabilization of BACE-1, the ß-secretase

which is rate-limiting for Aß generation (Puglielli et al., 2003). This process leads to accumulation of Aß, connecting IGF-1R signaling to neurotrophin action (Fig. g). Furthermore, Sotthibundhu *et al*. (Sotthibundhu et al., 2008) could recently show that treatment of wild type embryonic mouse hippocampal neurons with Aß (1-42) as a ligand for p75NTR resulted in significant cell death. In contrast, p75NTR deficient neurons are less affected by Aß (1-42) treatment. These data might provide a molecular link between aging, pathogenesis of AD and neuronal IR/IGF-IR signalling. Experiments in *Caenorhabditis elegans* revealed new insights into the role of IR/IGF-IR signaling in Aß toxicity and metabolism. Cohen and coworkers have shown that knocking down the DAF-2 pathway in *C. elegans*, which is orthologous to the mammalian insulin and IGF-1 signaling cascade, reduces Aß (1-42) toxicity (Cohen et al., 2006). Furthermore, this effect was mediated by the two downstream transcription factors, DAF-16 and HSF-1 (heat shock transcription factor-1) (Hsu et al., 2003). DAF-16 encodes a forkhead transcription factor (Ogg et al., 1997¸ Lin et al., 1997), which translocates into the nucleus (Lee et al., 2001) and modulates transcription when DAF-2 signalling is abrogated. The mammalian DAF-16 orthologs are Foxo1, 3, and 4 (Birkenkamp and Coffer, 2003). In the mammalian system, the IR/IGF-IR induces phosphorylation of Foxo1 and triggers its translocation outside the nucleus. Furthermore, in *C. elegans* the DAF-2 pathway is proposed to control longevity (Kimura et al., 1997). Similar findings were seen in *Drosophila melanogaster* where insulin signaling is mediated via *chico,* the ortholog of human IRS. If either the IR or *chico* is mutated, lifespan of these flies is prolonged (Tu et al., 2002; Clancy et al., 2001). Also, overexpression of dFoxO, the

ortholog of human FOXO, decreases mortality and increases lifespan in *Drosophila (*Giannakou et al., 2007).

To explore the influence of insulin and IGF-1 signaling on longevity in mammals, different rodent models with overexpression or deficiency in IR/IGF-IR signaling proteins have been studied. Peripheral injection of growth hormone (GH) leads to increased IGF-1 synthesis in the liver. It has been shown that GH-deficient, as well as GH-resistant mice, lived longer compared to their wild type littermates (Coschigano et al., 2003; Flurkey et al., 2001). Furthermore, GH seems to play an important role in age-associated cognitive decline (review in Gomez, 2008). Heterozygous deletion of the IGF-1R, and whole body IRS-1 knockout increases longevity in mice (Brown-Borg et al., 1996; Selman et al., 2008; Holzenberger, 2004). Interestingly, in mice, less IRS-2 signalling throughout the body or only in the CNS extended life span up to 18% (Taguchiet al., 2007), highlighting the role of IR/IGF-1R signalling in brain during aging. However, the different models feature remarkable differences concerning metabolism, especially insulin sensitivity, body weight and body fat mass. Taken together, these data from humans and different model organisms suggest an important role of IR/IGF-1R signaling in the regulation of APP cleavage and detoxification as well as aging.

5.8 Insulin and IGF-1 infkuence Aß clearance

Concerning insulin resistance, it has been shown that IDE expression is stimulated by the IR/IGF-IR cascade (Zhao et al., 2004). Furthermore, it has been suggested that increasing circulating IGF-1 levels lead to reduction of Aß burden in aging rats (Carro et al., 2002). In Tg2576 mice, serum IGF-1 levels are lower compared to controls (Carro et al.,

2002). IGF-1 administration resulted in reduction of cerebral Aß load in these mice, whereas Aß was elevated in the CSF suggesting an increased Aß elimination across the blood brain barrier (BBB) or the choroids plexus (Carro et al., 2002). Furthermore, it has been shown that the blockade of the IGF-IR in the choroid plexus triggers AD-like pathology (Carro et al., 2006). In contrast, Lanz *et al.* (Lanz et al., 2008) analyzed *in vivo* models using acute, subchronic and chronic IGF-1 treatment to evaluate Aß levels in brain, CSF and plasma of rats and Tg2576 mice. However, no changes in Aß were detected in any of these models. Furthermore, tau phosphorylation did not change significantly following chronic IGF-1 treatment in Tg2576 mice (Lanz et al., 2008). A possible explanation for this contradictory results concerning IGF-1 signaling could be that the chronic increase of IGF-1 by peripheral treatment might downregulate IGF-1R signaling. This hypothesis is supported by the finding that in a cohort of individuals with exceptional longevity, serum IGF-1 levels were high but IGF-IR activity was low leading to reduced IGF-IR signalling (Suh et al., 2008). Thus, increased serum IGF-1 levels do not mandatorily correlate with an upregulation of IGF-IR signaling. However, induction of insulin resistance by high fat diet (Ho et al., 2004) or intake of sucrose-sweetened water (Cao et al., 2007) leads to an aggravation of amyloid pathology in mouse models of AD. Furthermore, peripheral injection of supraphysiologically high insulin doses, but not of physiological doses, leads to transient cerebral tau phosphorylation (Freude et al., 2005), suggesting a dose dependent effect of IR/IGF-IR signaling in the pathogenesis of AD. Since the identification of neurofibrillary tangles and amyloid plaques as the most important neuropathological finding in brains of patients

suffering from AD (Ball, 1982), these two characteristics have been target for different therapeutic approaches. Drug development concentrates on inhibition of Aß production and aggregation or elimination of the Aß burden from AD brains by increasing ß-amyloid clearance (Torres-Aleman, 2007). A clinical trial where patients with AD were treated with the growth hormone secretagogue MK-0677 (Ryan et al., 2006) showed no beneficial effect on cognition despite elevated IGF-1 serum levels. However, there are still more studies needed to evaluate the beneficial or harmful impact of GH and IGF-1 administration as new treatment option of AD.

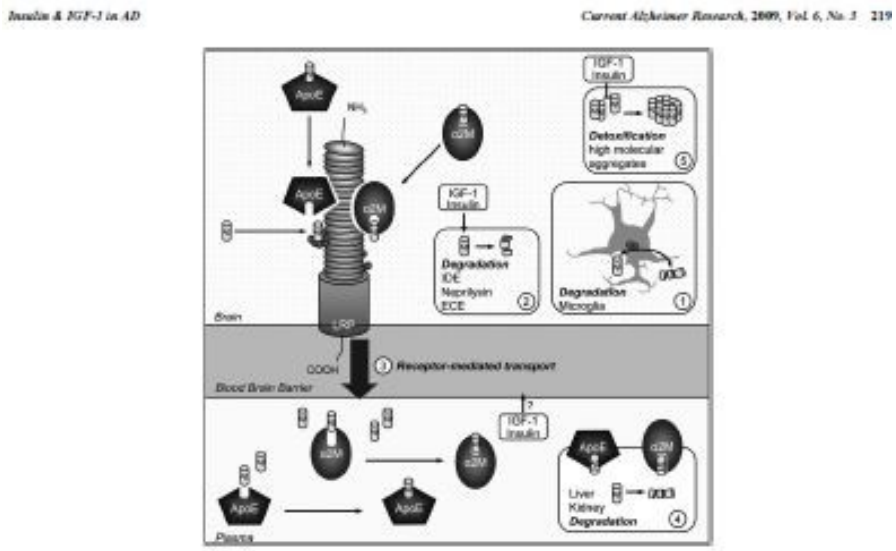

Fig. h. Aß clearance and detoxification. *Aß is removed from the brain by two different mechanisms: enzymatic degradation or receptor-mediated clearance. Degradation of Aß is enabled either by activated microglia (1) or by specific enzymes (2), e.g. IDE, neprilysin, or ECE. For receptor-mediated clearance Aß either binds directly to the LRP or is transported to the LRP by binding to LRP ligands, namely ApoE or α2M. The complexes are internalized (3) and Aß is released at the endothelial side of the BBB. Plasma ApoE and plasma α2M function as carriers to liver and kidney for degradation (4)..From Freude et al., 2009.*

CHAPTER I

Neurotoxic properties of the anabolic androgenic steroids, nandrolone and methandrostenolone, in primary neuronal cultures.

Filippo Caraci[1], V. Pistarà[2], A. Corsaro[2], Flora Tomasello[3], Maria Laura Giuffrida[1], Maria Angela Sortino[4], Ferdinando Nicoletti[5,6] & Agata Copani[1,7]

[1]Department of Pharmaceutical Sciences, [2]Department of Chemical Sciences, [3]PhD Program in Neuropharmacology, and [4]Department of Experimental and Clinical Pharmacology, University of Catania, Italy; [5]Department of Human Physiology and Pharmacology, University of Rome "Sapienza", Italy; [6]I.N.M. Neuromed, Pozzilli, Italy; [7]I.B.B., CNR-Catania, Italy.

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Corresponding author: Agata Copani, MD, PhD
Department of Pharmaceutical Sciences, University of Catania,
Viale Andrea Doria 6, 95125, Catania, Italy
Phone: + 39-095-7384028; fax: +39-095-222239
E-mail: acopani@katamail.com

Abstract

Anabolic-androgenic steroid (AAS) abuse is associated with multiple neurobehavioral disturbances. The sites of action and the neurobiological sequels of AAS abuse are unclear at present. We investigated whether two different AASs, nandrolone and methandrostenolone, could affect neuronal survival in culture. The endogenous androgenic steroid, testosterone, was used for comparison. Both testosterone and nandrolone were neurotoxic at micromolar concentrations, and their effects were prevented by blockade of androgen receptors (ARs) with flutamide. Neuronal toxicity only developed over a 48 hr exposure to the steroids. The cell-impermeable analogues, testosterone-BSA and nandrolone-BSA, which preferentially target membrane-associated ARs, were also neurotoxic in a time-dependent and flutamide-sensitive manner. Testosterone-BSA and nandrolone-BSA were more potent than their parent compounds, suggesting that membrane-associated ARs were the relevant sites for the neurotoxic actions of the steroids. As different from testosterone and nandrolone, toxicity by methandrostenolone and methandrostenolone-BSA was insensitive to flutamide, but was prevented by the glucocorticoid receptor (GR) antagonist, RU-486. Methandrostenolone-BSA was more potent than the parent compound, suggesting that its toxicity relied on the preferential activation of putative membrane-associated GRs. Consistent with the evidence that membrane-associated GRs can mediate rapid effects, a brief challenge with methandrostenolone-BSA was able to promote neuronal toxicity. Activation of putative membrane steroid receptors by non toxic (nanomolar) concentrations of either nandrolone-BSA or

methandrostenolone-BSA became sufficient to increase neuronal susceptibility to the apoptotic stimulus provided by ß-amyloid (the main culprit of AD). We speculate that AAS abuse might facilitate the onset or progression of neurodegenerative diseases not usually linked to drug abuse.

Keywords: testosterone, nandrolone, methandrostenolone, beta-amyloid, neuronal death

Introduction

Anabolic-androgenic steroids (AASs) are synthetic derivatives of testosterone that are abused in the world of sport to build muscle and boost athletic performance (Yesalis and Bahrke, 1995). Abuse of AASs causes serious side effects that involve the cardiovascular system, the liver, and the reproductive systems (Van Amsterdam et al. 2010). A major concern regards their neurobehavioral actions, which associate to stroke, mood disturbances and psychotic symptoms (Uzych et al. 1992; Hall et al. 2005; Santamarina et al. 2008).

At present, neurobiological mechanisms and sites of action of AASs are unclear. *In vitro*, low concentrations of AASs amplify excitotoxic neuronal death (Orlando et al. 2007). In male normal volunteers, high doses of AASs induce cognitive impairment (Daly et al. 2003; Su et al. 1993). The main endogenous androgenic steroid, testosterone, has both neuroprotective (Hammond et al. 2001; Pike et al., 2001; Nguyen et al., 2005; Pike et al. 2008) and neurotoxic effects (Estrada et al. 2006; Cunningham et al., 2009) depending on the experimental paradigm. Endogenous testosterone appears to

exhacerbate some types of neuronal injury, including ischemia-reperfusion injury (Yang et al., 2002) and methamphetamine-induced neurodegeneration of nigrostriatal dopaminergic neurons (Dluzen and McDermott 2006). In culture paradigms, a few studies indicate that testosterone can be directly neurotoxic both at supraphysiological (Estrada et al. 2006) and physiological (Cunningham et al., 2009) concentrations. Despite evidence that testosterone may be neurotoxic, there are conditions in which testosterone supports neuronal viability (Pike et al., 2009). In particular, it has been demonstrated that androgens selectively protect neurons against apoptosis-inducing insults (Nguyen et al., 2010), including ß-amyloid protein, the main contributor to Alzhemer's disease (AD) pathology.

Androgens mediate their classical genomic effects through binding to the androgen receptor (AR), a member of the nuclear receptor superfamily that functions as a ligand-activated transcription factor (Heinlein and Chang 2002). A CAG repeat expansion within the first exon of the AR gene is responsible for testosterone-dependent nuclear accumulation of ARs, with ensuing motor neuron degeneration in spinal and bulbar muscular atrophy (Katsuno et al. 2010). Androgens also mediate rapid non-genomic effects via the activation of signaling pathways (i.e., the MAPK pathway and the AKT pathway) triggered by either classical ARs (Heinlein and Chang 2002; Nguyen et al., 2005; Foradori et al. 2008) or by membrane-associated ARs (Gatson et al., 2006). How the different AR activities are related to the existing discrepancies as to whether androgens are protective or damage promoting is unclear.

Given that AAS abuse poses a significant public health problem, we investigated the potential neurotoxic effects of suprapharmacological concentrations of AASs, taking into consideration the existence of both classical and membrane-associated ARs, and focusing on two largely abused steroids with a different pharmacological profile, nandrolone and methandrostenolone. Nandrolone binds to ARs to a greater degree than testosterone, whereas methadrostenolone is a week agonist of the ARs (reviewed in Fragkaki et al., 2009). Hence, their neurotoxic properties were compared to those of testosterone, and the attempt to differentiate between intracellular and membrane functions of ARs was carried out by using cell-impermeable steroid analogues that preferentially bind to membrane-associated receptors.

Materials and Methods

All animal experimental procedures were carried out in accordance with the directives of the Italian and EU regulations for care and use of experimental animals, and approved by the ***Institutional Animal Care and Use Committee*** of the University of Catania.

Drugs

Testosterone, testosterone-BSA conjugate, nandrolone, methandrostenolone, RU-486, formestane, and flutamide were purchased from Sigma-Aldrich (Milan, Italy). Nandrolone- and methandrostenolone-3-(*O*carboxymethyloxime)-BSA conjugates were synthetized in two steps involving steroid-(O-carboxymethyl)-oxime derivatives, obtained in turn from the two steroids with hemihydrate carboxymethylamine in pyridine at room temperature. Derivatives

were covalently linked to BSA by a modified mixed anhydride method (Erlanger et al. 1957), using ethyl chloroformate and triethylamine in THF. Derivatives were then purified and characterized by IR, ^{1}H NMR and mass spectra.

Testosterone, nandrolone, methandrostenolone, flutamide, and formestane were dissolved in DMSO at the initial concentration of 10 mM. The final concentration of DMSO applied to the cultures was 0.1%. AAS-BSA conjugates were dissolved in 50 mM Tris-HCl (pH 8.5), at 0.2 -1 mg/mL.

Pure cultures of cortical neurons

Cultures of pure cortical neurons were obtained from rats at embryonic day 15 (Morini, s.a.s., Reggio Emilia, Italy). Briefly, cortices were dissected in Ca^{++}/Mg^{++} free buffer and mechanically dissociated. Cortical cells were plated at a density of 400 x 10^3/well on 24 multiwell plates precoated with 0.1 mg/ml poly-D-lysine in DMEM/Ham's F12 (1:1) medium supplemented with the following components: 10 mg/ml bovine serum albumin, 10 µg/ml insulin, 100 µg/ml transferrin, 100 µM putrescine, 30 nM selenium, 2 mM glutamine, 6 mg/ml glucose, 100 U/ml penicillin and 100 µg/Ml streptomycin. Cytosine-1ß-D-arabinofuranoside (10 µM) was added to the cultures 18 hr after plating to avoid the proliferation of non-neuronal elements and was kept for 3 days before medium replacement. This method yields >99% pure neuronal cultures (Copani et al. 1999).

Mixed cultures of cortical cells

Cells dissected from the cortices of rat embryos and dissociated as described above were grown into MEM supplemented with horse serum (10%), foetal calf serum (10%), glutamine (2mM), and glucose (6 mg/ml). Cultures were kept at 37°C in a humified 5% CO2 atmosphere. After 3-5 d *in vitro*, non-neuronal cell division was halted by 3 days exposure to Cytosine-1ß-D-arabinofuranoside (10 μM), and cultures were shifted to a maintenance medium identical to plating medium but lacking fetal serum. Subsequent partial medium replacement was carried out twice a week. Only mature cultures (12-14 days *in vitro*) were used for the experiments. Mature cultures cointained about 40% neurons.

Cultures of cortical astrocytes

Cortical glial cells were prepared from 1-3 day-old Sprague-Dowley rats (Morini, s.a.s.,). After removal of meninges and isolation of cortices, cells were dispersed by mechanical and enzymatic dissociation using a 0.25% solution of trypsin. Cells were plated onto 75 mm^2 flasks, and maintained in DMEM culture medium, supplemented with 10% FCS, penicillin/streptomycin (100 U/ml-100 μg/ml) and glutamine (2 mM). All medium constituents were from Invitrogen, and all plastic materials were from Corning Life Sciences, (Acton, MA). Confluent cultures at 8-10 days in vitro were shaked overnight at 37°C to remove microglia and oligodendrocytes. Astrocytes were collected by trypsin digestion, seeded onto 24 multiwell plates and used 6-8 days after re-plating.

Handling of Aß

Different lots of Aß(25-35) were tested, and the same batch was used throughout the entire study to rely on a consistent profile of toxicity. Peptides were solubilized in sterile, double distilled water at an initial concentration of 2.5 mM and stored frozen at -20°C. Aß(25–35) was used at a final concentration of 25 μM in the presence of the glutamate receptor antagonists MK-801 (1 μM) and DNQX (30 μM) to avoid the potentiation of endogenous glutamate toxicity.

Assessment of neuronal injury

In mixed cortical cultures, neuronal injury was estimated by examination of the cultures by phase contrast microscopy 24 h after the incubation with Aβ. Neuronal damage was quantitatively assessed in all experiments by estimation of dead neurons by trypan blue staining. Stained neurons were counted from three random microscopic fields per well. In pure neuronal cultures, neuronal injury was assessed by the 3-[4,5-dimethylthioazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Cultures were incubated with MTT (0.9 mg/ml final concentration) for 2 h at 37°C. A solubilization solution containing 20% sodium dodecyl sulphate was then added for an additional 1 h, and formazan production was evaluated in a plate reader (absorbance = 560 nm).

Western blot analysis

Cells were harvested in lysis buffer containing a cocktail of protease inhibitors (P2714, Sigma-Aldrich S.r.l., Milan, Italy). After sonication, an aliquot of the sample was processed for protein concentrations

according to the method of Bradford. Samples were concentrated and boiled for 5 min. Proteins were separated electrophoretically on polyacrylamide gel (30 mA/h) using 60-80 μg of cell proteins per lane. Proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences Europe GmbH, Milan, Italy) at room temperature using a transblot semidry transfer cell. After blocking, the membranes were incubated with rabbit anti-androgen receptor antibody (1:500, Ab3509, Abcam, Cambridge, UK) overnight at 4 °C. Membranes were then thoroughly washed and incubated with HRP-conjugated secondary antibodies. Specific bands were visualized using the SuperSignal chemiluminescent detection system (Pierce Biotechnology Inc., Rockford, IL).

Immunofluorescence labelling and confocal microscopy

Mixed cortical cultures were plated onto 35 mm dishes with glass slides (Amniodish Euroclone, Milan, Italy) precoated with 0.1 mg/ml poly-D-lysine. Cells were fixed for 20 min in paraformaldehyde and permeabilized with 0.1% Triton X-100 for 5 min. Non-specific binding was blocked by incubation with PBS containing 3% bovine serum albumin (BSA) at room temperature for 1 h. After blocking, rabbit anti-androgen receptor antibody (1:100 dilution) was added overnight at 4 ◦C. After washing, a Texas Red-conjugated donkey-anti-rabbit antibody was used for 1 h (1: 400 dilution, Santa Cruz Biotechnology). Samples were analyzed through a 60X objective on a confocal laser scanning microscope Olympus FV1000.

Results

To begin investigating the hypothesis that suprapharmacologic doses of AASs may be neurotoxic, we have compared the effects of two synthetic AASs (nandrolone and methandrostenolone) and their respective bovine serum albumine-conjugates (nandrolone-BSA and methandrostenolone-BSA) with those of testosterone, using as a model rat cortical cultures, both pure neuronal and mixed neuron-glia types. Consistent with *in vivo* evidence (Don Carlos et al., 2006; Sarkey S. et al., 2008), ARs were expressed by cultured astrocytes and neurons (Fig. 1A), and AR immunoreactivity was observed by confocal microscopy in neuronal (Fig. 1B) and glial processes (Fig. 1C). Initially, pure neuronal cultures were exposed to different concentrations (0.01-10 μM) of either testosterone or nandrolone and toxicity was assessed by MTT assay 48 h later. Testosterone and nandrolone exerted significant toxicity only at concencentrations of 10 μM (Fig. 2A). Instead, the cell-impermeable derivatives, testosterone-BSA and nandrolone-BSA, were toxic at 1 μM (Fig. 2B). As different from testosterone and nandrolone, methandrostenolone had a toxic effect at 1 μM, whereas its derivative, methandrostenolone-BSA, was toxic at concentrations as low as 100 nM (Fig. 2C). Toxicity by testosterone, nandrolone or their respective BSA-conjugates was abrogated by the AR antagonist, flutamide (Fig. 3). Instead, toxicity by methandrostenolone or methandrostenolone-BSA was insensitive to flutamide (not shown), but was prevented entirely by the glucocorticoid receptor antagonist, RU-486 (Fig. 3). Concentrations of nandrolone, methandrostenolone, or AAS-conjugates, which were neurotoxic following a 48 h exposure, were also applied to the cultures in a 20 min pulse and then washed out 24 h prior to the assessment of neuronal death. Under this experimental condition, only

methandrostenolone-BSA was toxic, and its toxicity was prevented again by RU486 (Fig. 4). Finally, testosterone, nandrolone, methandrostenolone or their respective BSA-conjugates were tested for their ability to increase neuronal vulnerability to the apoptotic insult provided by Aß protein (Loo et al., 1993). Because Aß is able to potentiate glutamate toxicity (Koh et al., 1990; Copani et al., 1991), and AASs might amplify glutamate toxicity (Orlando et al., 2007), experiments were carried out in the presence of a cocktail of ionotropic glutamate receptor antagonists (1 μM MK-801 + 30 μM DNQX) to exclude the contribution of endogenous excitotoxicity to the overall process of neuronal death. Aß(25-35) was used at concentrations (25 μM) able to induce a 45 % of neuronal death over a 24 hour period. Prior to the treatment with Aß(25-35), pure neuronal cultures were exposed for 24 h to the highest concentrations of the drugs devoid of intrinsic toxicity. Using this particular experimental protocol, methandrostenolone-BSA amplified Aß-induced toxicity at concentrations of 10 nM (Fig. 5).

The set of experiments described above was repeated in mixed rat cortical cultures, which include a glial component. In mixed cultures, neuronal death was assessed by Trypan blue staining. In the presence of glial cells, testosterone and nandrolone exerted significant toxicity already at 1 μM (Fig. 6A), and nandrolone-BSA became toxic at a concentration as low as 100 nM (Fig. 6B). Instead, the presence of glia cells did not appear to affect the toxicity profile of either methandrostenolone or methandrostenolone-BSA (Fig. 6C). Similar to what observed in cultures of pure neurons, flutamide abrogated toxicity by testosterone, nandrolone or their respective BSA-

conjugates (Fig. 7), whereas RU-486 prevented entirely toxicity by methandrostenolone or methandrostenolone-BSA (Fig. 7). When tested in the brief insult paradigm (i.e., 24 h after a 20 min pulse), methandrostenolone-BSA was toxic once more, and its toxicity was prevented by RU486 (Fig. 8).

Finally, all drugs were tested for their ability to affect neuronal susceptibility to Aß-induced toxicity in mixed cortical cultures. In hippocampal cultures, testosterone has been reported to be neuroprotective against Aß(25-35) at 10 nM concentrations (Pike et al., 2001). At these concentrations, testosterone protected mixed cortical cultures against Aß toxicity (Fig. 9), and protection was abolished by the combined addition of flutamide and the aromatase inhibitor, formestane (Fig. 9). However, at higher concentrations (100 nM), which were not toxic per se, testosterone did not influence Aß toxicity (Fig. 10). At the highest concentrations devoid of intrinsic toxicity, nandrolone-BSA, methandrostenolone and methandrostenolone-BSA all amplified Aß-induced toxicity in mixed cortical cultures (Fig. 10).

Discussion

We have demonstrated that suprapharmacologic doses of two different AASs, namely nandrolone and methandrostenolone, are toxic for cultured cortical neurons. However, the neurotoxic properties of nandrolone and methandrostenolone diverge.

Similar to testosterone, micromolar concentrations of nandrolone were detrimental to cortical neurons. The actions of testosterone and nandrolone were prevented by pharmacological

blockade of ARs with flutamide, suggesting that toxicity was dependent on ARs. The evidence that the neurotoxic effects of testosterone and nandrolone required a 48 hr exposure suggests that high concentrations of androgens may affect neuronal viability by acting through AR-mediated genomic mechanisms. Both testosterone and nandrolone were more potent in cortical cultures containing glia cells, suggesting that AR-expressing glia cells could be implicated in the regulation of neuronal survival. The role of AR activation in glia cells in currently unclear. Based on the evidence that androgens can promote the induction of NF-kB-dependent proinflammatory genes, which lead to brain inflammation (Gonzales et al. 2009), it cannot be excluded that glial response factors sinergize with androgens in reducing neuronal viability.

Confocal studies using an antibody against the classical nuclear AR revealed the localization of ARs to extranuclear sites, including neuronal axons and glia processes. AR immunoreactivity also profiled plasma membranes, indicating the presence of putative membrane receptors both in neurons and astrocytes. Evidence exists that membrane-associated ARs may be either related or unrelated to the classical nuclear receptors (reviewed in DonCarlos et al., 2006). We found that testosterone-BSA and nandrolone-BSA, which preferentially target membrane ARs, exerted neurotoxic effects both in pure neuronal and mixed cultures. These effects were prevented by flutamide, thus supporting the notion that membrane and intracellular ARs might share a similar pharmacological profile.

It has been proposed that activation of ARs may elicit opposite effects on cell survival (i.e., detrimental or beneficial) depending on whether membrane ARs or intracellular ARs are activated (Gatson et

al. 2006; Gatson and Singh, 2007). In pure neuronal cultures, both testosterone-BSA and nandrolone-BSA were toxic with a greater potency than their parent compounds. A similar effect was also observed following the exposure of mixed cultures to nandrolone-BSA, indicating that AAS-related toxicity depends on the preferential activation of putative membrane ARs over intracellular ARs. Interestingly, activation of putative membrane ARs by low nanomolar concentrations (10 nM) of nandrolone-BSA did not itself lead to neuronal death, but was sufficient to increase neuronal susceptibility to the apoptotic stimulus provided by Aß(25-35).

Consistent with the hypothesis that androgens may activate two competing pathways for the regulation of neuronal survival, physiological concentrations (10 nM) of testosterone were instead protective against Aß(25-35)-induced apoptosis in mixed cultures. The protective effect of 10 nM testosterone was fully blocked by combining flutamide with the aromatase inhibitor, formestane. We speculate that, under physiological conditions (i.e., low concentrations of the hormone in a neuron-glia interplay), testosterone might undergo aromatization to the neuroprotective molecule, 17ß-estradiol (Garcia-Segura et al., 2003). After that, the residual concentrations of testosterone might promote neuronal survival if intracellular ARs are abundant with respect to membrane-associated ARs.

A difference between methandrostostenolone and either nandrolone or testosterone could be observed when the drug was first tested for its intrinsic toxicity. The action of methandrostenolone was not blocked by flutamide, suggesting that toxicity was independent of AR activation. Consistent with the notion that methandrostenolone is only a week agonist of the ARs and most of its anabolic activity likely

comes from non-AR-mediated effects (Fragkaki et al., 2009), the neurotoxicity of the drug was fully prevented by the glucocorticoid receptor (GR) antagonist, RU486. RU486 has also a remarkable antiprogesterone activity (Baulieu, 1989); however, as different from other AASs (McRobb et al, 2008; Fragkaki et al., 2009), methandrostenolone lacks significant progestational properties (Wynn and Landon, 1961; Fragkaki et al., 2009), suggesting that progesterone receptors (PRs) were not involved in the neurotoxic action of the drug. Noteworthy, nandrolone is among the AASs endowed with progesterone-like actions (McRobb et al, 2008; Fragkaki et al., 2009); the evidence that flutamide prevented entirely the toxicity of nandrolone provides hints that PRs might not be relevant to the neurotoxic properties of AASs.

Activation of GRs has been shown to exacerbate a variety of neuronal insults, including excitotoxicity and Aß toxicity (Goodman et al., 1996). In mixed cultures, methandrostenolone was able to exacerbate Aß(25-35)-induced toxicity at concentrations that were not toxic *per se* (100 nM), and exhibited intrinsic toxicity at 1 µM concentrations independently of the presence of glia cells. Methandrostenolone-BSA, which likely binds membrane-associated GRs, was always more potent than the parent compound, suggesting that its toxicity relied on the preferential activation of putative membrane GRs over intracellular GRs. Recently, the activation of putative membrane-associated GRs has been shown to mediate rapid, non-genomic, effects able to potentiate NMDA-evoked toxicity in hippocampal neurons (Xiao et al., 2010). Consistent with this evidence, a brief challenge with methandrostenolone-BSA, but not

with nandrolone-BSA, was sufficient to promote neuronal toxicity both in pure and mixed neuronal cultures. The identity of membrane-bound GRs has not been elucidated yet, and either an unknown receptor type or classical intracellular GRs that associate with the membrane have been proposed (reviewed in Tasker et al., 2006). The "fast" toxicity induced by methandrostenolone-BSA was prevented by RU486, suggesting that the drug was acting at classical intracellular receptors associated with the plasma membrane.

Overall, we have provided evidence that two AASs with a different pharmacological profile, namely nandrolone and methandrostenolone, can affect neuronal survival at suprapharmacologic doses, raising a serious concern for steoroid abusers, who have micromolar concentrations of AASs in their brain (Lukas, 1996; Wu, 1997; Daly et al. 2001). The relevant sites for the neurotoxic action of nandrolone and methandrostenolone appear to be membrane-associated ARs and membrane-associated-GRs, respectively. Noteworthy, concentrations of the drugs that were not directly neurotoxic were, however, able to increase neuronal susceptibility to the apoptotic stimulus provided by Aß(25-35). Hence, *in vivo*, exposure to AASs may result in a compromised brain, more susceptible, later in life, to the onset or progression of diseases not usually linked to drug abuse, especially neurodegenerative diseases (e.g. Alzheimer's disease).

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Figure Legends

Fig. 1 AR expression in cultured neural cells. A) Western blot analysis of AR expression in pure cultures of rat cortical neurons or in cultured astrocytes. Samples have been loaded in duplicate. A single band of about 110 kD, corresponding to the molecular weight of ARs, was observed in both cases. Confocal images with 60X magnification of AR immunoreactivity in neurons (B) or astrocytes (C) from mixed cortical cultures.

Fig. 2 Neuronal death induced in pure neuronal cultures by increasing concentrations of testosterone and nandrolone (A), their respective BSA-conjugated analogues (B), and either methandrostenolone or methandrostenolone-BSA (C). All drugs were applied for 48 hours. Neuronal death is represented as the percentage reduction of neuronal survival measured by MTT assay. Values are means $\pm$ S.E.M. of 6-9 determinations. *$p < 0.05$ vs. controls (one-way ANOVA + Fisher's PLSD).

Fig. 3 Neuronal death induced in pure neuronal cultures by a 48 hour exposure to testosterone, nandrolone, methandrostenolone or their respective BSA-conjugated analogues alone, or combined with either 10 μM flutamide or RU486. Neuronal death is represented as the percentage reduction of neuronal survival measured by MTT assay. Values are means $\pm$ S.E.M. of 6 determinations. *$p< 0.05$ vs. controls and # $p<0.05$ vs. the values obtained in the absence of flutamide or RU486 (one-way ANOVA + Fisher's PLSD).

Fig. 4 Neuronal death by nandrolone (A), methandrostenolone (A), and their respective BSA-conjugated analogues (B) applied for 20 minutes to pure neuronal cultures. In B, RU486 (10 μM) was coadded with methandrostenolone-BSA during the 20 minute pulse. Neuronal death is represented as the percentage reduction of neuronal survival measured by MTT assay. Values are means ± S.E.M. of 4 determinations. *p< 0.05 vs. control and # p<0.05 vs. methandrostenolone-BSA alone (one-way ANOVA + Fisher's PLSD).

Fig. 5 Modulation of Aß(25-35)-induced toxicity in pure neuronal cultures by a 24 hour pre-exposure to testosterone, nandrolone, methandrostenolone or their respective BSA-conjugated analogues. Aß(25-35) was applied for 24 hours, and the amount of neuronal death induced by 25 μM Aß was set as 100%. Values are means ± S.E.M. of 6 determinations. *p< 0.05 vs. Aß alone (one-way ANOVA + Fisher's PLSD).

Fig. 6 Neuronal death induced in mixed cortical cultures by increasing concentrations of testosterone and nandrolone (A), their respective BSA-conjugated analogues (B), and either methandrostenolone or methandrostenolone-BSA (C). All drugs were applied for 48 hours. Neuronal death was assessed by Trypan blue staining. Following Trypan blue staining, dead neurons were counted in 3 random microscopic fields/well. Values are means ± S.E.M. of 6-9 determinations. *p < 0.05 vs. controls (one-way ANOVA + Fisher's PLSD).

Fig. 7 Neuronal death induced in mixed cortical cultures by a 48 hour exposure to testosterone, nandrolone, methandrostenolone or their respective BSA-conjugated analogues alone, or combined with either 10 μM flutamide or RU486. Following Trypan blue staining, dead neurons were counted in 3 random microscopic fields/well. Values are means ± S.E.M. of 6 determinations. *p< 0.05 vs. controls and # p<0.05 vs. the values obtained in the absence of flutamide or RU486 (one-way ANOVA + Fisher's PLSD).

Fig. 8 Neuronal death by nandrolone (A), methandrostenolone (A), and their respective BSA-conjugated analogues (B) applied for 20 minutes to mixed cortical cultures. In B, RU486 (10 μM) was coadded with methandrostenolone-BSA during the 20 minute pulse. Neuronal death was assessed by Trypan blue staining. Values are means ± S.E.M. of 4 determinations. *p< 0.05 vs. control and # p<0.05 vs. methandrostenolone-BSA alone (one-way ANOVA + Fisher's PLSD).

Fig. 9 Low concentrations of testosterone protected against Aß(25-35)-induced toxicity in mixed cortical cultures. Testosterone was applied 24 hour before the addition of 25 μM Aß(25-35) alone, or together with flutamide (10 μM), formestane (1 μM) or a combination of flutamide + formestane.

Aß(25-35) was applied for 24 hours, and the amount of neuronal death induced by 25 μM Aß was set as 100%. Values are means ± S.E.M. of 4 determinations. *p< 0.05 vs. Aß alone, and #p< 0.05 vs. Aß + testosterone (one-way ANOVA + Fisher's PLSD).

Fig. 10 Modulation of Aß(25-35)-induced toxicity in mixed cortical cultures by a 24 hour pre-exposure to testosterone, nandrolone, methandrostenolone or their respective BSA-conjugated analogues. Aß(25-35) was applied for 24 hours, and the amount of neuronal death induced by 25 µM Aß was set as 100%. Values are means ± S.E.M. of 6-9 determinations. *p< 0.05 vs. Aß alone (one-way ANOVA + Fisher's PLSD).

Fig.1

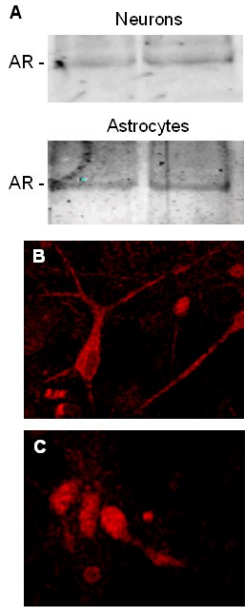


Fig.2

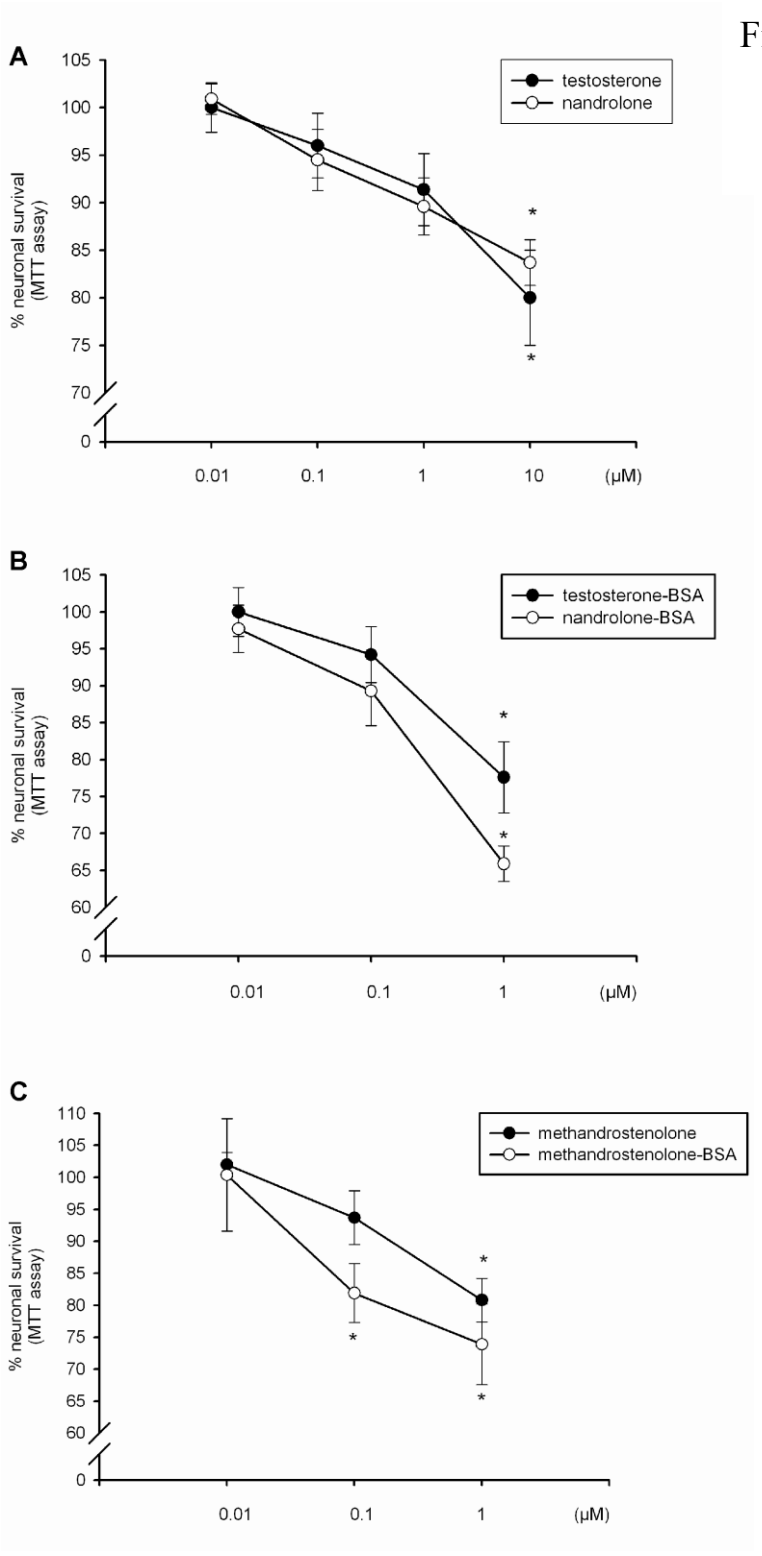

A
% neuronal survival (MTT assay)
105
100
95
90
85
80
75
70
0
0.01
0.1
1
10
(μM)
testosterone
nandrolone
*
*
B
% neuronal survival (MTT assay)
105
100
95
90
85
80
75
70
65
60
0
0.01
0.1
1
(μM)
testosterone-BSA
nandrolone-BSA
*
*
C
% neuronal survival (MTT assay)
110
105
100
95
90
85
80
75
70
65
60
0
0.01
0.1
1
(μM)
methandrostenolone
methandrostenolone-BSA
*
*
*

Fig.3

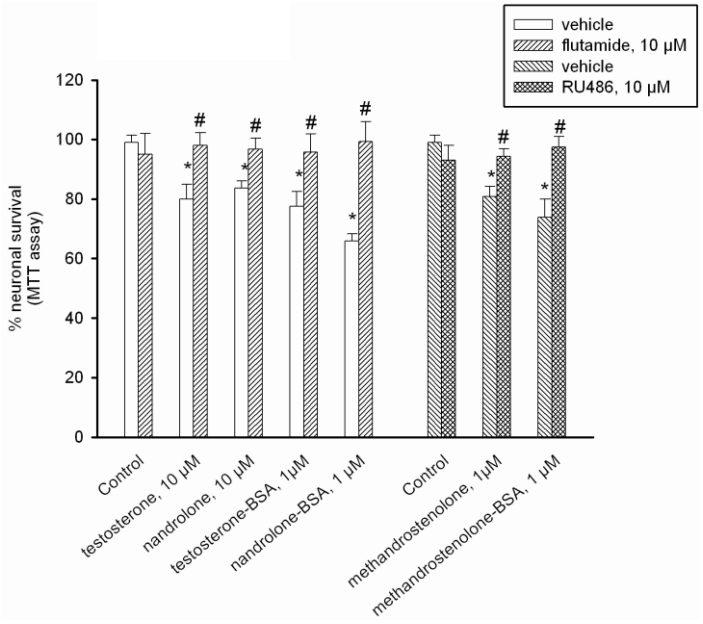

vehicle
flutamide, 10 μM
vehicle
RU486, 10 μM
% neuronal survival
(MTT assay)
120
100
80
60
40
20
0
Control
testosterone, 10 μM
nandrolone, 10 μM
testosterone-BSA, 1μM
nandrolone-BSA, 1 μM
Control
methandrostenolone, 1μM
methandrostenolone-BSA, 1 μM

Fig.4

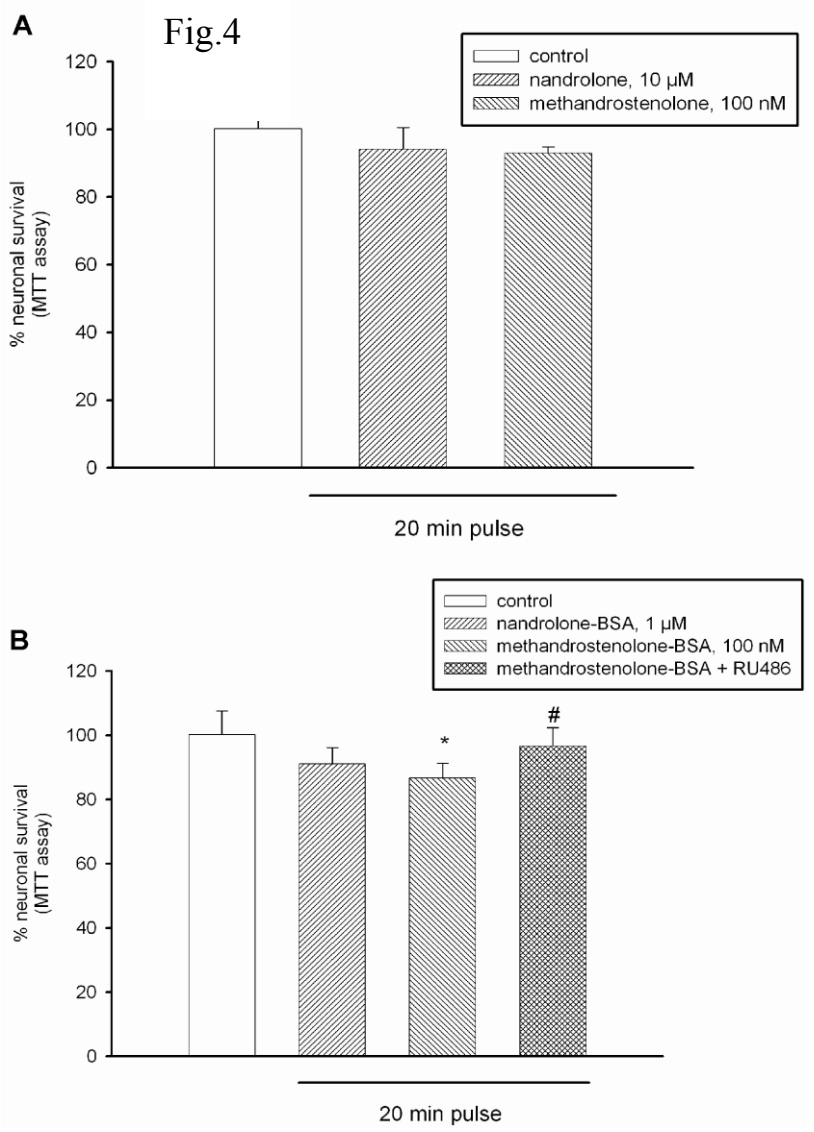

A
control
nandrolone, 10 μM
methandrostenolone, 100 nM
% neuronal survival
(MTT assay)
120
100
80
60
40
20
0
20 min pulse
B
control
nandrolone-BSA, 1 μM
methandrostenolone-BSA, 100 nM
methandrostenolone-BSA + RU486
% neuronal survival
(MTT assay)
*
#
20 min pulse

Fig.5

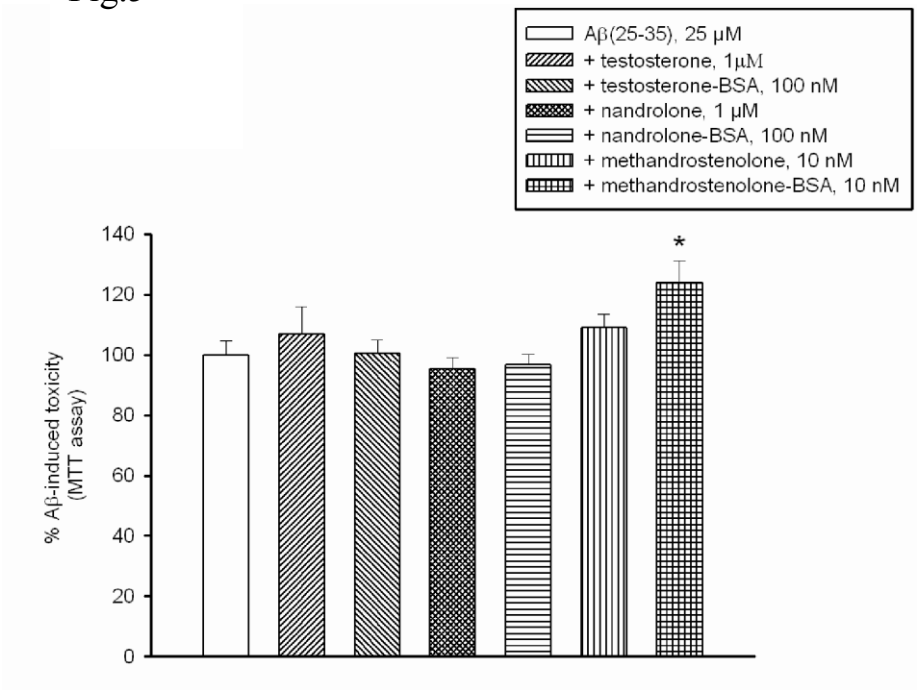

Aβ(25-35), 25 μM
+ testosterone, 1μM
+ testosterone-BSA, 100 nM
+ nandrolone, 1 μM
+ nandrolone-BSA, 100 nM
+ methandrostenolone, 10 nM
+ methandrostenolone-BSA, 10 nM
% Aβ-induced toxicity (MTT assay)
140
120
100
80
60
40
20
0
*

Fig.6

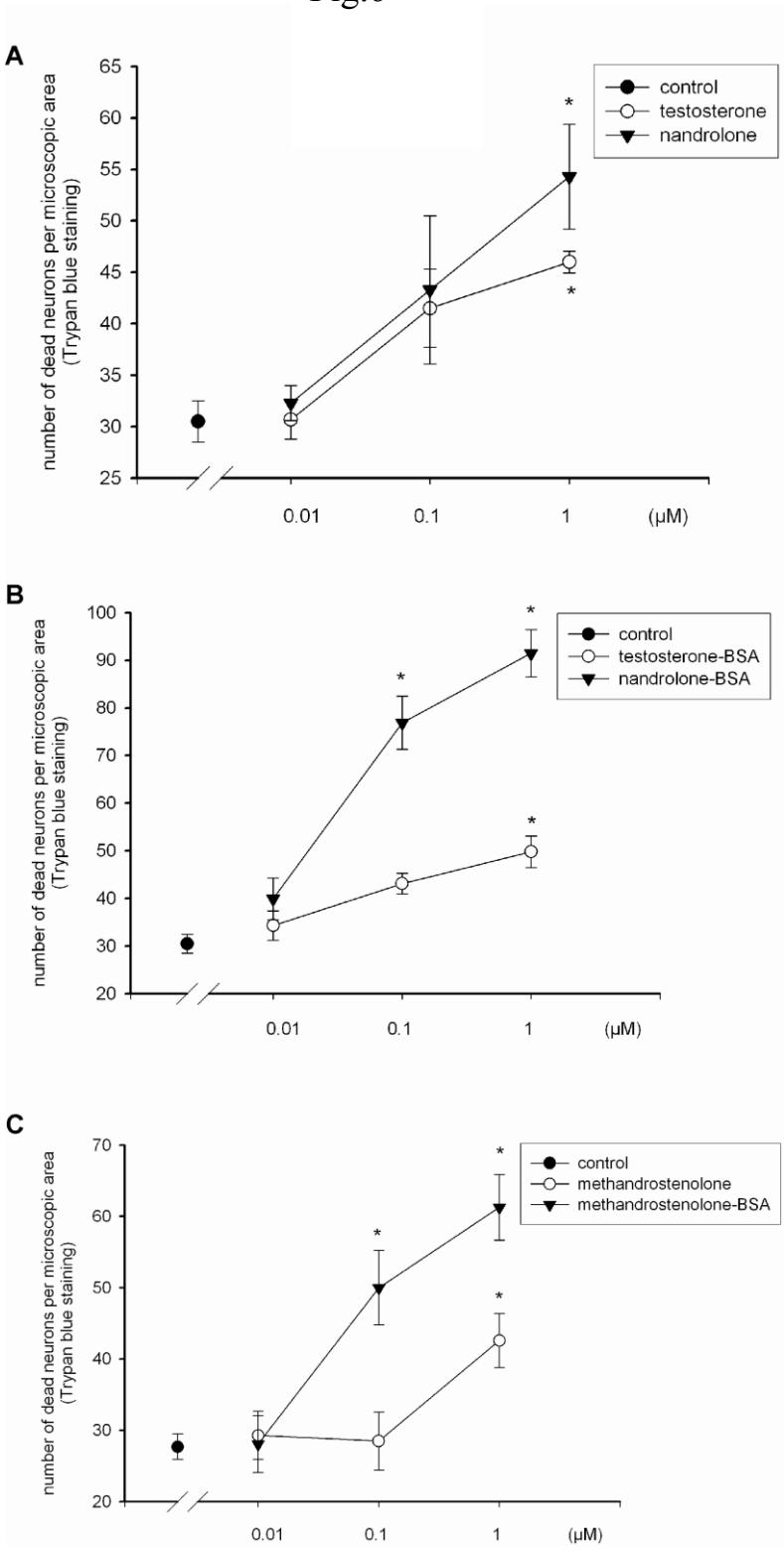
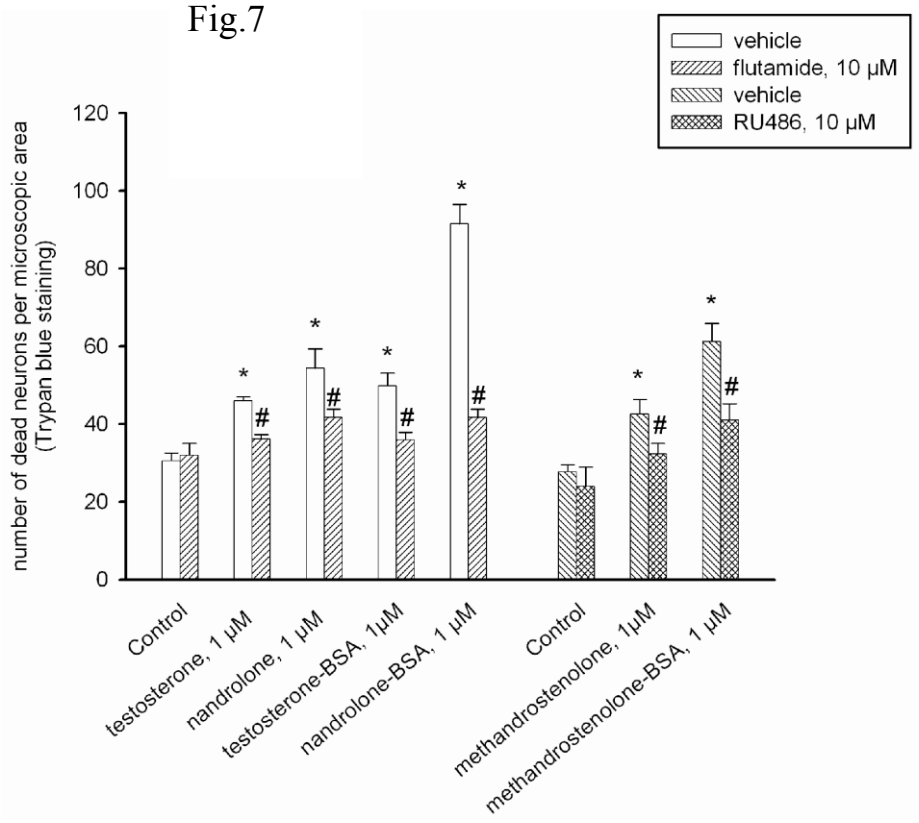

A
number of dead neurons per microscopic area
(Trypan blue staining)
65
60
55
50
45
40
35
30
25
control
testosterone
nandrolone
*
*
0.01
0.1
1
(μM)
B
number of dead neurons per microscopic area
(Trypan blue staining)
100
90
80
70
60
50
40
30
20
control
testosterone-BSA
nandrolone-BSA
*
*
*
0.01
0.1
1
(μM)
C
number of dead neurons per microscopic area
(Trypan blue staining)
70
60
50
40
30
20
control
methandrostenolone
methandrostenolone-BSA
*
*
*
0.01
0.1
1
(μM)

Fig.7


vehicle
flutamide, 10 μM
vehicle
RU486, 10 μM
number of dead neurons per microscopic area
(Trypan blue staining)
120
100
80
60
40
20
0
*
#
Control
testosterone, 1 μM
nandrolone, 1 μM
testosterone-BSA, 1μM
nandrolone-BSA, 1 μM
Control
methandrostenolone, 1μM
methandrostenolone-BSA, 1 μM

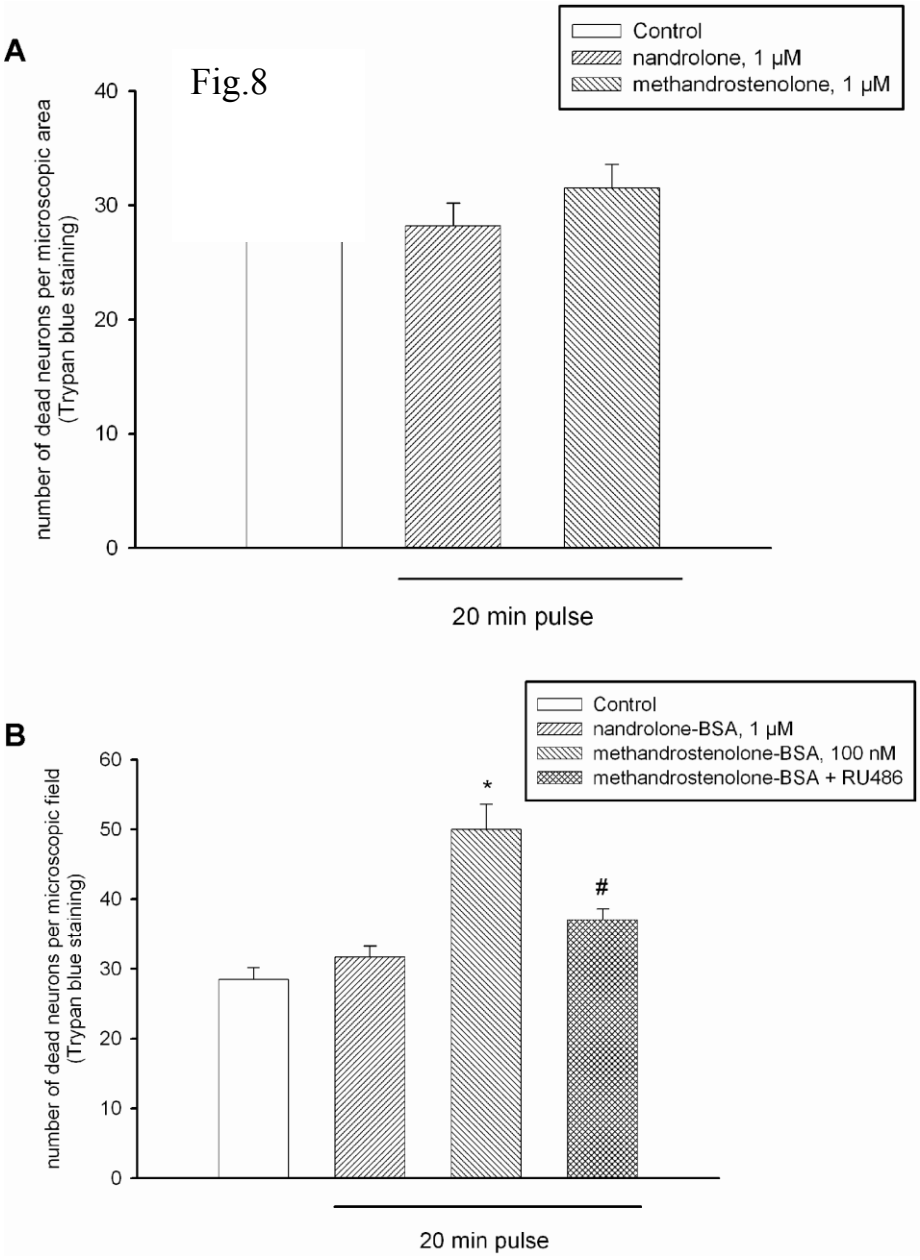

A
Control
nandrolone, 1 μM
methandrostenolone, 1 μM
number of dead neurons per microscopic area
(Trypan blue staining)
40
30
20
10
0
20 min pulse
B
Control
nandrolone-BSA, 1 μM
methandrostenolone-BSA, 100 nM
methandrostenolone-BSA + RU486
number of dead neurons per microscopic field
(Trypan blue staining)
60
50
40
30
20
10
0
*
#
20 min pulse

Fig.8

Fig.9

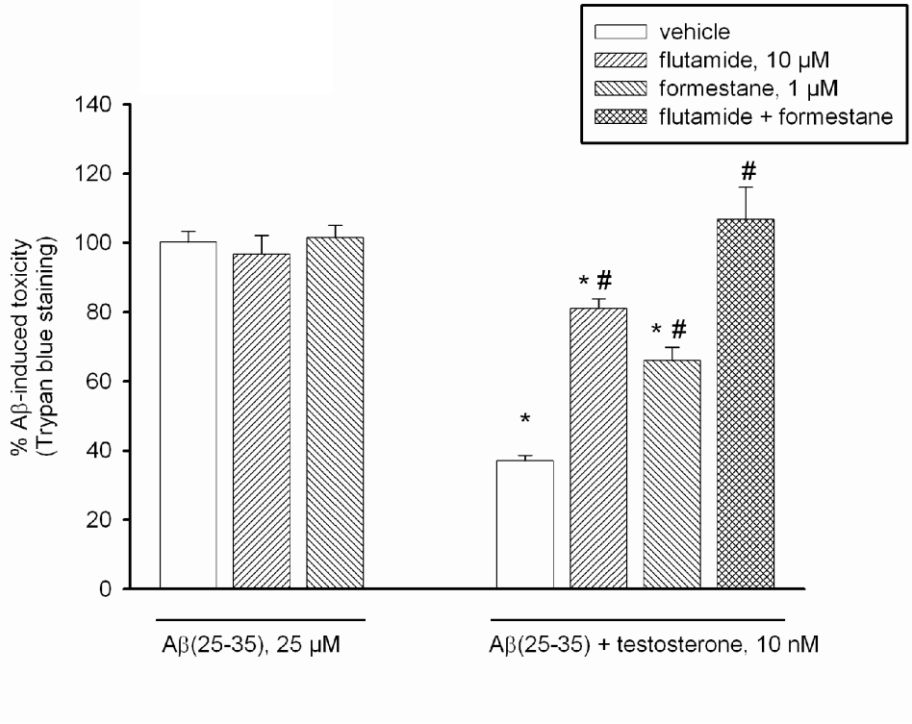
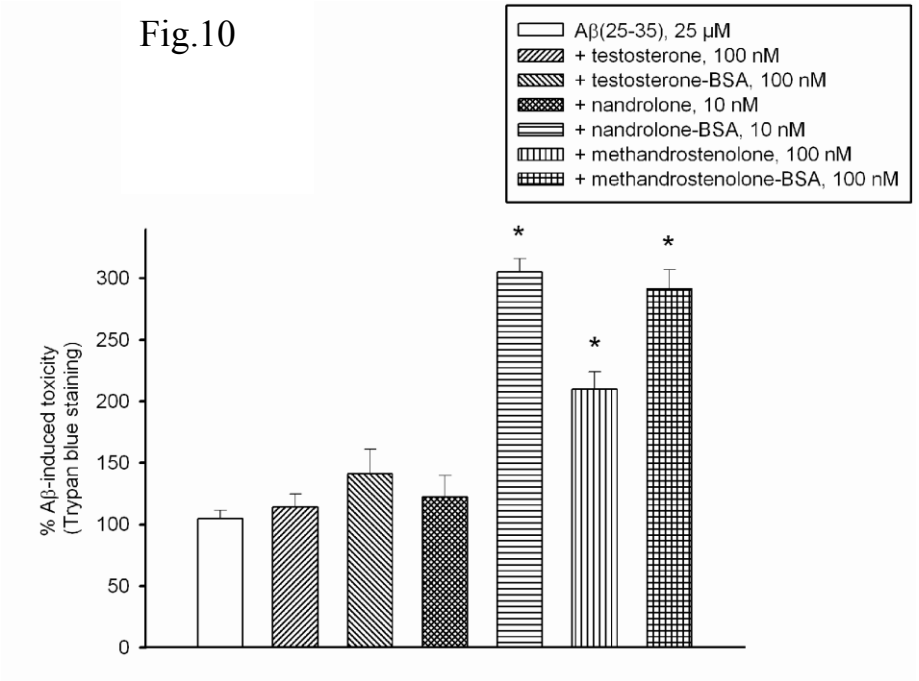

vehicle
flutamide, 10 μM
formestane, 1 μM
flutamide + formestane
% Aβ-induced toxicity
(Trypan blue staining)
140
120
100
80
60
40
20
0
*
* #
* #
#
Aβ(25-35), 25 μM
Aβ(25-35) + testosterone, 10 nM

Fig.10


Aβ(25-35), 25 μM
+ testosterone, 100 nM
+ testosterone-BSA, 100 nM
+ nandrolone, 10 nM
+ nandrolone-BSA, 10 nM
+ methandrostenolone, 100 nM
+ methandrostenolone-BSA, 100 nM
% Aβ-induced toxicity
(Trypan blue staining)
0
50
100
150
200
250
300
*
*
*

CHAPTER II

Beta-amyloid monomer and insulin/IGF-1 signaling in Alzheimer'S disease

Maria Laura Giuffrida[1], Flora Tomasello[2], Filippo Caraci[3], Santina Chiechio[4], Ferdinando Nicoletti[5,6*], Agata Copani[1,4*].

[1]Institute of Biostructure and Bioimaging, National Research Council, Viale Andrea Doria, Catania 95125, Italy; [2]PhD Program in Neuropharmacology, [3]Department of Formative Processes, and [4]Department of Drug Sciences, University of Catania, Viale Andrea Doria, Catania 95125, Italy; [5]Department of Human Physiology and Pharmacology, University of Rome "La Sapienza", Piazzale Aldo Moro, Rome 00185, Italy; [6]Instituto Neurologico Mediterraneo, Neuromed, Località Camerelle, Pozzilli 86077, Italy;

*Address correspondence to:

Dr. Ferdinando Nicoletti (ferdinandonicoletti@hotmail.com; phone: +39-06-49912969)

or

Dr. Agata Copani (acopani@katamail.com; phone: +39-095-7384212)

Abstract

Alzheimer's disease is the most common form of dementia among older people and is still untreatable. While ß-amyloid protein is recognized as the disease determinant with a pivotal role in inducing neuronal loss and dementia, an impaired brain insulin signaling seems to account in part for the cognitive deficit associated with the disease. The origin of this defective signaling is uncertain. Accumulating toxic species of ß-amyloid, the so-called oligomers, have been proposed to be responsible for a down-regulation of neuronal insulin receptors. We have found that the non-toxic form of ß-amyloid, the monomer, is able to activate the insulin/IGF-1 receptor signaling and thus behaves as a neuroprotectant agent. Our suggestion is that the depletion of ß-amyloid monomers, occurring in the preclinical phase of Alzheimer's disease, might be the cause of early insulin/IGF-1 signaling disturbances that anticipate the cognitive decline.

Keywords: ß-amyloid, insulin, insulin-like growth factor 1, Alzheimer's disease

Introduction

Alzheimer's disease (AD) is the most frequent form of dementia and the most common neurodegenerative disease [1]. The two classical lesions of the disease originally described by Alois Alzheimer, namely senile plaques and neurofibrillary tangles, are made from proteins (ß-amyloid and tau, respectively) that have a pivotal role in inducing dementia, with tau alterations occurring downstream of Aß build-up [2]. Early-onset forms of the disease, occurring before the age of 65, have a familiar aggregation and some of these are caused by rare autosomal dominant mutations in the genes encoding the amyloid precursor protein (APP), and presenilin-1 and –2 (PSEN1 and PSEN-2), which are all invoved in ß-amyloid (Aß) production [3]. The majority of AD cases have a late onset and are sporadic [3], likely resulting from complex interactions of disease determinants with age-related risk factors (e.g., loss of sex hormones [4] or decline of insulin-like growth factor-1 (IGF-1) function [5]) and systemic disease conditions (e.g., hypercholesterolemia [6] or diabetes [7]), which progressively overcome the brain physiological cognitive reserve. Stranahan and Mattson [8] have recently suggested that the cognitive reserve relies on insulin/neurotrophic factor signaling and glucose metabolism that set the brain metabolic efficiency.

Approximately 20% of neurodegenerative disorders have been linked to some sort of altered insulin action. Isolated peripheral insulin resistance is rare (e.g., in ataxia-teleangectasia), and either type I (e.g., in Turner syndrome, Wolfram syndrome, thiamine-responsive megaloblastic anemia syndrome, and maternally inherited diabetes and deafness), or type II (e.g., in narcolepsy, Prader-Willi syndrome, and

Werner syndrome) diabetes are observed. An altered peripheral glucose metabolism has been reported also in Parkinson's disease and Huntington's chorea [reviewed in 9]. Apart from those cases in which evident genetic or biochemical factors indicate a unifying mechanism for both diabetes and neurodegeneration (e.g., mutations of mitochondrial tRNAs directly affecting mitochondria metabolism, [9]), overall the high prevalence of diabetes in people suffering from neurodegenerative disorders points to the relevance of insulin signaling in the brain capacity to compensate for neuropathology.

In the specific case of AD, a relatively high percentage of affected individuals have peripheral insulin resistance or type II diabetes, but the vast majority of AD patients do not have these diseases [10]. Interestingly, even in the absence of a systemic disease, the AD brain shows impairments in insulin/IGF-1 signaling mechanisms [10] and a deficit of glucose metabolism that anticipates the cognitive decline [11].

The present review discusses first the role of insulin/IGF-1 receptors in the adult brain and the possibility that the function of these receptors might exceed mediation of insulin/IGF-1 actions; then suggests that an impairment of insulin/IGF-1 receptor signaling contributes to AD via a disease-specific mechanism involving the loss of receptor activation by monomers of Aß.

INSULIN AND THE ADULT BRAIN

Both insulin and insulin receptors (IRs) are present in the brain. Concentrations of insulin in the different brain regions range from

10 to 100-fold greater than in plasma [12], from where insulin is transported in the cerebrospinal fluid (CSF) through a IR-based saturable transport occurring mostly in the olfactory bulb and in the hypothalamus [12]. Evidence for insulin synthesis in the CNS is less solid; neuronal synthesis has been found in animals [13], but it is unknown whether brain-derived insulin has a significant role in the adult human brain. IRs are highly abundant and localized on both astrocytes and neurons. Glial cells express typical IRs, whereas a brain specific IR is present in neurons [12]. This brain specific IR is a IR-A isoform that is less glycosylated than the corresponding peripheral receptor [14]. As different from the IR-B isoform, which has exquisite metabolic actions, IR-A also has mitogenic and anti-apoptotic actions during development [14]. It is peculiar to the adult brain this predominant expression of the IR-A, since IR-B is the main receptor in all adult peripheral tissues that depend on insulin for glucose metabolism [14]. The evidence that IR-A, but not IR-B, is a low specificity receptor that is activated with high affinity by ligands other than insulin (i.e., IGF-1 and IGF-2) [15] suggests that many of the effects observed with insulin administration (e.g., neuronal survival or memory enhancement [16-17]) may physiologically depend on locally produced substances. Accordingly, IR density and insulin contents do not correlate well in the different brain areas [12]. Based on the evidence that dendritic areas receiving rich synaptic inputs have a high IR density [18], it has been suggested a possible correlation between IRs and synaptic activity [19]. The obvious functional sequel would be the cognition enhancing properties of insulin. There are several mechanisms by which insulin may affect memory; these include

modulation of neurotransmitter release and enhanced expression of postsynaptic NMDA receptors, which are responsible for the induction of long-term potentiation, the molecular substrate of learning and memory [20]. Mechanisms directly related to the modulation of glucose uptake have also been suggested. Insulin does not affect whole-brain glucose use, but it increases glucose metabolism in selected brain regions [21-22] where discrete neuronal populations express insulin-sensitive glucose transporters, namely GLUT4 and GLUT8 [12, 23-24]. Because of their somatic cellular localization [25], GLUT4 and GLUT8 are likely to support the metabolic requirements of neuronal cell bodies, but the fact remains that an insulin-insensitive glucose transporter, GLUT3, is present in the neuropil [25], and is likely to uphold synaptic energy provision.

Another level of complexity is added by the evidence that IR is highly homologous to IGF-1R [14], with nearly identical signal transduction pathways potentially leading to the same neuronal effects. The two receptors are receptor tyrosine kinases that, after ligand-induced autophosphorylation, associate with insulin receptor substrate (IRS) adapter proteins. IRS proteins bind to tyrosine phosphate docking sites on the activated receptors, undergo phosphorylation themselves, and then recruit additional SH2-containing signaling proteins. Among these, the phosphatidylinositol-3 kinase (PI-3K), *via* phosphorylation of protein kinase B (PKB)/AKT, leads to the translocation of facilitative GLUTs from the intracellular pool to the plasma membrane [reviewed in 14]. AKT/ PKB also induces the inhibitory serine phosphorylation of glycogen synthase kinase-3β (GSK-3β), which

relieves the inhibition of the glycogen synthase and the translation initiation factor eIF2B, thus promoting glycogen and protein synthesis [26]. In addition, PI-3K/AKT activation may result into: i) an activation of regulatory-associated protein of mTOR (raptor) – mammalian target of rapamycin (mTOR) pathway, which regulates cell growth and metabolism [27]; ii) an inhibition of the pro-apoptotic BAD functions and iii) a suppression of the transcriptional program of FoxO proteins [reviewed in 14]. Finally, another signal transduction protein interacting with IRS proteins is GRB2, an adaptor that in turn elicits the activation of the (extracellular-regulated kinase) ERK cascade leading to mitogenic responses [reviewed in 14].

IR and IGF-1R expression overlaps in many brain regions [28], and also hybrid insulin/IGF-1 receptors, with an unclear physiological role, are highly present in the brain [29]. Noteworthy, insulin has a low affinity for both IGF-1R and hybrid receptors that, instead, are bounded by IGF-1 with higher affinity than insulin [reviewed in 14]. Specifically, at least in purified receptors from human placenta, the concentration of unlabelled IGF-I for half-maximal inhibition of ^{125}I-IGF-I binding appears to be 0.1-0.2 nM for hybrids and 0.05-0.01 for IGF-1R. By contrast, unlabelled insulin required for half-maximal inhibition of ^{125}1-insulin binding is 3-5 nM for hybrids and 0.3-0.5 nM for IRs, confirming the relatively low affinity of hybrids for insulin [30]. The evidence that IGF-1 inhibits 1^{25}1-insulin binding to hybrid receptors or IGF-1R more effectively than insulin (1 nM and 0.04 nM [IGF-I] vs. 4 nM and 4 nM [insulin] for hybrid receptors and IGF-IR, respectively) [30], and also stimulates the kinase activity of hybrid receptors more significantly than insulin [31] suggests that hybrid

insulin/IGF-1 receptors might have the funtional properties of an IGF-1R.

IGF-1 AND THE ADULT BRAIN

The insulin homologue, IGF-1, is highly produced in the developing brain by IGF-1R expressing neurons, suggesting local autocrine/paracrine actions of neuronal IGF-1 [32]. In the adult brain the local production of IGF-1 is low, but serum IGF-1 gets access to the brain through the blood-brain-barrier (BBB) [33]. Thus, the first question arises of whether brain IGF-1 and peripheral IGF-1 play different roles in the nervous tissue. Genetic manipulation of IGF-1 contents in transgenic mice has determined the fundamental role of neuronal IGF-1 in the regulation of brain growth and glucose utilization during development [34]. In contrast, brain IGF-1 actions in the adult, and particularly in the control of cerebral glucose metabolism, are not fully understood. Brain IGF-1 does not seem to participate in glucose utilization under normal condition in the adult; however, IGF-1 induction (both neuronal and glial) in response to injury correlates with increases in local glucose utilization [34], suggesting that brain IGF-1 functions at least to provide glucose for biosynthetic and reparative processes. Under these conditions, IGF-1 is believed to substitute for insulin by promoting GLUT4 activity [34]. More ample effects have been reported for serum IGF-1, including modulation of adult neurogenesis [35], neuronal excitability [36], neuroprotection by exercise [37], and cognitive functions [38]. Because of the need for serum IGF-1 in the brain, a peculiar mechanism of regulated passage through the BBB (beside a tonic

input) exists, according to which neuronal activity is coupled to the entrance of serum IGF-1 and, in turn, this peripheral input of IGF-1 to the brain might sustain the activity of already active neurons [39]. This feed-forward mechanism has been named "neuro-trophic coupling" and suggested to be a determinant of the cognitive reserve of the brain [33].

The interrelationship between IGF-1 and insulin actions remains to be established. As in the periphery, brain insulin signaling could depend on proper IGF-1 signaling via hybrid receptors [40], and/or direct facilitation of insulin signaling could occur *via* IGF-1 co-stimulation of IRs [41].

Interpretation remains open until a more comprehensive analysis will be available, which includes the potential context-specific role of IGF-binding proteins (IGFBPs) [42] in setting IGF-1 activity with respect to insulin, the specific properties of hybrid insulin/IGF-1 receptors, and pathophysiological conditions that may affect hybrid assembly [29].

INSULIN, IGF-1 AND THE AD BRAIN

Possible defects in insulin/IGF-1 signaling have been investigated in post-mortem AD brains mainly by immunohistochemical analysis [43-44]. Hoyer and colleagues first reported a reduction of IRs and receptor-kinase activity markers in the tissue [45]. Recently, a more detailed analysis carried by Moloney et al. [46] has revealed that the localization of both IRs and IGF-1Rs in AD neurons is away from the plasma membrane and concentrated in the cytosol, suggesting that

these neurons become resistant to insulin/IGF-1 signaling in the course of the disease. Accordingly, decreased levels of IRS-1 and IRS-2, key adaptors for both IR and IGF-1R signaling, are disease-stage related and correlate strongly with neurofibrillary tangle pathology [46]. Since insulin and IGF-1 engage the same downstream adaptors (i.e., IRS-1/IRS-2 and Shc) to drive the activation of PI-3 kinase and Ras/ERK kinase pathways [14], determining the relative contribution of IRs and IGF-1R to this defective signaling system is particularly challenging. One difference stays in the evidence that IGF-1R is highly expressed in AD astrocytes, and increasing IGF-1R levels, but not IR levels, accumulate within and around plaque pathology both in the AD brain and in 18-month-old Tg2576 mice, a transgenic model of AD [46]. This finding is consistent with a reparative role of IGF-1 under injury conditions [34], and might reflect the AD brain attempt to cope with progressing neuropathology by activating the IGF-1 signaling system.

Genetically engineered model targeting either the IGF-1 or the insulin signaling might help to identify relevant steps for AD pathology in the absence of a systemic disease condition (i.e., diabetes). Heterozygous inactivation of IGF-1R in the mouse brain has confounding effects resulting from a reduced somatotopic tone with ensuing decelerated animal growth and delayed mortality [47]. Even so, it is interesting that the IGF-1R-deficient brain shows a compensatory over-activation of the remaining IGF-1Rs so that the animals exhibit only a subtle impairment of exploratory behaviour [47]. On the other hand, brain-specific IR knock-out mice exhibit the features of a reduced insulin signaling, including the lack of activated PI-3K and the presence of

activated glycogen synthase kinase-3ß (GSK-3ß) and phosphorylated tau protein, but not cognitive dysfunctions [48].

Both spontaneous and experimentally-induced animal models of diabetes have been used to search for the presence of AD-like pathology [49]. The limit of this approach is that mice, due to the intrinsic nature of their own Aß, cannot produce the extracellular Aß aggregates [50] that have a pivotal role in AD; therefore, all data are biased by the lack of evident Aß-related neuronal pathology.

Instead, the induction of both type 1 and type 2 diabetes in transgenic mouse models of AD appears to exacerbate brain pathology [49], confirming the notion that diabetes-related metabolic disturbances are intervening promoting factors in the pathogenic cascade leading to AD [51].

The diabetogenic substance streptozotocin has been found to induce an isolated insulin-resistant brain state (IRBS) months after a single i.c.v. injection in rats [52]. After STZ i.c.v. administration, regionally specific alterations have been reported in the rodent brain, including a reduced IGF-1R gene expression in the cortex and striatum [53], a reduced IR gene expression in the frontoparietal cerebral cortex and hippocampus [54] and an increase in the non-phoshorylated active GSK-3 in the hippocampus [52]. Interestingly, when the IRBS is induced in AD transgenic mice by the i.c.v. injection of streptozotocin, mice neuropathology is exacerbated [55].

A unifying interpretation of the data is that the impairment of IRs and IGF-1Rs , which *per se* represents a neuronal stressor, is a contributing factor in the pathogenesis of AD (see after). That said, we still need to

determine what is directly responsible for the defects in the insulin/IGF-1 system, which have been found in post-mortem AD brains.

THE NATURE OF Aß

Aß (a 40-42 amino acid peptide) is normally produced by neuronal cells through the endo-proteolytic cleavage of APP [56], and is exported outside the brain by the low density lipoprotein receptor related protein-1 (LRP-1). Aß is also synthesized in the periphery and gets into the brain *via* the receptor for advanced glycation end-products [57]. This tightly regulated bidirectional trafficking of Aß across the blood brain barrier, together with Aß clearance by different metalloproteases [58-59], is aimed at maintaining the peptide into a specific range of concentrations. As other aggregation-capable molecules, Aß has a defined equilibrium state between monomers and oligomers such that it is primarly monomeric below a certain concentration [60]. Thermodynamic studies predict that, at the estimated *in vivo* concentrations [61], soluble Aß is mainly monomeric [60], and thus oligomers must originate in localized compartments (e.g., cell membranes) and under pathological conditions. *In vitro*, many different types of Aß assembly forms, including protofibrils, annular structures, paranuclei, Aβ-derived diffusible ligands, and globulomers have been described [62]. *In vivo*, pre-fibrillar assemblies of Aß, known as soluble Aß oligomers, have been demonstrated to correlate better with dementia than plaques [63], suggesting that oligomers represent the primary neurotoxic species in AD. Natural oligomers of human Aß disrupt synaptic functions when added *in vitro*

to hippocampal slices [64] or microinjected in living rats [65], where also interfere rapidly and reversibly with the memory of a learned behaviour [66]. The neurotoxicity of Aß oligomers has been confirmed by distinct experimental approaches, including the use of synthetic or native Aß peptides, cell cultures over-expressing APP, and APP transgenic mice [65, 67].

Mechanisms of Aß toxicity have been largely investigated [68-69]. However, since the majority of the reviews on the topic of cell death in AD is largely focused on the toxic actions of Aß**,** there is no need to repeat the knowledge here.

On the contrary, few studies have addressed the physiological activities of Aß, although indirect evidence for the implication of Aß in the normal neuronal metabolism occasionally appears in published papers. Thus, the *in vitro* inhibition of either ß- or γ-secretase (the two enzymes required for APP metabolism and Aß production) has been reported to affect the viability of cortical neurons, which are rescued by adding picomolar concentrations of Aß [70]. The addition of Aß to cultured neurons has been shown to enhance metabolism *via* the induction of hypoxia-inducible factor-1 [71]. Finally, it dates back to 1989 the finding that the 1-28 fragment of Aß has a neurotrophic activity [72].

More recent findings provide hints towards the concept of a physiological role for Aß. ß-Amyloid precursor protein cleavage enzyme (BACE 1) knock-out mice, which lack Aß formation, have behavioral deficits [73] and synaptic dysfunctions [74-75]. Along this line, picomolar concentrations of synthetic Aß, which likely

approximate the endogenous level of the peptide, have been shown to enhance synaptic plasticity and memory in the rat hippocampus [76].

To date, several lines of evidence indicate that Aß is released in normal brains during synaptic activity. Kamenetz and colleagues first reported that Aß is secreted from neurons in response to neuronal activity, and that in turn it down-regulates excitatory synaptic transmission [77], thus providing a physiological homeostatic control of neuronal activity. In rodent hippocampal cells and slices, acute increases in Aß levels expand reversibly the number of active synapses [78]. In the same system, enduring inhibition of Aß clearance results into a reduction in the number of synapses [78], suggesting that Aß functions at least as a modulator of synaptic activity requiring a fine balance between production and clearance. Although the nature of the endogenous released Aß has not been determined, it seems reasonable to assume that it is released in its non-toxic monomeric state.

Aß AND THE INSULIN/IGF-1 SYSTEM IN AD

The existence of different Aß forms (i.e., non-toxic monomers and toxic oligomers) adds complexity to the understanding of the link between the dysregulation of the insulin/IGF-1 signaling, which has been reported in AD [44], and Aß peptide itself. One level of interaction is established by the fact that insulin and IGF-I have a direct effect on the metabolism and clearance of Aß. Insulin directly increases Aß secretion from neurons by accelerating peptide trafficking to the plasma membrane [79], and promotes Aß degradation by regulating the expression of the insulin degrading enzyme (IDE), a metalloprotease that catabolizes both insulin and Aß

[80-81]. On its side, IGF-1 increases Aß clearence from the brain by enhancing transport of Aß-carrier proteins (e.g., albumin and transthyretin) into the brain [82]. Hence, insulin and IGF-1 seem to act in conjunction as regulators of brain Aß content, and systemic conditions altering their interplay could indirectly promote Aß oligomerization. For example, aging (the main risk factor for AD) is a associated with low serum levels of IGF-1 [5], and type 2 diabetes is associated with peripheral hyperinsulinemia and low brain insulin levels that could result in a reduced Aß clearance [83].

Another level of interaction is at the receptor level. Zhao and co-workers have suggested that the state of insulin resistance observed in the AD brain is a response to Aß oligomers, which downregulate neuronal surface IRs [84]. In contrast, IR activation would promote the reduction of oligomers to monomers via the insulin-degrading enzyme (IDE) activity [86]. Our own contribution to the field is the demonstration that Aß monomers support the survival of developing neurons under conditions of trophic deprivation and protect mature neurons against excitotoxic death. Both effects result from the stimulation of a receptor of the insulin/IGF-1 system, and are mediated by the activation of the PI-3K pathway [86]. Among the survival pathways that are stimulated by insulin/IGF-1, such as the ERK1/2 pathway and the PI-3K pathway [14], Aß monomers appear to specifically activate the last one, thus leading to an enhanced phosphorylation of Akt and also to an enhanced Ser9 phosphorylation (inhibition) of the Akt substrate, GSK-3ß [86]. Inhibition of GSK-3ß promotes cell survival through a variety of mechanisms including a reduced degradation of ß-catenin, which activates the transcription of

protective genes [87]. Accordingly, the neuronal levels of ß-catenin show a rapid and substantial increase in response to Aß monomers [86]. By inhibiting GSK-3ß, Aß monomers could also decrease the the overall phosphorylation of tau [88], a process that appears to be facilitated by decreased tau O-GlcNAcylation [89]. Interestingly, O-GlcNAcylation processes depend on glucose metabolism and a reduced O-GlcNAcylation seems to be the link between low brain glucose metabolism and tau pathology in AD [89]. Whether or not Aß monomers can increase the O-GlcNAcylation of tau protein, by supporting neuronal glucose provision, remains to be established.

A possible model of interaction between the different Aß species and the IR/IGF-1R system could be the following: accumulating Aß oligomers impair the insulin/IGF-1 signaling, which exacerbates Aß oligomerization and toxicity within a feed-foward mechanism. Aß monomers, by sustaining the insulin/IGF-1 signaling, promote survival, impede oligomerization and contribute to the homeostatic control of the system (figure 1).

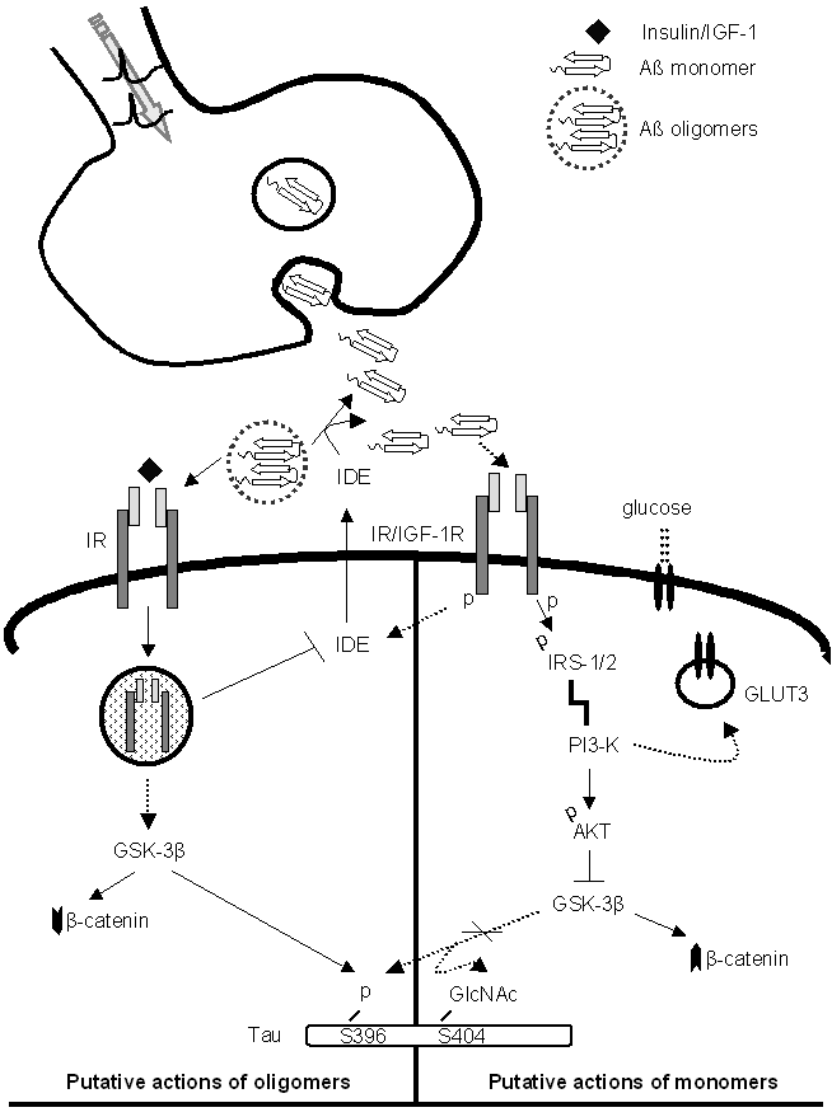
At present, it is unknown whether Aß monomers bind directly to IRs and/or IGF-1Rs, although evidence suggests that monomers have specific recognition sites on the neuronal surface [86]. The unheralded importance of the finding that Aß monomers are able to activate IRs/IGF-1Rs would be the evidence that the peptide is produced and released to sustain transient needs in synaptic modeling, neuronal energy provision and protection in the absence of brain insulin/IGF-l fluctuations.

Conclusion

The non-toxic form of Aß, the monomer, appears to behave as a brain protective factor able to regulate synaptic activity and to activate the insulin/IGF-1 receptor signaling. Depletion of Aß monomers in the preclinical phase of AD, resulting from pathological Aß aggregation, could be responsible for early defects of insulin/IGF-1 receptor signaling (including the deficit of glucose metabolism that anticipates cognitive decline [11]), thus participating to the overall AD pathology.

FIGURE CAPTURE

Fig 1 Possible interactions between Aß and the insulin/IGF-1 receptor signaling. Aß monomers released at the synapse (right panel) promote the activation of the insulin/IGF-1 signaling pathway, resulting into: i) self-maintained levels of Aß monomers (via the activity of the insulin-degrading enzyme – IDE -), ii) sustained neuronal survival (via ß-catenin-regulated gene transcription), iii) and decreased tau phosphorylation (via GSK-3ß inhibition). Aß monomers could also be responsible for synaptic glucose provision (via GLUT3 translocation) and increased O-GlcNAcylation of tau protein, which opposes tau hyperphosphorylation. On the other side, accumulating Aß oligomers (left panel) induce the downregulation of insulin/IGF-1 receptors that will exacerbate Aß oligomerization with ensuing neurotoxicity. Dashed lines refer to proposed but not proven mechanisms.


Insulin/IGF-1
Aß monomer
Aß oligomers
IDE
IR
IR/IGF-1R
glucose
p
p
p
IDE
IRS-1/2
GLUT3
PI3-K
p
AKT
GSK-3β
β-catenin
GSK-3β
β-catenin
p
GlcNAc
Tau
S396
S404
Putative actions of oligomers
Putative actions of monomers

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CHAPTER III

Monomeric β-Amyloid interacts with type-1-Insulin-Like- growth fsctor Receptors to provide energy supply to neurons

M. L. Giuffrida[1,9], F. M. Tomasello[2,9], F. Caraci[3], G. Pandini[4], G. Pappalardo[1], F. Attanasio[1], S. Chiechio[5], R. Vigneri[4], E.Rizzarelli[1,6], F. Nicoletti[7,8], A. Copani[1,5*]

[1]National Research Council, Institute of Biostructure and Bioimaging, Catania, Italy.

[2]PhD Program in Neuropharmacology, University of Catania, Italy.

[3]Department of Formative Processes, University of Catania, Italy.

[4]Department of Clinical and Molecular Biomedicine, University of Catania, Italy.

[5]Department of Drug Sciences, University of Catania, Italy.

[6]Department of Chemical Sciences, University of Catania, Italy.

[7]Department of Human Physiology and Pharmacology, University "La Sapienza", Rome, Italy.

[8]I.R.C.C.S Neuromed, Pozzilli, Italy.

*Correspondence to: A. Copani (acopani@katamail.com).

[9]Equally contributed to the work.

We found that the Alzheimer's disease protein ß-amyloid (Aß), in its non-toxic monomeric state, activates type-1 insulin-like growth factor (IGF) receptors, and mimics the metabolic actions of IGFs in neurons and peripheral cells. In neurons, endogenous Aß release was required to uphold glucose uptake during activation, and exogenously added Aß monomers caused the translocation of type-3 glucose transporters to the plasma membrane with ensuing glucose uptake. Pathological aggregation of Aß monomers might impair neuronal ability to cope with transient needs in energy provision.

The 42 amino acid-long Aß protein ($Aß_{1-42}$) is produced by proteolytic cleavage of the transmembrane type-1 protein, amyloid precursor protein (APP). Under pathological conditions, $Aß_{1-42}$ self-aggregates into oligomers, which are believed to be the culprit of Alzheimer's disease (AD) by causing synaptic dysfunction and neuronal loss[1]. However, $Aß_{1-42}$ is mainly monomeric at physiological concentrations[2], and the precise role of monomeric $Aß_{1-42}$ in neuronal function is largely unknown. We reported a protective activity of monomeric human $Aß_{1-42}$ in cultured cortical neurons, which was sensitive to inhibitors of insulin/insulin-like growth factor (IGF) receptor signaling[3]. We now show that the protective effect was shared by rat/mouse $Aß_{1-42}$ (**Fig. 1a, b**), which is resistant to oligomerization[4]. Because oligomerization of human $Aß_{1-42}$ results into neurotoxicity rather than neuroprotection [3], we hypothesized that the peptide

sequence recruited in the aggregation process might be engaged in mechanisms of neuroprotection. The 16-20 amino acid sequence of $A\beta_{1-42}$ (KLVFF) is critically involved in $A\beta_{1-42}$ oligomerization, and is used as template for the design of beta-sheet breakers[5]. Synthetic Ac-KLVFF-NH_2 maintained into a monomeric form (**Supplementary Fig. 1 and supplementary methods**) shared the protective activity of monomeric $A\beta_{1-42}$, and its action was prevented by the insulin/IGF receptor inhibitor, AG1024, or by the selective type-I IGF receptor (IGF-IR) inhibitor, PPP (**Fig. 1a, b**). Neither the D-isomer, klvff, nor the scrambled peptide, FKLVF, caused neuroprotection, whereas the retroinverse ffvlk peptide, which maintains the overall spatial topology of KLVFF, was protective, albeit to a lesser extent. The 1-16 amino acid sequence of human $A\beta_{1-42}$ was inactive (**Fig. 1a, b**). These data suggested that monomeric human $A\beta_{1-42}$ could interact with insulin/IGF receptors *via* the 16-20 KLVFF sequence, thereby causing neuroprotection.

Both monomeric human $A\beta_{1-42}$ and Ac-KLVFF-NH_2 stimulated Tyr-phosphorylation of native IGF-IR in cultured cortical neurons (**Fig. 1c**), without affecting Tyr-phosphorylation of insulin receptor (IR) (**Supplementary Fig. 2a**). We searched for a direct peptide-receptor interaction in 3T3-like mouse fibroblasts with a disrupted IGF-IR gene and transfected with either the human IGF-IR (R^+ cells), or with type-A IR (IR-A) (R^-IR-A cells) cDNA[6]. On immunoadsorbed IGF-IRs derived from R^+ cells, monomers of $A\beta_{1-42}$ or Ac-KLVFF-NH_2 potentiated the ability of IGF-1 to promote autophosphorylation of the receptor-kinase domain (**Fig. 1d, e**), with $A\beta_{1-42}$ displaying appreciable efficacy at IGF-IR by its own (**Fig. 1e**). $A\beta_{1-42}$ monomers

per se had negligible activity on immunoadsorbed IR-A, and failed to affect receptor response to insulin (**Supplementary Fig. 2b**).

IGF-1 is known to stimulate glucose uptake in neurons by mechanism(s) similar to those used by insulin in the periphery, including membrane translocation of glucose transporters (GLUTs)[7]. In our model, neuronal dependence on a steady supply of glucose was assessed by depriving the cultures from glucose for 75 minutes prior to exposure to the fluorescent non-hydrolyzable glucose analogue, 6-(N-(7-nitrobenzen-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose (6-NBDG). During starvation, neurons were exposed for 30 min to recombinant rat IGF-1 at concentrations (5 ng/ml) that selectively activate IGF-I and lie within a physiological range in the CSF[8]. This treatment caused a significant increase in 6-NBDG uptake, as assessed by confocal microscopy or flow-cytometry (**Fig. 2b, d**). Consistent with its ability to engage IGF-IRs, monomeric $A\beta_{1-42}$ (100 nM) increased the population of 6-$NBDG^{+}$ neurons following starvation, and its action was inhibited by the IGF-IR inhibitor, PPP (**Fig. 2c, d**). Thus, $A\beta_{1-42}$ monomers diplayed IGF-1-like metabolic activity in cultured neurons. This evidence, together with the knowledge that $A\beta_{1-42}$ is released from neurons in response to synaptic activity[9], provided the hint for testing the hypothesis that $A\beta_{1-42}$ monomers might function to increase glucose uptake during neuronal activation. A depolarization pulse with KCl (40 mM for 15 minutes) caused a significant increase in glucose uptake, which occluded any additional effect of $A\beta_{1-42}$ monomers (**Fig. 2e**). Depolarization-induced glucose uptake was prevented by a pretreatment with PPP (500 nM for 20 minutes), suggesting that endogenous activation of IGF-IRs was required for

activity-dependent energy supply (**Fig. 2e**). Addition of a γ-secretase inhibitor (γ-secretase inhibitor IX, 100 nM) to block the endogenous production of $A\beta_{1-42}$ (**Supplementary table 1 and supplementary methods**), blunted depolarization-induced glucose uptake, which was re-established by the exogenous application of $A\beta_{1-42}$ monomers (**fig. 2e**). Glucose transport in neurons is mediated by the membrane transporters, Glut3, Glut4, and Glut8, of which Glut3 has a neuropil localization[10] that might enable neurons to uphold gluocose demand during synaptic activity. Depolarization is known to promote the fusion of Glut3 vesicles with the cell surface in neurons[11]. In our cultures, K^+-induced depolarization increased Glut3 immunoreactivity in neuronal threads, as well as in perikarya and axon hillocks (**Fig. 2n, o**). $A\beta_{1-42}$ monomers induced a similar pattern of Glut3 immunoreactivity (**Fig. 2h, i**), which was prevented by pretreatment with PPP (**Fig. 2l, m**). The intense Glut3 immunoreactivity profiling neuronal perikaryon was paralleled by a reduction of Glut3 signal spread assayed in a z-stack series of neuronal slices (**Fig. 2p**), suggesting that $A\beta_{1-42}$ monomers were promoting Glut3 translocation. Glut3 translocation was further assessed by immunolabeling neurons with an antibody raised against the exofacial epitope of Glut3 in the absence of membrane permeabilization. Both monomeric $A\beta_{1-42}$ and Ac-KLVFF-NH_2 increased immunoreactivity for exofacial Glut3 as assessed by immunocytochemistry and cytofluorimetric analysis (**Supplementary Fig. 3 and supplementary methods**).

To strengthen the evidence that monomeric $A\beta_{1-42}$ has IGF-1-like activity, we extended the analysis to classical peripheral actions of IGF-1. L6 rat skeletal muscle cells show a prevalence of IGF-IRs over

IRs[12] and also express Glut3[13]. In differentiated L6 myotubes both monomeric $A\beta_{1-42}$ and Ac-KLVFF-NH_2 stimulated Tyr-phosphorylation of IGF-IRs (**Fig. 3a**), and enhanced the amount of phophorylated high-molecular weight eIF-4E binding protein, 4E-BP1 (**Fig. 3a**), used as an indicator of IGF-IR signaling. Similarly to IGF-1 (2 ng/ml), monomeric $A\beta_{1-42}$ and Ac-KLVFF-NH_2 (both at a 100 nM) stimulated 6-NBDG uptake (**Fig. 3b, c**) and $A\beta_{1-42}$ monomers also increased Glut3 immunoreactivity (**Fig. 3d**) in myotubes. Physiological concentrations of IGF-1 are known to inhibit insulin secretion from pancreatic ß cells[14,15]. Similarly to IGF-1, monomers of $A\beta_{1-42}$ and Ac-KLVFF-NH_2 (injected i.p. to obtain plasma concentrations of 100 nM) caused a transient increase in blood glucose levels in mice undergoing a glucose tolerance test, which reflects the inhibition of insulin secretion (**Supplementary Figure 4a and supplementary methods**). We also used INS-1E insulinoma cells, which secrete insulin in response to glucose[16]. We observed a 1.2 - 2 fold increase in insulin release by shifting the cells from low (3 mM) to high (15 mM) glucose concentrations. Monomers of $A\beta_{1-42}$ and Ac-KLVFF-NH_2 inhibited glucose-stimulated insulin secretion, and the effect was prevented by PPP (**Supplementary Figure 4b and supplementary methods**). Hence, monomeric $A\beta_{1-42}$ and its functional epitope, KLVFF, exhibited IGF-1-like effects in different cell types. The endogenous tone of IGF-1[17,18] (see also **supplementary methods**) should be relevant to KLVFF effects, but could be dispensable in the case of $A\beta_{1-42}$ monomers.

Our results provide evidence that monomeric $A\beta_{1-42}$ acts as a positive allosteric modulator of IGF-IR endowed with intrinsic

agonist-like activity. This activity serves to meet the neuronal need of glucose. Hence, glucose uptake during neuronal activation might relay on naturally secreted monomers of Aß protein acting on IGF-IRs. These data pose two questions: i) whether the entrapment of $Aß_{1-42}$ monomers into diffusible oligomers at synapses impairs the brain ability to cope with transient needs in neuronal energy provision, and ii) whether reduced amounts of $Aß_{1-42}$ monomers may be responsible for the unexplained resistance of IGF-IRs to be activated in post-mortem AD brain tissue[19].

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AUTHOR CONTRIBUTIONS

M.L.G., F.M.T., F.C., G.P., G.P., F.A., S.C., R.V., E.R., F.N., and A.C. designed research; M.L.G., F.M.T., F.C., G.P., G.P., F.A., S.C., and A.C. performed research; M.L.G., F.M.T., F.C., G.P., G.P., F.A., S.C., R.V., E.R., F.N., and A.C. analyzed data; M.L.G., F.M.T., F.N., and A.C. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no conflicts of interests.

FIGURE LEGENDS

Figure 1 $Aß_{1-42}$ monomers activated IGF-IRs both in primary neurons and in recombinant cells. The selective inhibitor of the IR superfamily,

AG1024, and the preferential IGF-IR inhibitor, PPP, prevented the neuroprotective activity of $A\beta_{1-42}$ monomers, either human (mAß) or rat/mouse (R/M mAß), and of Ac-KLVFF-NH_2 monomers (KLVFF) against NMDA-induced toxicity (a) or insulin-deprivation (b). In (a) and (b) values are means $\pm$ S.E.M. of eight determinations from two independent experiments. Significantly different from NMDA (*), or from the respective peptide condition (#) at $p < 0.05$ (one-way ANOVA + Fisher's LSD test). (c) Representative western blot analysis of immunoprecipitated IGF-IR beta subunit (IGF-IR i.p.) in neuronal extracts from control cultures (CTRL) or cultures exposed for 15 min to 100 nM of either mAß or KLVFF, both in the absence and in the presence of AG1024 (100 nM). Levels of activated IGF-IR beta subunit, p(Y1161) IGF-IR, were increased by either mAß or KLVFF but not in the presence of the receptor antagonist, AG1024. IGF-IR bands are shown as control for loading. (d) Autophosphorylation of immunocaptured human IGF-IR in response to IGF1, mAß, or a combination of both. (e) Autophosphorylation of immunocaptured human IGF-IR in response to IGF1, KLVFF, or a combination of both. The lack of effects of the scrambled peptide, Ac-VFLKF-NH_2 (VFLKF), is also shown. Phosphorylation was quantitated by ELISA as described under Methods. Both in (d) and (e) data are the means $\pm$ S.E.M. of three independent experiments and are expressed as % over basal receptor phosphorylation. *Significant at $p < 0.05$ vs. IGF-1 alone, or vs. basal (#) (one-way Anova + Fisher's LSD test).

Figure 2 $A\beta_{1-42}$ monomers stimulated glucose uptake and membrane translocation of Glut3 in neurons by activating IGF-IRs. Confocal images of 6-NBDG uptake in live neurons exposed to either IGF-1

(5ng/ml) (b) or monomeric $A\beta_{1-42}$ (mAß) (c) for 30 min after starvation, scale bar = 10 μm. Percentage of 6-NBDG$^+$ neurons following treatments was scored by flow cytometry in (d) and (e). (d) The IGF-IR antagonist, PPP, prevented neuronal 6-NBDG uptake induced by $A\beta_{1-42}$ monomers (mAß). Values are means ± S.E.M. of two independent experiments. *Significant at $p < 0.05$ vs. controls (CTRL), or vs. mAß (#) (one-way Anova + Fisher's LSD test). (e) The IGF-IR antagonist, PPP, and the γ-secretase inhibitor IX (γ-Sec inh) prevented neuronal 6-NBDG uptake induced by a 15 min depolarization pulse with KCl. Values are means ± S.E.M. of two independent experiments. *Significant at $p < 0.05$ vs. controls (CTRL), or vs. KCl (**), or vs. γ-Sec inh + KCl (#) (one-way Anova + Fisher's LSD test). Confocal images of neurons co-immunolabeled for Glut3 and ß-actin following 10 min stimulation with 100 nM $A\beta_{1-42}$ monomers (mAß) both in the absence (h, i) and presence of the IGF-IR antagonist, PPP (l, m), or with 40 mM KCl (n, o). Before stimulations, neurons were glucose-deprived for 30 min. Arrows point to Glut3 immunoreactivity profiling neuronal perikarya in (h), (i) and (n); arrowheads point to Glut3 immunoreactivity profiling neuronal threads in (i) and (o). Scale bars indicate low magnification (f, h, l, n) and high magnification (g, i, m, o) images. (p) Glut3 signal spread assayed in a z-stack series of neuronal slices as described under Methods. Ten random fields for each experimental condition were imaged, and tens neurons/field were scored. Each treatment was repeated twice in three separate experiments. Bars represent fold decrease of signal spread compared to controls (CTRL). *Significant

at $p < 0.05$ vs. controls (CTRL), or vs. mAß (#) (one-way Anova + Fisher's LSD test).

Figure 3 IGF-1-like actions of $Aß_{1-42}$ monomers in L6 rat skeletal myotubes. (a) Left side: representative western blot analysis of immunoprecipitated IGF-IR beta subunit (IGF-IR i.p.) in L6 extracts from control cultures (CTRL) or cultures exposed for 15 min to 100 nM of either monomeric $Aß_{1-42}$ (mAß) or monomeric Ac-KLVFF-NH_2 peptide (KLVFF). Levels of activated IGF-IR beta subunit, p(Y1161) IGF-IR, were increased by either mAß or KLVFF. IGF-IR bands are shown as control for loading. Right side: representative western blot analysis of phophorylated 4E-BP1 (p4E-BP1) in L6 extracts from control cultures (CTRL) or cultures exposed for 15 min to 100 nM of either monomeric $Aß_{1-42}$ (mAß) or monomeric Ac-KLVFF-NH_2 peptide (KLVFF). Levels of high-molecular weight p4E-BP1 isoform (see arrow) were increased by either mAß or KLVFF. ß-actin bands are shown as control for loading. IGF-1 (2 g/ml) was used as a positive control within the experiments. (b, c) $Aß_{1-42}$ monomers promoted glucose uptake in L6 rat skeletal myotubes. Confocal images of 6-NBDG uptake in live L6 cells exposed to either monomeric $Aß_{1-42}$ (mAß) or IGF-1 (2ng/ml) for 15 min after starvation are shown in (b); scale bar = 510 µm. In (c), 6-NBDG fluorescence intensity was quantified by confocal imaging and represented as fold change with respect to basal. Fluorescence intensity was calculated from 300 cells/experiments in two independent experiments. *Significant at $p < 0.05$ vs. basal, or vs. the respective control condition (#) (One-Way Anova + Fisher's LSD test). D) $Aß_{1-42}$ monomers increased Glut3

immunoreactivity in L6 cells. Confocal images of L6 myotubes co-immunolabeled for Glut3 and ß-actin following 15 min stimulation with 100 nM $Aß_{1-42}$ monomers (mAß) or 2ng/ml IGF-1 are shown; scale bar = 140 μm.

METHODS

IGF-IR and IR phosphorylation assay

Clones of R^- cells (3T3-like mouse fibroblasts with a disrupted IGF-IR gene), stably transfected with either the human IGF-IR (R^+) or the human IR-A cDNA (R^- IR-A), were obtained as previously described[7]. Cell lysates from R^-IR-A cells or R^+ cells (40 μg protein/well) were immunocaptured in Maxisorp Break-Apart immunoplates (Nunc) coated with antibodies MA-20 (Novus Biologicals), which recognizes the IR α-subunit, and αIR-3 (Calbiochem), which recognizes the IGF-IR α-subunit, at a concentration of 2 μg/ml and 1 μg/ml, respectively, in 50 mM sodium bicarbonate (pH 9.0) overnight at 4°C. After washing, the immunocaptured receptors were incubated with increasing concentrations of either porcin insulin (Sigma-Aldrich) or recombinant human IGF-I (PeproTech) (in 50 mM HEPES-buffered saline (pH 7.6), 150 mM NaCl, 0.1% TritonX 100, BSA 0.05%, containing 10 μM ATP, 10 mM $MgCl_2$ and 2 mM $MnCl_2$) in the presence or absence of $mAß_{1-42}$. After 2 h at RT, the plates were washed and the captured phosphorylated proteins were incubated with biotin-conjugated anti-phosphotyrosine antibody 4G10 (0.3 μg/ml in 50 mM HEPES (pH 7.6), 150 mM NaCl, 0.05% Tween 20, 1% BSA, 2 mM sodium orthovanadate, 1 mg/ml bacitracin) for 2 h at RT and then with

peroxidase-conjugated streptavidin. The peroxidase activity was determined colorimetrically by using the TMB microwell peroxidase substrate system (KPL). The reaction was stopped by the addition of 1.0 M H_3PO_4, and the absorbance was measured at 450 nm.

Neuronal cultures: preparation and treatments

Animal care and experimentation was in accordance with institutional guidelines. Cultures of pure cortical neurons were obtained from rats at embryonic day 15 as described previously[3]. Cultures of mixed cortical cells, contaning both neurons and glia, were obtained from rats at embryonic day 17 and grown onto poly-D-lysine coated 16 mm multiwell vessels ($4x10^5$ cells/well) as described previously[3]. Mature cultures, at 14-16 days *in vitro* (DIV), were used for the study. All experiments were performed always after extensive washing of the cultures to avoid any interference with serum IGF-1/IGFBP3/ALS ternary complexes.

Mature pure neuronal cultures at 7 DIV were deprived from insulin and, where required, peptide monomers were added and maintained for 48 h. AG1024 (100 nM) and PPP (500 nM) were applied for 30 min before insulin deprivation. Neuronal survival was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay.

Mixed cortical cultures at maturation were exposed to 300 μM NMDA for 10 min at room temperature in a HEPES-buffered salt solution. Neuronal toxicity was examined 24 h later by light microscopy and quantified after staining with trypan blue (0.4% for 5 min). Stained neurons were counted from three-random fields/well.

Peptide monomers were added in combination with NMDA. Where required, AG1024 (100 nM) and PPP (500 nM) were applied 15 min before the excitotoxic pulse.

Imaging of 6-NBDG up-take in neurons by laser scanning confocal microscopy (LSM)

Neurons were plated on glass bottom culture dishes and were used at 6-8 DIV. For the experiments, cultures were rinsed with glucose-free HCSS (120mmol/l NaCl, 5.4 mmol/l KCl, 1.8mmol/l $CaCl_2$, 20mmol/l HEPES pH 7.4) and kept for 45 min under glucose deprivation followed by exposure for 30 min to either $mA\beta_{1-42}$ (100 nM), or recombinant rat IGF-1 (5ng/ml, R&D Systems). The non-hydrolyzable glucose analog 6-NBDG was allowed to be internalized into neuronal cells at 37°C and 5% CO_2 for 10 min. 6-NBDG^+ neurons were imaged by using an Olympus FV1000 LSM. Images were captured at 488 excitation/505-550 emission.

Assessment of 6-NBDG uptake in neurons by cytofluorimetric analysis

Neurons were grown onto 35 mm dishes and the experiments were performed at 7 DIV. Cultures were rinsed in glucose-free HCSS and maintained for 45 min under glucose deprivation followed by exposure to either $mA\beta_{1-42}$ (100 nM) or IGF-1 (5ng/ml) for 30 min, or to KCl (40 mM) for 15 min. PPP (500nM) was added 15 min before $mA\beta_{1-42}$. When required, a γ-secretase inhibitor (γ-sec-Inhibitor IX, Calbiochem, 100 nM) was added 2h before glucose deprivation and maintained troughout the experiment. 6-NBDG (100μM) was added 10 min before ending the experiment by rinsing the cells twice with

ice-cold phosphate buffered saline (PBS). Neurons were scraped into ice-cold PBS and maintained at 4°C for the cytofluorimetric analysis (cytomics FC500, Beckman Coulter). 20.000 Events for experimental condition (each in triplicate) were collected.

L6 cell cultures

L6 rat skeletal muscle cells (EACC) were cultured in 5% CO2 in DMEM (Invitrogen) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin. Once the cells reached 80% confluence, they were split (using 0.25% trypsin-EDTA) into fractions and propagated or seeded to be used in the experiments. To induce differentiation into the myotubes that were used for experiments, cells were made confluent and the concentration of FBS was then reduced to 1% for 24 – 48 h. Cells were passaged biweekly. Passages 10–30 were used for all the experiments.

Measurement of 6-NBDG uptake in L6 cells

The uptake of the fluorescent 6-NBDG was assayed in L6 myotubes by a modification of methods used previously in other mammalian cells[20]. Briefly, L6 myotubes, seeded in 22 mm glass bottom dishes, were placed in Krebs'Ringer buffer (136 mM NaCl, 20 mM HEPES, 2 mM NaHCO3, 0.5 mM NaH2PO4, 3.6 mM KCl, 0.5 mM MgCl2, and 1.25 mM CaCl2, pH 7.4) without glucose for 45 min. When required, inhibitors were added for 30 min during the glucose starvation time. The stimulation of glucose uptake was obtained by incubating cells for 15 more min with either IGF-1, mAβ_{1-42}, □or KVLFF. Cells were then loaded with 100 μM 6-NBDG for 15 min. The concentration and incubation time were chosen as the best for

giving an adequate signal/noise ratio. After the loading period, culture dishes were washed twice and placed on the stage of a FV1000 LSC microscope. Cultures were excited at 488 nm, and 6-NBDG was imaged at 505–550 nm emission wavelengths.

Indirect Glut3 immunofluorescence analysis

To mimic the experimental conditions under which glucose uptake was observed, cells seeded in round coverslips (pure cortical neurons or L6 myotubes) were washed repeatedly and glucose starved for 30 min. When required, PPP was added during the glucose starvation time. Cells were then washed again and stimulated for 10 min with monomeric $A\beta_{1\text{-}42}$ (100 nM), KCl (40 mM), or KLVFF monomers (100 nM) in the presence of glucose. Cells were then fixed in 2% formaldehyde and permeabilized using 0.1% Triton X-100. Unspecific binding was blocked by 30 min of incubation in 4% bovine serum albumin (BSA) in 0.1% Triton X-100-PBS. Glut-3 was detected by incubating over-night cells with rabbit anti-Glut-3 antibody (1:100, Abcam). Counterstaining was obtained by over-night incubation with mouse anti-actin (1:200, Sigma-Aldrich). After PBS washing, cells were exposed for 1 h at RT to the respective secondary antibody (anti-rabbit AlexaFluor 546 or anti-mouse AlexaFluor 680). Coverslips were mounted with the ProLong Gold antifade mounting medium (Invitrogen) and examined under a FV1000 LSC microscope using the Fluoview Olympus image software.

Imaging was carried out using a 63 Plan-Apo/1.4-NA oil-immersion objective. Standard 3 confocal channel (3 photomultiplier detectors) acquisitions were made by using the following lasers,

mounted on a laser combiner: Multi-line Argon laser (457nm, 488nm, 515nm), total 30mW HeNe-Green laser (543nm), 1.5mW HeNe-Red laser (633nm), 10mW. Single or multiple optical sections (0.42 µm *z* axis) through the middle of the cells were acquired for each field. The pinhole was adjusted to keep the same size of *z*-optical sections for all the analysis. Sequential mode imaging was performed to ensure that there was no crosstalk between the channels. Ten random fields for each treatment were imaged, with each treatment repeated twice in three separate experiments. Quantitative analysis was carried out using the FV1000 single particle analysis software (release 2). Glut3 signal spread was calculated as follows: for each microscopic field a z-stack series, made up of 20 slices (0,42 µm thickness each), was acquired. Then, the difference between the average fluorescence intensity/pixel, measured as z-projection in the z-stack series, and the average fluorescence intensity/pixel, measured for a single slice corresponding to the middle of the neuron, was calculated.

Western Blotting analysis

Western blotting analysis for phospho-eIF-4E binding protein (p4EBP1) was performed on total protein extracts (50µg) from differentiated L6 cells treated with IGF-1 (2 ng/ml), mAß$_{1\text{-}42}$ (100nM), or KLVFF (100nM) for 15 min. Samples were loaded onto 4-12% bis-Tris Glycine gel (NuPAGE, Invitrogen). After separation, proteins were transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Italia) using a transblot semi-dry transfer cell. Membranes were blotted at 4°C o.n. with the following primary antibodies: rabbit anti p4EBP1 (1:1000, Cell Signaling), and mouse anti-ß-actin (1:1000, Sigma Aldrich). Secondary goat anti-rabbit

labeled with IR dye 680 (1:14.000 Li-COR Biosciences) and goat anti-mouse labeled with IRdye 800 (1:12.000 Li-COR Biosciences) were used at RT for 45 min. Hybridization signals were detected with the Odyssey Infrared Imaging System (LI-COR Biosciences).

For the analysis of phospho-IGF-IR or phospho-IR, cell lysates were obtained either from pure neuronal culture or L6 cells following a 15 min stimulation. Cell lysates (150 μg) were incubated for 4 h at 4°C with the following primary antibodies: rabbit anti-IGF-IR β (10 μg/mg protein lysate, Cell Signaling Technology) or rabbit anti-Insulin Receptor β (10 μg/mg protein lysate, Cell Signaling Technology). Then, 20μl of protein G Plus-agarose were added, and samples were incubated overnight at 4°C on a rotating device. Immunoprecipitates were pelleted, washed and resuspended in 25μl of 1x electrophoresis sample buffer containing dithiothreitol. Following SDS-PAGE and transfer, blots were probed with a primary rabbit antibody to both p(Y1161) IGF-IR and p(Y1185) IR (1:500, Abcam). Specific hybridization signals were obtained by using horseradish peroxidase-conjugated secondary antibodies, followed by the enhancing chemiluminescence detection system (Immobilon Western, Millipore). In all cases, after probing with the phospho-specific antibody, blots were stripped for 30 min in 25 mM glycine-HCl, pH 2.0, containing 1% SDS and 0.1% Tween, and reprobed with anti-IGF-IR β or anti-Insulin Receptor β to control for loading.

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Fig 1

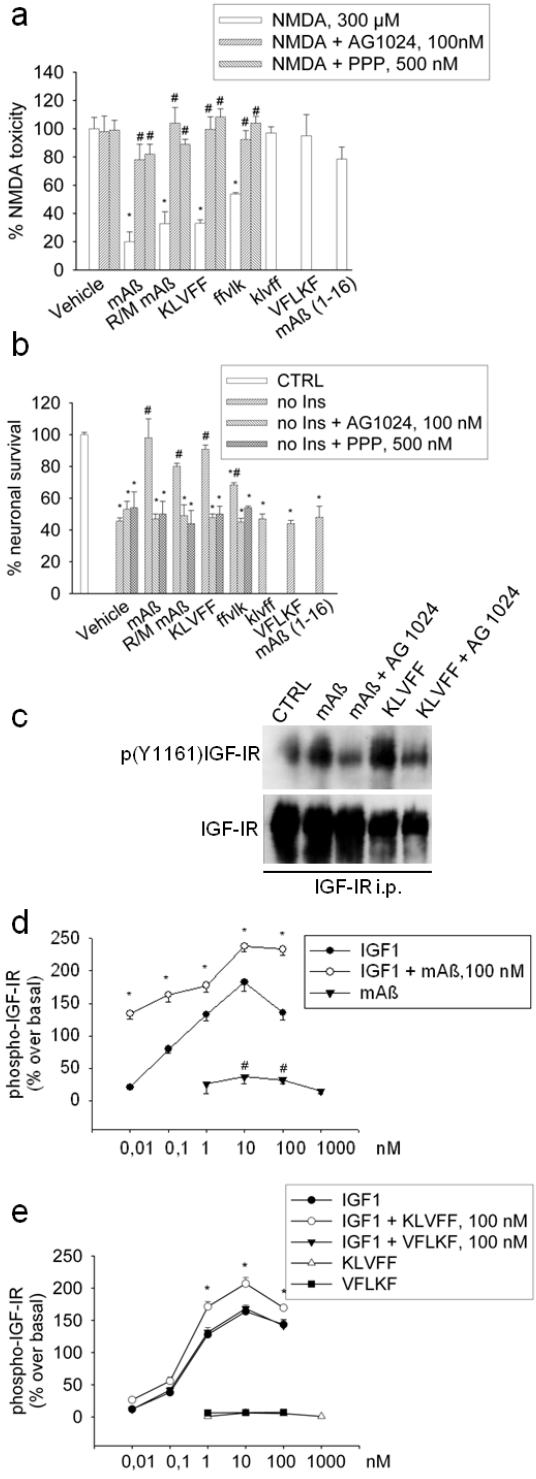

a
NMDA, 300 µM
NMDA + AG1024, 100nM
NMDA + PPP, 500 nM
% NMDA toxicity
Vehicle
mAß
R/M mAß
KLVFF
ffvlk
klvff
VFLKF
mAß (1-16)
b
CTRL
no Ins
no Ins + AG1024, 100 nM
no Ins + PPP, 500 nM
% neuronal survival
c
CTRL
mAß
mAß + AG 1024
KLVFF
KLVFF + AG 1024
p(Y1161)IGF-IR
IGF-IR
IGF-IR i.p.
d
IGF1
IGF1 + mAß,100 nM
mAß
phospho-IGF-IR (% over basal)
nM
e
IGF1
IGF1 + KLVFF, 100 nM
IGF1 + VFLKF, 100 nM
KLVFF
VFLKF
phospho-IGF-IR (% over basal)
nM

Fig 2/3

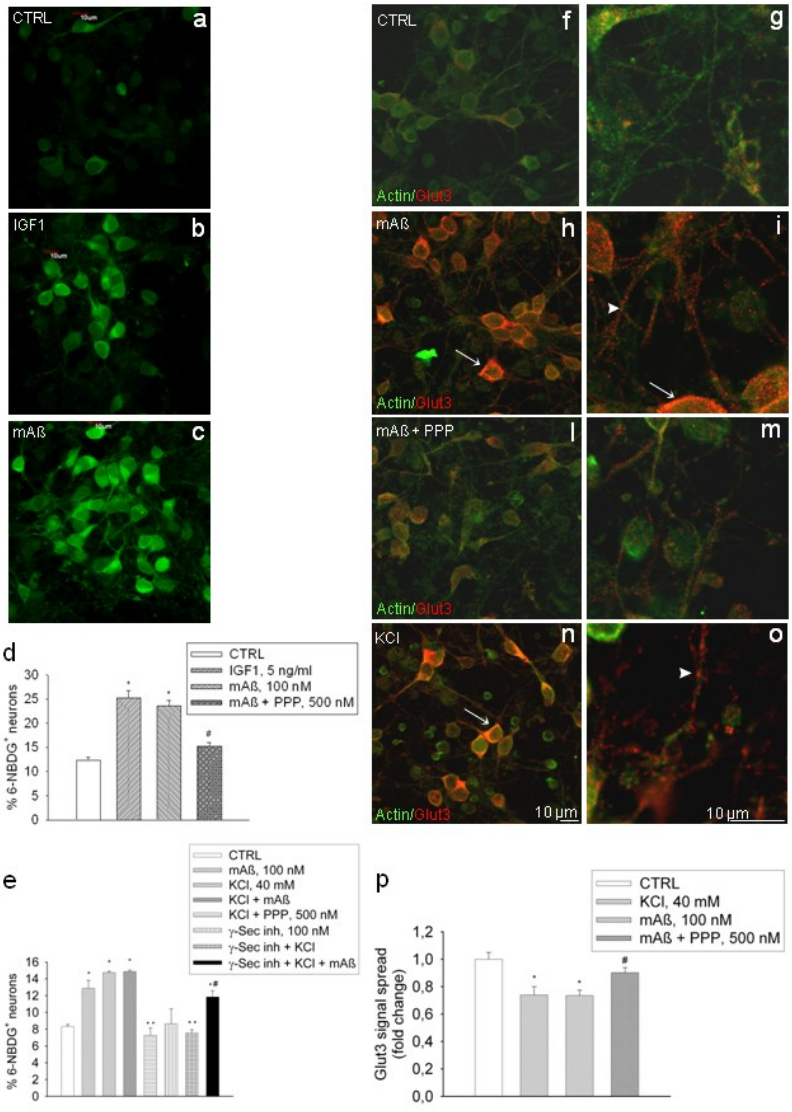
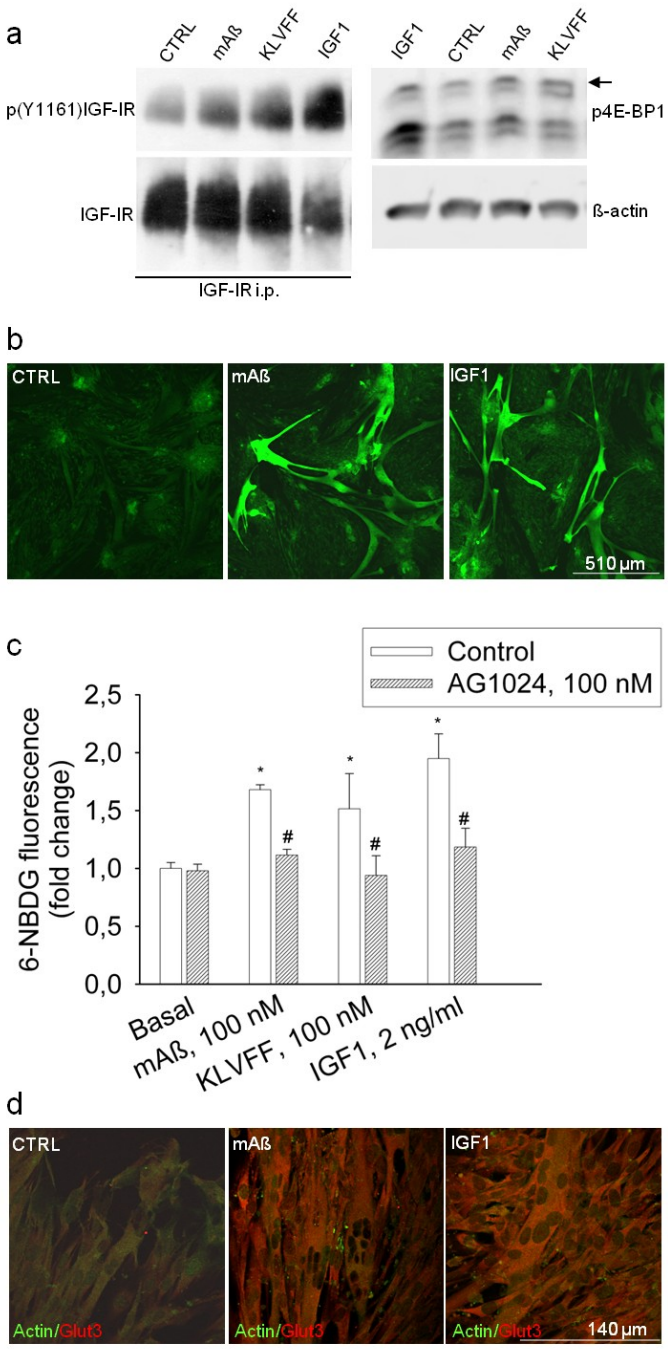

CTRL
a
IGF1
b
mAß
c
CTRL
f
g
Actin/Glut3
mAß
h
i
Actin/Glut3
mAß + PPP
l
m
Actin/Glut3
KCl
n
o
Actin/Glut3
10 µm
10 µm
d
% 6-NBDG+ neurons
CTRL
IGF1, 5 ng/ml
mAß, 100 nM
mAß + PPP, 500 nM
e
% 6-NBDG+ neurons
CTRL
mAß, 100 nM
KCl, 40 mM
KCl + mAß
KCl + PPP, 500 nM
γ-Sec inh, 100 nM
γ-Sec inh + KCl
γ-Sec inh + KCl + mAß
p
Glut3 signal spread (fold change)
CTRL
KCl, 40 mM
mAß, 100 nM
mAß + PPP, 500 nM

Fig 4


a
CTRL
mAß
KLVFF
IGF1
IGF1
CTRL
mAß
KLVFF
p(Y1161)IGF-IR
p4E-BP1
IGF-IR
ß-actin
IGF-IR i.p.
b
CTRL
mAß
IGF1
510 µm
c
Control
AG1024, 100 nM
6-NBDG fluorescence (fold change)
2,5
2,0
1,5
1,0
0,5
0,0
Basal
mAß, 100 nM
KLVFF, 100 nM
IGF1, 2 ng/ml
d
CTRL
mAß
IGF1
Actin/Glut3
Actin/Glut3
Actin/Glut3
140 µm

SUPPLEMENTARY INFORMATION

MONOMERIC ß-AMYLOID INTERACTS WITH TYPE-1 INSULIN-LIKE GROWTH FACTOR RECEPTORS TO PROVIDE ENERGY SUPPLY TO NEURONS

M. L. Giuffrida[1,9], F. M. Tomasello[2,9], F. Caraci[3], G. Pandini[4], G. Pappalardo[1], F. Attanasio[1], S. Chiechio[5], R. Vigneri[4], E.Rizzarelli[1,6], F. Nicoletti[7,8], A. Copani[1,5*]

SUPPLEMENTARY METHODS

Synthesis of pentapeptides

Pentapeptides (KLVFF, FKLVF, klvff, ffvlk) were synthesized by means of microwave-assisted solid phase peptide synthesis on a CEM "Liberty" peptide synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Peptides were cleaved off from the solid support using a mixture of Trifluoro-acetic acid (TFA)/water (H_2O)/tri-isopropyl-silane (TIS) 95/2.5/2.5 (v/v/v), then precipitated with cold freshly distilled diethyl ether. Crude peptides were purified by preparative RP-HPLC. Samples identity was confirmed by ESI-MS (Calculated mass for $C_{37}H_{55}N_7O_6$: 693.42; Observed $[M+H]^+$: 694.58).

To control for KLVFF effects, in addition to the retroinverse ffvlk, which maintains the overall spatial topology of KLVFF, we chose to synthesize both the scrambled peptide, FKLVF, and the D-enatiomer klvff. The latter is very likely to bind $Aß_{1-42}$ as the parent KLVFF[21], thus ruling out the possibility that KLVFF acts by stabilizing endogenous Aß monomers.

Peptide sample preparation

$Aß_{1-42}$ was purchased from Bachem Distribution Services GmbH, Germany. All peptides were dissolved in trifluoroacetic acid (TFA) at a concentration of 1mg/ml and sonicated for 10 min. TFA was removed by gentle streaming of argon. Peptides were then

dissolved in 1,1,1,3,3,3-hexa-fluoro-2-propanol (HFIP) and incubated at 37 ^{0}C for 1 h. Following argon streaming, peptides were dissolved again in HFIP, lyophilized and then resuspended in 5 mM anhydrous dimethyl sulfoxide (DMSO) prior to dilution to 100μM in ice-cold cell culture medium DMEM-F12.

Circular Dichroism measurements

CD spectra were recorded at 37 °C under a constant nitrogen flow on a JASCO model J-810 spectropolarimeter, equipped with a Peltier thermostatted cell holder. CD spectra were run in the far-UV region (200-260 nm) using 1 cm path length cuvettes. CD spectra were acquired every 30 min over a time course of 1200 min. Buffer contribution to the CD intensity was subtracted from peptide CD spectra.

Thioflavin T (ThT) fluorescence measurements

Fluorescence measurements were performed on a Perkin Elmer LS 55 spectrophotofluorimeter equipped with a thermostatic cell holder. The experiments were carried out at 37 °C using a 1 cm light path quartz. ThT (45μM) emission fluorescence was followed for 1200 min by monitoring the increase in the dye intensity at 480 nm, with a 440 nm excitation wavelength. The excitation and emission slit widths were set at 5 nm.

Rayleigh Scattering measurements

Rayleigh scattering measurements were performed on a Perkin Elmer LS 55 spectrophotofluorimeter at 37°C in a 1 cm path-length cell. Peptide samples were excited at 400 nm and scattering was

monitored for 1200 min at 400 nm. Both excitation and emission slits were fixed at 5 nm.

Detection of the Glut3 exofacial domain

Specific detection of the N-terminal extracellular domain of Glut3 was achieved by indirect immunofluorescence of non-permeabilized neurons, using a goat antibody raised against a peptide mapping within this region (1:25, Santa Cruz). Glut3 immunoreactivity was revealed with the donkey anti-goat IgG-Texas Red, Santa Cruz). Quantitative analysis was carried out by flow cytometry. Briefly, at the end of each treatment, neurons were harvested, washed once with PBS and pelleted. The cell pellet was fixed by incubation with 200 µl of 2% formaldehyde for 1h at RT. Fixed cells were blocked with 4% BSA, and stained by 2 h incubation at RT with the exofacial Glut3 antibody. Stained cells were then washed twice, and finally revealed by 1 h incubation with the secondary Texas Red-conjugated antibody. Immunostained samples were checked by cytofluorimetric analysis with a CyFlowML flow cytometer system (Partec). Neurons were excited by an air-cooled argon 488nm laser and Texas Red signal was read on FL3 detector. The data acquired (20,000 cells per sample) were compensated, gated and analysed using FlowMax software (Partec). Each experimental condition was repeated in triplicate.

Quantitative determination of $A\beta_{1-42}$ in neuronal culture supernatants

To mimic the experimental conditions under which 6-NBDG was observed, neuronal cultures were rinsed in glucose-free HCSS and

maintained for 30 min under glucose deprivation. KCl (40 mM) was added 15 min before ending the experiment. When required, □-sec-Inhibitor IX (100 nM) was added 2 h before glucose deprivation. $Aß_{1-42}$ was quantitated in the collected HCSS by using the Wako human/rat $Aß_{42}$ ELISA kit, high-sensitive. This kit detects human/rat $Aß_{x-42}$ in the 0.1-20 pmol/L range, with a sensitivity of 0.024 pmol/L; 100 µl of undiluted cell supernatants from equivalent cultures were used for the assay.

Assessment of IGF-1 release in neuronal cultures

IGF1-release in neuronal cultures was assessed by the IGFBP-blocked IGF-I ELISA (ALPCO Diagnostics). Cultures were exposed to the washing protocol utilized for all the experiments and IGF-1 content was quantitated in the culture buffer within 2 h. Low levels of IGF-1 were generally found under these conditions (about 0.003 nM). However, IGF-1 levels strictly depended on the experimental condition. For example, a brief NMDA pulse lead to a 5 fold increase in the release (up to 0.017 nM). Since the assay detected total IGF-1 (free and IGFBP-bound), measurements could not provide indications about the active quote of the factor.

Assessment of insulin release in pancreatic INS-1E cells

Rat INS-1E ß-cells were maintained in RPMI-1640 medium with 11.1 mmol/l D-glucose, supplemented with 10% FBS, 100U/ml penicillin, 100 µg/ml streptomycin, 10 nmol/l HEPES, 2nmol/l L-glutamine, 1nmol/l sodium pyruvate and 50 µmol/l β-mercaptoethanol. Cells were grown in T-75 flask at 37°c and 5% CO_2 and passaged every 5 days by using 0.05% trypsin-EDTA. For the experiments, cells

were plated onto 24-well plates at a density of 0.5x 10^6 cells/well and grown to 100% confluence. 18 h Before the experiment, growing medium was replaced with fresh medium containing 5mmol/l glucose. Insulin secretion assay was performed in HEPES balanced salt solution (HBSS) (114 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.16 mmol/l $MgSO_4$, 20mmol/l HEPES, 2.5 mmol/l $CaCl_2$, 25.5 mmol/l $NaHCO_3$, 0.2% bovine serum albumin), pH 7.2. Cells were washed and maintained in 3 mM glucose HBSS for 2 h before the switch in 15 mM glucose either in the absence or in the presence of m$Aß_{1-42}$ (100nM) and KLVFF (100nM). When required, PPP (500 nM) was added 15 min before the switch. The supernatant was collected 15 min after switching, and the total insulin content was determined by the use of rat/mouse Insulin Enzyme Linked Immunosorbent Assay (ELISA) kit (Millipore).

Intra-peritoneal glucose tolerance test (GTT)

CD1 male mice (33-35 g body weight) were housed up to five for cage and fasted for 16 h prior to the test. For the GTT, a solution of glucose (20% in 0.9% NaCl) was administered by intra-peritoneal (i.p.) injection (100 µl/10 g body weight) and blood glucose was measured at different time points during the following 4h. Blood glucose was obtained from the paw and measured by using the blood glucose meter One Touch Vita, J&J. Assuming a plasma volume of 1.5 ml/mouse, stock solution of $Aß_{1-42}$ monomers, Ac-KLVFF-NH_2 monomers or recombinant rat IGF-1 were prepared freshly in saline so to reach the plasma concentrations of 100 nM for monomers, and 2 ng/ml for IGF-1. After basal blood glucose measurements, where appropriate, monomers and IGF-1 were i.p. injected 5 min before glucose loading.

Animal care and experimentation was in accordance with institutional guidelines.

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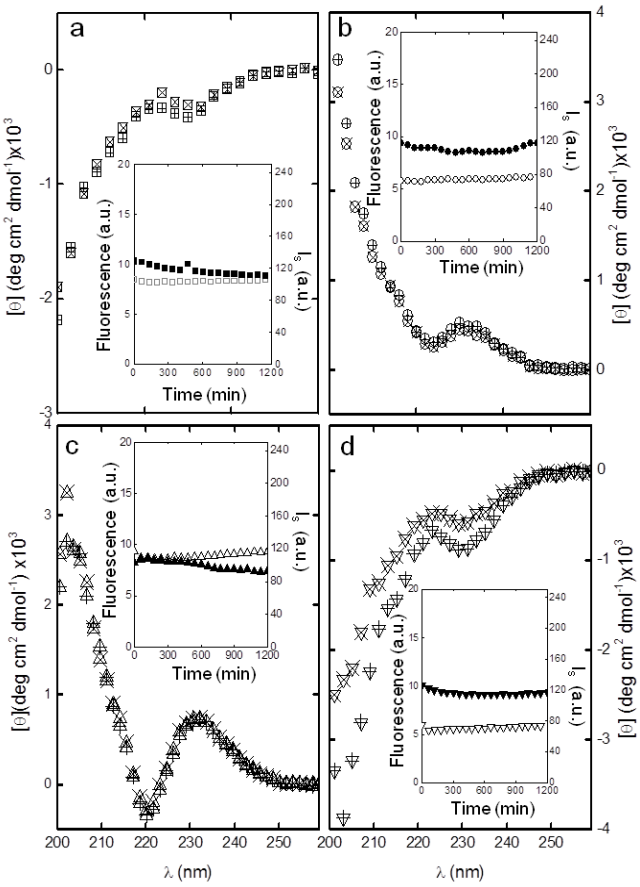
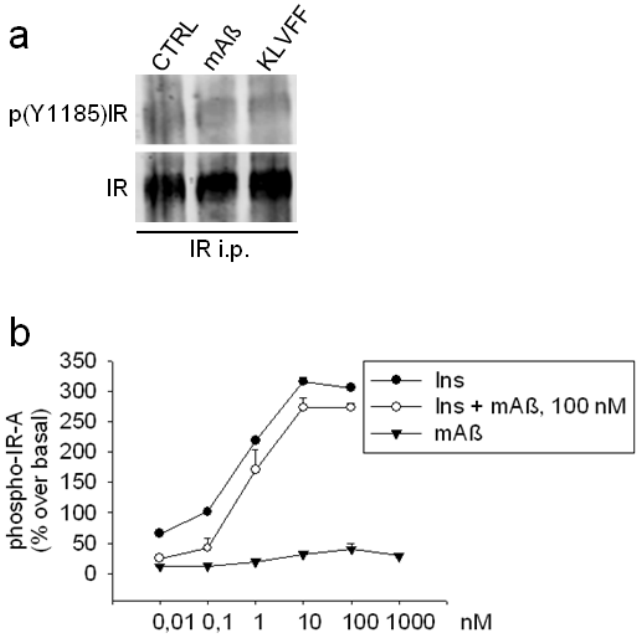
Supplementary Figure 1



Supplementary Figure 1. Pentapeptides maintained a monomeric, yet flexible conformational state overtime. Circular Dichroism spectra of (a) KLVFF (square), (b) klvff (circle), (c) ffvlk (triangle up) and (d) VFLKF (triangle down) peptide solutions, incubated at 37 °C, at t = 0 min (x symbol) and t = 1200 min (+ symbol), respectively. All the CD spectra exhibited dichroic bands (either positive or negative depending on the peptide stereo-configuration) around 200 - 230 nm, suggesting that the pentapeptides did not adopt any preferred secondary structure

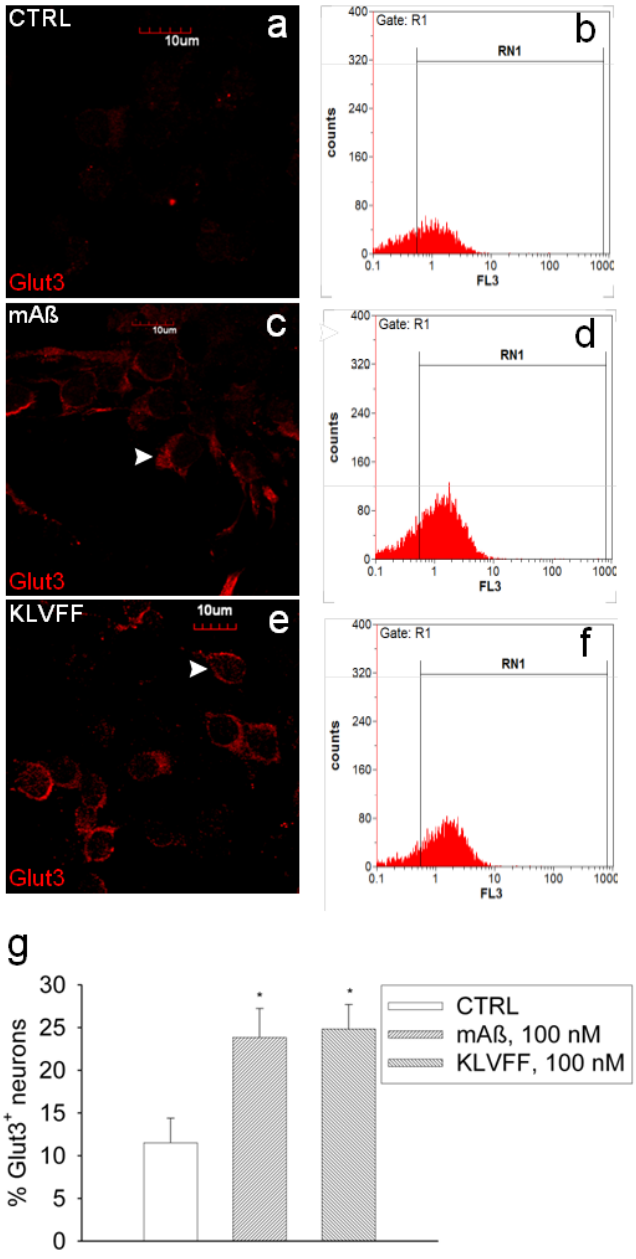
in aqueous solution. Inset: ThT fluorescence intensities (open symbol) at 480 nm and Rayleigh scattering intensities (filled symbol) at 400 nm as a function of time for peptide samples incubated at 37 °C. Both Th-T fluorescence and Rayleigh scattering measurements confirmed the un-aggregated condition and featureless conformational state of the peptide chains.

Supplementary Figure 2



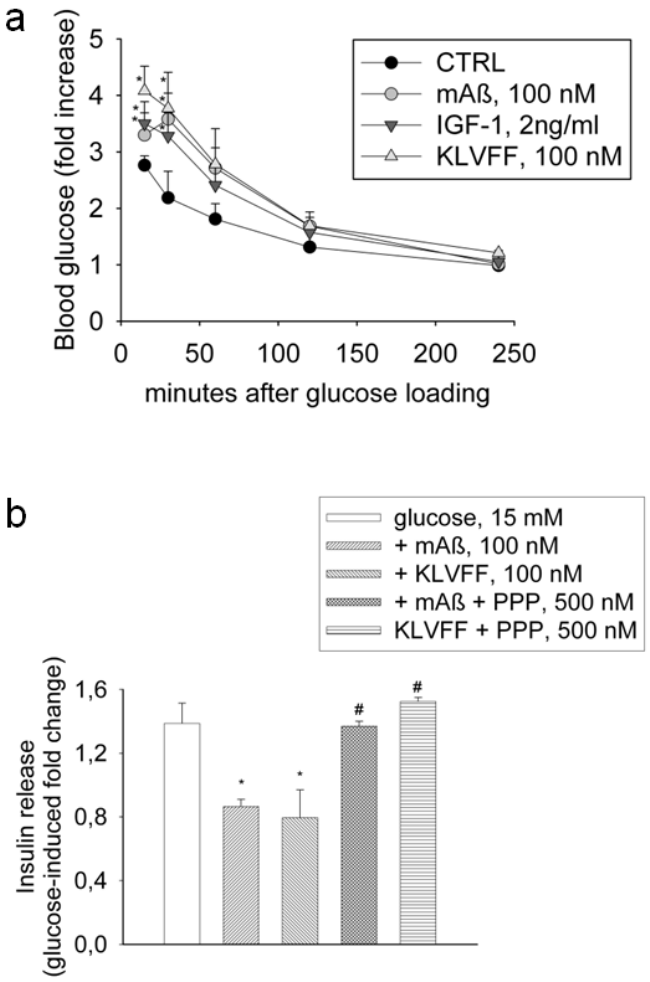
Supplementary Figure 2. $A\beta_{1-42}$ monomers did not activate insulin receptors. (a) Representative western blot analysis of immunoprecipitated IR beta subunit (IR i.p.) in neuronal extracts from control cultures (CTRL) or cultures exposed for 15 min to 100 nM of either monomeric $A\beta_{1-42}$ (mAß) or monomeric KLVFF peptide. Baseline levels of activated IR beta subunit, p(Y1185) IR, were unaffected. IR bands are shown as control for loading. (b) Autophosphorylation of immunocaptured human IR-A in response to insulin (Ins), $A\beta_{1-42}$ monomers (mAß) or a combination of both. Phosphorylation was quantitated by ELISA as described under Methods. Data are representative of two experiments and are expressed as % over basal receptor phosphorylation.

Supplementary Figure 3



Supplementary Figure 3. Monomers of $A\beta_{1-42}$ and Ac-KLVFF-NH_2 induced Glut3 translocation at neuronal plasma membrane. Confocal imaging of the N-terminal extracellular domain of Glut3 in adherent non-permeabilized neurons (a, c, e). Immunostaining was barely visible under control conditions (CTRL). Arrowheads point to plasma membrane profiles in neurons that were exposed to 100 nM of either monomeric $A\beta_{1-42}$ (mAß) (c) or Ac-KLVFF-NH_2 monomers (KLVFF) (e) for 10 min after glucose starvation. Scale bar = 10 µm. (b, d, f, g) Glut3 immunostaining carried-out in harvested non-permeabilized neurons and quantified by flow cytometry. Representative histograms displaying Glut3 fluorescence intensity (FL3) on the x-axis and the numer of events (counts) on the y-axis are shown for controls (b), mAß (d) and KLVFF (f). The percentage of positive neurons is gated under RN1. (g) Bars represent means $\pm$ S.E.M of three determinations. 20,000 Neurons/determination were acquired. *Significant at $p < 0.05$ vs. controls (CTRL) (one-way Anova + Fisher's LSD test).

Supplementary Figure 4



Supplementary Figure 4. Peripheral effects of $A\beta_{1-42}$ monomers. (a) $A\beta_{1-42}$ monomers transiently increased blood glucose levels in CD1 male mice undergoing a glucose tolerance test. This effect was mimicked by the Ac-KLVFF-NH_2 peptide and by IGF-1. Fasted mice were i.p. injected with either $A\beta_{1-42}$ monomers (mAß), KLVFF monomers or IGF-1 five minutes before glucose loading (2g/kg). Plots represent the fold increase of glucose levels over basal in 4 animals per experimental condition. *Significantly different from control (CTRL) at $p < 0.05$ (One-Way Anova + Fisher's LSD test). Data are from one experiment repeated three times with similar results. (b) $A\beta_{1-42}$ monomers reduced glucose-stimulated insulin release in pancreatic INS-1E cells. This effect was mimicked by the Ac-KLVFF-NH_2 peptide and was prevented by the IGF-IR antagonist, PPP. Data, representative of three experiments, are fold change of glucose-stimulated insulin release. Significantly different from the 15 mM glucose condition (*), or the respective controls (#) at $p < 0.05$ and (One-Way Anova + Fisher's LSD test).

Supplementary Table 1

Condition	**Aß$_{1-42}$ release (pmol/L)**
Control	3.593 ± 0.569
γ-Sec Inh, 100 nM	0.257 ± 0.068*
KCl, 40 mM	14.033 ± 1.486**
γ-Sec Inh + KCl	4.237 ± 0.917#

Supplementary Table 1. γ-Secretase inhibitor IX blocked the endogenous production of Aß$_{1-42}$ under both basal and depolarizing conditions. Values are means ± S.E.M. of three determinations from one representative experiment that was carried out under conditions similar to those utilized for the assessment of neuronal 6-NBDG uptake. γ-Secretase inhibitor was present in the cultures for 2 h before washing and shifting into a glucose-free buffer for 30 min. Glucose-free buffer collected after 30 min, in the absence of γ-sec inh., is

reported as control condition. Where indicated, KCl was added 15 min before collecting the buffer. $A\beta_{1-42}$ was quantitated in the collected buffer by using the Wako human/rat $A\beta_{42}$ ELISA kit, high-sensitive. Significant at *$p < 0.05$ and at **$p < 0.01$ vs. control, and at #$p < 0.01$ vs. KCl alone (one-way ANOVA + Fisher's LSD test).

GENERAL DISCUSSION

Since the discovery of Aβ peptide as the principal component of amyloid plaques, AD research has focused on events associated with Aβ production, aggregation and toxicity. Despite a great effort to clarify the mechanism(s) underlying toxic effects of Aβ, little is known about the biological function of this peptide. However, in order to develop effective therapeutics, a detailed molecular and cellular understanding of Aß function(s) is mandatory. The evidence that Aß is present into the brain and in the cerebral spinal fluid of normal individuals suggests that the peptide is physiologically active (Shoji, 2002). This is in line with our group's hypothesis that Aß(1-42), in normal small amounts that favor the persistence of the peptide in its non-toxic monomeric state, may have a physiological role in the brain (Giuffrida et al., 2010). Indirect evidence for a neuroprotective activity of Aß has been obtained in patients who underwent invasive intracranial monitoring after acute brain injury. The results of the study show a strong correlation between Aß levels in the cerebral interstitial fluid and the patient's neurological status, with increased Aß concentrations when the neurological status improves and fall when the neurological status declines (Brody et al., 2008). Mention should also be made of the older findings that the 1-28 fragment of the Aß peptide has a neurotrophic activity (Whitson et al, 1989), and that the endogenous production of Aß is essential for the survival of cultured neurons (Plant et al., 2003). In addition, since the pathology of AD seems to be mainly a result of synaptic dysfunction, understanding how Aβ production alters normal synaptic functions and what types of

synaptic functions are differentially affected by different Aβ species, becomes important in developing effective therapeutics for disease intervention.

This PhD thesis focuses on Aβ activity *in vitro*, with special attention to the relationship between the structure/aggregation state and the neurotoxic/biological activity. In **paper I** we have addressed the issue of Aβ toxicity, whereas in **paper II and III** the non-toxic form of Aß, the monomer, has been studied with respect to its function as a brain protective factor able to regulate synaptic activity and to activate the insulin/IGF-1 receptor signalling.

The majority of AD cases have a late onset and are sporadic, likely resulting from complex interactions of disease determinants with age-related risk factors (e.g., change of sex hormones (Barron et al., 2012)), the decline of insulin-like growth factor-1 (IGF-1) functions (Piriz et al., 2011) and systemic disease conditions such as hypercholesterolemia (Ledesma end Dotti, 2012) or diabetes (de la Monte, 2012). Data from AD brain (Shankar et al., 2009) transgenic APP over-expressing mice (Lesne et al., 2006) and neuronal cultures indicate that the self association of Aß(1-42) monomers into soluble oligomers is required for neurotoxicity (Pike et al., 1993). To date, many different mechanisms underlying the toxic effects of Aß have been proposed and are still being studied.

In **paper I** (Chapter I) we have addressed the neurotoxic activity of Aß in a particular model in which anabolic-androgenic steroid (AAS) sensitize neurons to the toxicity of Aβ oligomers. AAS abuse is in fact associated with multiple neurobehavioral disturbances

that might facilitate the onset or progression of neurodegenerative diseases (as AD) not usually linked to drug abuse.

In vitro, low concentrations of AASs amplify excitotoxic neuronal death (Orlando et al. 2007) and, in male normal volunteers, high doses of AASs induce cognitive impairment (Daly et al. 2003; Su et al. 1993). It has been proposed that activation of android receptors (ARs) may elicit opposite effects on cell survival depending on whether membrane ARs or intracellular ARs are activated (Gatson and Singh, 2007). In pure neuronal cultures, we found that, both testosterone-BSA and nandrolone-BSA, which preferentially target membrane associated ARs, were toxic with a greater potency than their parent compounds, indicating that AAS-related toxicity depends on the preferential activation of putative membrane ARs over intracellular ARs. Interestingly, activation of putative membrane ARs by low nanomolar concentrations of nandrolone-BSA did not itself lead to neuronal death, but was sufficient to increase neuronal susceptibility to the apoptotic stimulus provided by Aß.

Activation of glucocorticoid receptors GRs has also been shown to exacerbate a variety of neuronal insults, including excitotoxicity and Aß toxicity (Goodman et al., 1996). We also found that methandrostenolone was able to exacerbate Aß-induced toxicity at concentrations that were not toxic *per se*. Methandrostenolone-BSA, which likely binds membrane-associated GRs, was always more potent than the parent compound, suggesting that its toxicity relied on the preferential activation of putative membrane GRs over intracellular GRs. Recently, the activation of putative membrane-associated GRs has been shown to mediate rapid, non-genomic, effects able to

potentiate NMDA-evoked toxicity in hippocampal neurons (Xiao et al., 2010).

Overall in **paper I**, we have provided evidence that two AASs with a different pharmacological profile, namely nandrolone and methandrostenolone, can affect neuronal survival at suprapharmacologic doses, raising a serious concern for steoroid abusers, who have micromolar concentrations of AASs in their brain (Lukas, 1996; Wu, 1997). The relevant sites for the neurotoxic action of nandrolone and methandrostenolone appear to be membrane-associated androgen receptors (ARs) and membrane-associated-glucocorticoid receptors (GRs), respectively. Noteworthy, concentrations of the drugs that were not directly neurotoxic were, however, able to increase neuronal susceptibility to the apoptotic stimulus provided by Aß. Hence, *in vivo*, exposure to AASs may result in a compromised brain, more susceptible, later in life, to the onset or progression of diseases not usually linked to drug abuse, especially neurodegenerative diseases (e.g. Alzheimer's disease).

While Aß is recognized as the disease determinant with a pivotal role in inducing neuronal loss and dementia, an impaired brain insulin/IGF-1 signaling seems to account, in part, for the cognitive deficit associated with the disease. The origin of this defective signaling is uncertain. On one side, Aß oligomers have been proposed to be responsible for a down-regulation of neuronal insulin receptors; on the other side, our group has previously demonstrated that the non-toxic form of Aß, the monomer, is able to activate the insulin/IGF-1 receptor signaling and thus behaves as a neuroprotectant agent (Giuffrida et al., 2009); moreover, insulin/IGF-1 signalling has also

been reported to influence Aß metabolism (Cohen et al., 2006). Based on these findings, in **paper II** (Chapter II), we have advanced the hypothesis that the depletion of Aß monomers, occurring in the preclinical phase of Alzheimer's disease, might be the cause of early insulin/IGF-1 signaling disturbances that anticipate the cognitive decline.

Several studies point to an intriguing relationship between disturbances of glycemic control, cognitive impairment, and AD (Roriz-Filho et al, 2009). AD has been linked to peripheral hyperinsulinaemia with ensuing reduction in CNS insulin concentrations (Steen et al., 2005), a blunted neuronal insulin response (Wang et al., Soc. Neurosci. 2010, Abs. 725.2), and also to reduced levels of IGF-I (Messier and Teutenberg, 2005). All these disturbances might well be related to the pattern of reduced cerebral metabolic rate and cognitive impairment observed in early AD. However, both oral anti-diabetic agents, which acts primarily by increasing insulin sensitivity, and the potent inducer of IGF-1 secretion, MK-0677, have been ineffective at slowing the rate of progression of symptoms in AD patients (Tzimopoulou et al., 2010; Sevigny et al., 2008). These caveats suggest that factors other than insulin/IGF-1 might function to increase neuronal glucose uptake expecially during synaptic activity. In **paper II** we discuss the possibility that the function of Insulin/IGF-1 receptors might exceed the mediation of insulin/IGF-1 actions; we propose that an impairment of insulin/IGF-1 receptor signalling may contribute to AD via a disease-specific mechanism involving the loss of receptor activation by monomers of Aß. We suggest a possible model of interaction between the different Aß species and the IR/IGF-

1R system: accumulating Aß oligomers impair the insulin/IGF-1 signaling, which exacerbates Aß oligomerization and toxicity within a feed-foward mechanism. Aß monomers, by sustaining the insulin/IGF-1 signaling, promote survival, impede oligomerization and contribute to the homeostatic control of the system.

The relevance of the finding that Aß monomers are able to activate IRs/IGF-1Rs would be the evidence that the peptide is produced and released form neurons to sustain transient needs in synaptic modeling, neuronal energy provision and protection in the absence of brain insulin/IGF-l fluctuations. With the intention of uncovering a novel physiological role for Aß42, in **paper III** we have investigated whether Aß42 monomers function to regulate peripheral glucose homeostasis and/or neuronal glucose uptake.

In **paper III** (Chaper III) we show that the protective effect of Aß (1-42) is shared by the rat/mouse Aß(1-42), which is resistant to oligomerization. Because oligomerization of human Aß42 results into neurotoxicity rather than neuroprotection (Shivers et al., 1998), we investigated whether the 16-20 amino acid sequence of Aß42 (KLVFF), critically involved in Aß(1-42) oligomerization (Tjernberg et al., 1996), is involved in mechanisms of neuroprotection. We found that synthetic Ac-KLVFF-NH_2, maintained into a monomeric form, shared the protective activity of monomeric Aß42, and its action was prevented by the insulin/IGF receptor inhibitor, AG1024, or by the selective IGF-IR inhibitor, PPP. These results are indicative of a ligand-receptor interaction, and suggest that the KLVFF sequence of Aß(1-42) makes up the binding surface for IR and/or IGF-1R. To our knowledge, at present, data indicating whether Aß monomers bind

directly to IRs and/or IGF-1Rs are lacking, although evidence suggests that monomers have specific recognition sites on the neuronal surface (Giuffrida et al., 2009). Searching for a direct peptide-receptor interaction we found that monomers of Aß(1-42) or Ac-KLVFF-NH_2 stimulated Tyr-phosphorylation of native IGF-IR and potentiated the ability of IGF-1 to promote autophosphorylation of the receptor-kinase, with Aß42 displaying appreciable efficacy at IGF-IR by its own. Conversely Aß42 monomers *per se* failed to affect receptor response to insulin.

IGF-1 is known to stimulate glucose uptake in neurons by mechanism(s) similar to those used by insulin in the periphery, including membrane translocation of glucose transporters (GLUTs) (Bondy and Cheng, 2004). We reasoned that, Aß(1-42) monomers, which are released during neuronal transmission and activate many of the molecules within the insulin/IGF-I signaling pathway, might function to increase neuronal glucose uptake during synaptic activity. Consistent with its ability to engage IGF-IRs, monomeric Aß(1-42) increased glucose uptake in neurons. Thus, Aß(1-42) monomers diplayed IGF-1-like metabolic activity in cultured neurons. This evidence, together with the knowledge that Aß(1-42) is released from neurons in response to synaptic activity (Cirrito et al., 2005), provided the hint for testing the hypothesis that Aß(1-42) monomers might function to increase glucose uptake during neuronal activation. A depolarization pulse with KCl caused a significant increase in glucose uptake, prevented by PPP, meaning that endogenous activation of IGF-IRs is required for activity-dependent energy supply. Moreover, addition of β-secretase inhibitors, to block the endogenous production

Aß42, blunted depolarization-induced glucose uptake, which was re-established by the exogenous application of Aß42 monomers.

Glucose transport in neurons is mediated by the membrane transporters, Glut3, Glut4, and Glut8. Both IRs and IGF-1Rs colocalize with the insulin-sensitive GLUT4 (McEwen and Reagan, 2004), which is expressed primarily in neuronal cell bodies and it is likely to fulfill the metabolic requirements of neurons under basal conditions. The GLUT3 transporter, which is found in the neuropil, is considered to be insulin-insensitive. Although, insulin is able to promote the translocation of GLUT3 under neuronal depolarizating conditions (Uemura and West Greenlee, 2006), evidence for insulin/IGF-1 level fluctuations in parallel with synaptic activity is missing. Here we found that K^+-induced depolarization and Aß (1-42) monomers increased Glut3 immunoreactivity in neuronal threads and axon hillocks, an effect prevented by PPP.

To strengthen the evidence that monomeric Aß (1-42) has IGF-1-like activity, we extended the analysis to classical peripheral actions of IGF-1. Studies addressing this issue might clarify the puzzling relationship between fluctuations of plasma Aß42 levels and either glucose loading (Takeda et al., 2009), or insulin injection (Fishel et al., 2005), and might provide a novel possible explanation for the relationship between insulin resistance and AD (de La Monte and Wands, 2005). L6 rat skeletal muscle cells show a prevalence of IGF-IRs over IRs (Beguinot et al., 1985) and also express Glut3 (Bilan, et al., 1992). In differentiated L6 myotubes both monomeric Aß42 and Ac-KLVFF-NH_2 stimulated IGF-IR signaling and glucose uptake. Regulation of peripheral glucose uptake depends mainly on insulin,

whereas IGF-1 is required for normal insulin sensitivity (Clemmons, 2004), and also for proper basal and glucose-regulated insulin secretion (Kulkarni et al., 2002). Physiological concentrations of IGF-1 are, indeed, known to inhibit insulin secretion from pancreatic ß cells (Van Schravendijk et al., 1990). We found that, similarly to IGF-1, monomers of Aß (1-42) and Ac-KLVFF-NH_2 caused a transient increase in blood glucose levels in mice undergoing a glucose tolerance test, which reflects the inhibition of insulin secretion. This was confirmed by using INS-1E insulinoma cells, in which monomers of Aß (1-42) and Ac-KLVFF-NH_2 inhibited glucose-stimulated insulin secretion. Hence, monomeric Aß (1-42) and its functional epitope, KLVFF, exhibited IGF-1-like effects in different cell types.

Overall, the results reported in **paper III** provide evidence that monomeric Aß(1-42) acts as a positive allosteric modulator of IGF-IR endowed with intrinsic agonist-like activity. This activity serves to meet the neuronal need of glucose. Hence, glucose uptake during neuronal activation might relay on naturally secreted monomers of Aß protein acting on IGF-IRs. Depletion of Aß monomers in the preclinical phase of AD, resulting from pathological Aß aggregation, could be responsible for early defects of insulin/IGF-1 receptor signaling (including the deficit of glucose metabolism that anticipates cognitive decline), thus participating to overall AD pathology.

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PUBBLICATIONS DURING THE PHD PROGRAMM

1. Caraci F, Pistarà V, Corsaro A, **Tomasello F**, Giuffrida ML, Sortino MA, Nicoletti F, Copani A. Neurotoxic properties of the anabolic androgenic steroids nandrolone and methandrostenolone in primary neuronal cultures. J Neurosci Res. 2011 Apr;89(4):592-600. doi: 10.1002/jnr.22578. Epub 2011 Feb 2. PMID: 21290409.

2. Giuffrida ML, **Tomasello F**, Caraci F, Chiechio S, Nicoletti F, Copani A. Beta-Amyloid Monomer and Insulin/IGF-1 Signaling in Alzheimer's Disease. Mol Neurobiol. 2012 Dec;46(3):605-13. doi: 10.1007/s12035-012-8313-6. Epub 2012 Aug 12. PMID:22886436.

3. **F. M. Tomasello**, M. L. Giuffrida, F. Caraci, G. Pandini, G. Pappalardo, F. Attanasio, S. Chiechio, R. Vigneri, E.Rizzarelli, F. Nicoletti, A. Copani. Monomeric β-amyloid interacts with type-1 Insulin-like growth factor receptors to provide energy supply to neurons. Submitted to Nature Neuroscience.