Linköping University Medical Dissertation No. 1288

# **Neuroprotective Effect of Genistein**

Studies in Rat Models of Parkinson's and Alzheimer's Disease

Maryam Bagheri



# Linköping University FACULTY OF HEALTH SCIENCES

Department of Clinical and Experimental Medicine, Linköping University, SE-581 85, Linköping, Sweden

Linköping 2012

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Cover illustration shows the hippocampus of a rat with  $A\beta_{1\text{-}40}$  injection.

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Printed by LiU-Tryck, Linköping, Sweden, 2012.

ISBN: 978-91-7519-984-9 ISSN: 0345-008

To Sajjad

## LIST OF PAPERS

This thesis includes the following papers:

Paper I.

Baluchnejadmojarad T, Roghani M, Nadoushan MR, Bagheri M.

Neuroprotective effect of genistein in 6-hydroxydopamine hemi-parkinsonian rat model.

Phytotherapy Research, 2009; 23(1):132-5.

Paper II.

Bagheri M, Joghataei MT, Mohseni S, Roghani M.

Genistein ameliorates learning and memory deficits in amyloid beta (1-40) rat model of Alzheimer's disease.

Neurobiology of Learning and Memory, 2011; 95(3):270-6.

Paper III.

Bagheri M, Roghani M, Joghataei MT, Mohseni S.

Genistein inhibits aggregation of exogenous amyloid-beta1-40 and alleviates astrogliosis in the hippocampus of rats.

Brain Research, 2012; 1429:145-54.

Paper IV.

Bagheri M, Rezakhani A, Roghani M, Joghataei MT, Mohseni S.

Genistein inhibits  $A\beta_{1-40}$  induced astrogliosis – A three dimensional confocal morphometric study.

Submitted

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### ABSTRACT

Parkinson's disease (PD) and Alzheimer's disease (AD) are neurodegenerative disorders that mainly affect the elderly population. It is believed that oxidative stress is involved in development of both these diseases and that estrogen deficiency is a risk factor for development of AD. Genistein is a plant-derived compound that is similar in structure to estrogen and has anti-oxidative properties. The general objective of the present research was to evaluate the effects of genistein on neurodegeneration in rat models of PD and AD.

Using a rat model of PD, we found that a single intraperitoneal dose of genistein 1 h before intrastriatal injection of 6-hydroxydopamine (6-OHDA) attenuated apomorphine-induced rotational behavior and protected the neurons of substantia nigra pars compacta against 6-OHDA toxicity.

To produce an animal model of AD, we injected  $A\beta_{1-40}$  into the hippocampus of rats. Using groups of these  $A\beta_{1-40}$ -lesioned animals, the involvement of estrogen receptors (ERs) was evaluated by intracerebroventricular injection of the estrogen receptor antagonist fulvestrant, and the role of oxidative stress was studied by measuring levels of malondialdehyde (MDA), nitrite, and superoxide dismutase (SOD) activity. The results showed that intrahippocampal injection of  $A\beta_{1-40}$  caused the following: lower spontaneous alternation score in Y-maze tasks, impaired retention and recall capability in the passive avoidance test, and fewer correct choices and more errors in a radial arm maze (RAM task), elevated levels of MDA and nitrite, and a significant reduction in SOD activity in the brain tissue. Furthermore, hippocampus in theses rats exhibited  $A\beta_{1-40}$  immunoreactive aggregates close to the lateral blade of the dentate gyrus (DGlb), extensive neuronal degeneration in the DGlb, high intracellular iNOS<sup>+</sup> and nNOS<sup>+</sup> immunoreactivity, and extensive astrogliosis.

Genistein pretreatment ameliorated the A $\beta$ -induced impairment of short-term spatial memory, and this effect occurred via an estrogenic pathway and through attenuation of oxidative stress. Genistein also ameliorated the degeneration of neurons, inhibited the formation of A $\beta_{1-40}$ -positive aggregates, and alleviated A $\beta_{1-40}$ -induced astrogliosis in the hippocampus.

## SAMMANFATTNING

Parkinsons och Alzheimers sjukdom är de vanligaste hjärnsjukdomarna hos äldre. Hög ålder och ärftlighet är riskfaktorer för båda sjukdomarna och man tror att det kvinnliga könshormonet östrogen har en skyddande effekt. Genistein är en substans som utvinns ur växter och finns exempelvis i soja. Det har en struktur som liknar den hos östrogen. I denna studie undersökte vi huruvida behandling med genistein kunde minska beteendemässiga och strukturella störningar i djurmodeller för Parkinsons och Alzheimers sjukdom. Vi använde oss av en råttmodell av Parkinsons sjukdom i vilken toxinet 6-hydroxydopamin injiceras i en viss del av hjärnan. För att efterlikna Alzheimers sjukdom injicerade vi amvloid-beta i hjärnan på råttor eftersom ackumulering av amyloid-beta tros vara huvudorsaken till skadorna vid Alzheimers sjukdom. Djuren gavs en hög dos genistein en timme innan operationen och vi studerade sedan vilka effekter genistein hade på minnesfunktion, hjärnstruktur och inflammation i dessa modeller. I Parkinson-modellen räknade vi hur många rotationer råttorna utförde samt hur många celler som fanns i specifika delar av hjärnan två veckor efter operationen. Antalet rotationer ökade signifikant och antalet celler minskade markant. Genistein minskade ökningen i antalet rotationer och skyddade delvis nervcellerna mot 6hydroxydopamin. Djurmodellen för Alzheimers sjukdom hämmade inlärning och minne i olika beteendetest. Förbehandling med genistein lindrade störningarna i korttidsminnet genom att påverka östrogensystemet och minska bildandet av kroppsegna toxiska ämnen. Vidare tycktes genistein hämma den inflammatoriska reaktionen i hjärnan. Vi drar slutsatsen att genistein kan lindra funktionella och strukturella störningar i råttmodeller för Parkinsons och Alzheimers sjukdom.

## ABBREVIATIONS

Αβ	amyloid beta
AChE	acetylcholinesterase
AD	Alzheimer's disease
APP	amyloid precursor protein
BACE1	beta-site amyloid precursor protein-cleaving enzyme 1
BACE2	beta-site amyloid precursor protein-cleaving enzyme 2
BDNF	brain-derived neurotrophic factor
Cr-EL	Cremophor-EL
COX	cyclooxygenase
DAPI	4,6-diamidino-2-phenylindole
DGlb	lateral blade of dentate gyrus
DGmb	medial blade of dentate gyrus
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
GFAP	glial fibrillary acidic protein
IL	interleukin
iNOS	inducible nitric oxide synthase
MDA	malondialdehyde
MAPK	mitogen-activated protein kinase
MAO-B	monoamine oxidase B
NFκB	nuclear factor kappa light-chain-enhancer of activated B
	cells
NFT	neurofibrillary tangle
NMDA	N-methyl D-aspartate
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NSAID	non-steroidal anti-inflammatory drug
PD	Parkinson's disease
РКА	protein kinase A
PSI	gene encoding the protein presenilin 1
PS2	gene encoding the protein presenilin 2
RAM	radial arm maze
ROS	reactive oxygen species
sAPP	soluble amyloid precursor protein
STL	step-through latency
SNC	substantia nigra pars compacta
SOD	superoxide dismutase
ΤΝFα	tumor necrosis factor alpha
6-OHDA	6-hydroxy dopamine
3D	Three-dimensional

## INTRODUCTION

#### Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, and it was named after the British surgeon James Parkinson, who published the first detailed description of six cases of shaking palsy in 1817 (Parkinson, 1817). PD is clinically characterized by motor symptoms such as resting tremor, bradykinesia and rigidity of skeletal muscle, postural instability, stooped posture, and freezing of gait. Furthermore, patients with this disease can show non-motor symptoms including cognitive and behavioral problems, as well as sensory impairment, and they may also suffer from sleep disorders or autonomic dysfunction (Chaudhuri and schapira, 2009). In industrial countries, PD has a prevalence of approximately 0.3% in the general population and affects about 1% of those older than 60 (de Lau and Breteler, 2006). This disease rarely occurs before the age of 50, and men are at higher risk than women. In Europe, PD affected 1.2 million people in 2010, resulting in costs per patient of EUR 5,626 for direct health care and EUR 4,417 for non-medical care. In 30 European countries, the total cost of all care for patients with PD in 2010 was EUR 13.9 billion (de Lau and Breteler, 2006).

#### Hallmarks of Parkinson's disease

Multiple neuronal systems are affected in PD, but the basic clinical motor symptoms mentioned above result primarily from severe loss of dopaminergic neurons in the substantia nigra pars compacta (SNC) of the basal ganglia. This decline is accompanied by the presence of intraneuronal inclusions called Lewy bodies, which are composed largely of  $\alpha$ -synuclein, a protein that is found chiefly in presynaptic terminals and plays a key role in vesicular release of neurotransmitters, axonal transport, and mechanisms of autophagy (Ben Gedalya et al., 2009; Koprich et al., 2011; Perez et al., 2002). The occurrence of Lewy bodies is one of the criteria used to diagnose PD, and gliosis in the substantia nigra (SN) is also often observed in the brain of patients affected by this disease. The non-motor symptoms are related to a general degeneration of noradrenergic, cholinergic, or serotonergic neurons in different parts of the brain.

#### **Risk factors**

Both genetic and non-genetic factors can contribute to an increased or decreased risk of PD.

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#### Genetic factors

A positive family history of PD is correlated with a higher risk of incidence of the disorder, and 5–10% of patients with clinical signs of PD carry mutations in genes associated with the disease. A mutation in the gene encoding α-synuclein was the first gene-related mechanism that was suggested to underlie the initiation of PD. Today, 16 loci designated PARK1 to PARK16 and 11 genes on different chromosomes are known to be associated with a higher risk of PD (for review, see Corti et al., 2011). Mutations in these loci affect the expression of several proteins, such as ubiquitin ligase, UCHL-1, DJ-1, PTEN-induced kinase, dardarin, and nuclear receptor, which are involved in protection against oxidative stress, mitochondrial dysfunction, and survival of dopaminergic cells (Devine et al., 2011).

#### Non-genetic factors

*Occupational exposure* to toxins and heavy metals increase the risk of PD. In 1983, many people exhibited typical signs of PD after taking an opioid called Desmethylprodine or MPPP (1-methyl-4-phenyl-4-propionoxypiperidine), and it was discovered that the drug was contaminated with N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) during manufacturing. This observation led to the finding that MPTP selectively damages dopaminergic neurons in the SNC, which in turn resulted in the hypothesis that some environmental toxins can increase the risk of developing PD. Since that time, numerous studies have been performed to examine the role of other environmental factors in the pathogenesis of this disease. Today, we know that exposure to agricultural chemicals (e.g., the pesticide rotenone and the herbicide paraquat) is associated with a higher risk of developing PD, because these substances have harmful effects on dopaminergic neurons (Betarbet et al., 2000; de Lau and Breteler, 2006). The risk of PD has also been reported to be greater after exposure to certain heavy metals, including iron, manganese, zinc, and copper, presumably because these elements induce oxidative stress, which in turn causes dopaminergic neuronal depletion in the SNC (Lai et al., 2002; Tanaka et al., 2011).

*Smoking* decreases the risk of PD, and several hypotheses have been proposed to explain the neuroprotective effect of this practice. For example, it has been suggested that nicotine, the chief constituent of tobacco, can stimulate the release of dopamine, act as an antioxidant, and alter the activity of monoamine oxidase B activity (MAO-B) (Heman et al., 2000).

*Coffee consumption* has been found to be inversely related to the risk of developing PD (Ross et al., 2000). The active component of coffee is caffeine, which is an adenosine  $A_2$  inhibitor that improves motor deficits in mouse models of PD. Interestingly, the effect of caffeine is stronger in men than in women, because estrogen is a competitive inhibitor of caffeine (Ross et al., 2000).

*Fat and fatty acids* consumed daily in large amounts have been shown to be associated with greater incidence of PD. It is plausible that a high lipid content increases peroxidation of lipids and thereby raises levels of oxygen radicals, which are harmful to neurons. Accordingly, scientists have focused more attention on the neuroprotective effect of unsaturated fatty acids (de Lau et al., 2005).

*Homocysteine* is an amino acid that is synthesized by cells in the body and may have a toxic effect on neurons and accelerate cell death in general. Therefore, recent investigations have examined the relationship between development of PD and higher intake of vitamin B, a substance that is associated with lower plasma levels of homocysteine (de Lau and Breteler, 2006). Some, but not all, of these studies demonstrated that high consumption of vitamin B6 is correlated with a decreased risk of PD, whereas no such impact was found for vitamin B12 or folate (Murakami et al., 2010).

*Mitochondrial dysfunction and increased oxidative stress* may also play an essential role in the pathogenesis of PD (for review, see Henchcliffe and Beal, 2008). Oxidative stress to lipids, proteins, and DNA, and also reduced levels of the antioxidant glutathione have been observed in post-mortem brain samples from individuals with PD. Antioxidants such as vitamins E and C can protect dopaminergic cells against free radicals, although it seems that the influence of these agents is more pronounced during very early stages of the disease (Devore et al., 2010).

*Inflammatory cytokines* and activated glial cells have been detected in clinical samples from patients with PD, which suggests that inflammatory mechanisms are involved in pathogenesis of the disease. Several investigations have shown that use of non-steroidal anti-inflammatory drugs (NSAIDs) decreases the risk of PD. According to Wahner et al. (2007), both aspirin and non-aspirin NSAID users are less likely to contract PD. However, Ton et al. (2006) conducted a clinical study of 206 patients with newly diagnosed idiopathic PD and 383 randomly

selected controls exposed to anti-inflammatory drugs, and these authors found only limited support for the hypothesis that use of aspirin can reduce the risk of PD and no data indicating that other NSAIDs offer such protection. In agreement with this finding, a large case-control study including 22,007 physicians aged 40–84 years provided no evidence that NSAIDs can reduce the risk of PD (Driver et al., 2011).

*Estrogen* deprivation may cause the death of dopaminergic neurons, and there is evidence to suggest that the low levels of estrogen present in men can explain why the incidence of PD is higher in men than in pre-menopausal women. This female sex hormone somehow protects neurons against degeneration. According to the Parkinson Study Group POETRY investigators (Investigator PSGP, 2011), estrogen replacement therapy in post-menopausal women with PD may be associated with improvement in motor symptoms. The neuroprotective effect of this hormone probably occurs through antioxidant mechanisms and interactions with growth factors (e.g., insulin-like growth factor 1). Estrogen also activates cascades of signaling molecules, such as the phosphatidylinositol-3 kinase/Akt and mitogenactivated protein kinase (MAPK) pathways (Bourque et al., 2009).

#### Evaluation of drugs

Currently, treatment of patients diagnosed with PD is restricted to relief of symptoms, because, unfortunately, attempts to prevent initiation or progression of the disease have failed. In as much as dopamine deficiency leads to development of symptoms of PD, most treatment strategies have been focused on restoration of dopamine activity and the mechanisms related to the metabolic pathways that include this catecholamine.

*Levodopa or L-dopa* is a dopamine precursor that has long been considered to be the gold standard drug for treatment of PD. L-dopa can improve motor function, daily activities, and quality of life in PD patients, whereas other non-motor symptoms such as postural instability, freezing, mood and sleep disorders, autonomic dysfunction, and dementia do not respond to this drug. Sadly, chronic treatment with L-dopa is also associated with some motor complications, motor fluctuation, and dyskinesia, and hence there is an urgent need to find new drugs to treat this disease.

*Dopamine agonists* can directly stimulate the postsynaptic receptors in the striatum. This category of drugs includes two basic groups: ergot derivative (bromocriptine) and non-ergot dopamine agonist (pramipexole). Side effects of dopamine agonists include hallucination, sleepiness during daytime, and compulsive disorders (Kalinderi et al., 2011). Furthermore, the frequency of valvulopathy, which entails fibrosis of the heart-valve resulting in thickening, retraction, and stiffening of a heart valve, is higher in patients receiving ergolinic dopamine agonists (reviewed by Antonini and Poewe, 2007).

*Catechol-O-methyltransferase inhibitors* block the enzyme that catalyzes conversion of Ldopa to 3-omethyl-dopa, and thus they prolong the action of L-dopa in the brain. The most serious side effect of this category of drugs is the potential to induce hepatic toxicity (reviewed by Kalinderi et al., 2011).

*MAO-B inhibitors* can slow the catabolism of dopamine and thus improve the symptoms in PD patients (reviewed by Kalinderi et al., 2011).

*Anticholinergic agents* can maintain the balance between dopamine and acetylcholine activity in the striatum, and their most important feature is a beneficial effect on tremor. Today, use of these drugs is limited, especially in the elderly, due to side effects on the central and peripheral cholinergic systems. In a recent cohort study, Ehrt et al. (2010) found that cognitive decline was more pronounced in patients who had been taking drugs with anticholinergic activity over the 8-year follow-up period.

*Stem cell therapy* has received much attention from the scientific community over the past 20 years. The aim of such treatment is to replace the lost dopamine-producing neurons with new cells taken from fetal ventral midbrain tissue. However, clinical trials have had varying degrees of success, and thus this technique must be further developed before it can be deemed appropriate for use in patients (for a recent review, see Brundin et al., 2010). Also, the main concern in this context is indeed the ethical aspects of using human fetal tissue.

As discussed above, various drugs are used to treat PD, but all of these agents are aimed at ameliorating the symptoms, and none of them can cure the disease. The lack of effective therapy causes immense suffering for the patients and their families, and hence there is hope that scientists will soon develop a drug that can successfully combat this debilitating disorder.

#### Animal models of Parkinson's disease

Animal models represent potential tools in our attempts to understand the pathophysiology of PD, and such systems have played an important role in the development of new treatment strategies.

*Pharmacologic animal models* of selective damage of dopaminergic neurons have been used for many years in PD research, and 6-hydroxy dopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, paraquat, lipopolysaccharide, and manganese are the toxins that have been applied most widely in rats, mice, and monkeys (Klivenyi and Vecsei, 2011). These toxins can induce mitochondrial dysfunction, which leads to energy deficits, oxidative stress, and finally neuronal degeneration in specific parts of the brain. The compound 6-OHDA is structurally similar to dopamine and norepinephrine, and binds to the plasma membrane transporters of these catecholamines. Furthermore, 6-OHDA does not cross the blood brain barrier, but, when injected into the brain, it kills neurons containing dopamine and norepinephrine by producing hydrogen peroxidase (Javoy et al., 1976). The 6-OHDA animal model is particularly useful for evaluating the effects of new drugs on motor skills. The concentration of toxins, the type of vehicle, and the methods of administration employed can vary according to the animal species used. In general, pharmacological animal models are reproducible and have made important contributions to our current knowledge about PD.

*Transgenic animals* have been and are being used extensively in attempts to produce models of PD exhibiting pathology close to that observed in humans. In most cases, a group of mice are genetically engineered to develop loss of dopaminergic neurons in the SN. Another group of transgenic animals has been created that has mutations in genes related to a familiar form of PD, and a third model was developed based on virally expressed genes in the SN (for review, see Meredith et al., 2008). For example, a mouse model that carries a double-stranded mitochondrial DNA (mt-DNA) break and is deficient in oxidative phosphorylation has been produced through expression of mitochondria-targeted restriction enzyme PstI or mito-PstI (Pickrell et al., 2011). This model has most of the features of PD, including motor dysfunction and degeneration of dopaminergic neurons in the SN, and it enables evaluation of the role of mitochondria in the pathophysiology of the disease. Genetically engineered mice have also been used to develop two models that generate progressive neuronal loss in the SNC. Furthermore, there are Pitx3 -/- mice with a spontaneous mutation in the homeobox transcription factor Pitx3, and engrailed knockout mice with SNC neuronal loss accompanied

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by cerebellar pathology (Meredith et al., 2008). In addition, mutations in genes encoding the proteins  $\alpha$ -synuclein, parkin, DJ1, and LRRK2 have been used to create models of the familial form of PD. Animals with a mutation in  $\alpha$ -synuclein primarily display the symptoms of the disease and do not exhibit neuronal loss, and thus, disappointingly, they are not very useful for investigating PD. Mutations in the gene encoding parkin can cause proteasomal dysfunction and induce early onset of familial PD. In mice and flies, mutations in the genes for DJ1 can lead to decreased cell resistance to oxidative stress but not loss of cells. Mutations in LRRK2 can elicit late onset of familial PD, and a transgenic mouse model comprising these aberrations is currently being developed (Meredith et al., 2008).

*Viral-based animal models* can be obtained by acute delivery of virally expressed genes such as recombinant adeno-associated virus (rAAV) into the SN, and these animals often exhibit neuronal loss. For example, animals with overexpression of  $\alpha$ -synuclein show neuronal loss as well as behavioral deficits. Thus, these models are more useful than other engineered mouse models, if the goal is to acquire animals with the hallmarks of PD (Dehay and Bezard, 2011; Meredith et al., 2008).

The nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* have also been used to elucidate the cellular and molecular pathways involved in different forms of familial PD. *Drosophila melanogaster* can show duplication or triplication of the  $\alpha$ -synuclein gene, which makes these flies a good model for investigating synucleinopathies. The disadvantage of these two invertebrate species is that they do not produce Lewy bodies, which are the predominant feature of PD in humans (Meredith et al., 2008).

It can be mentioned that none of the animal models described above express the genotype and/or phenotype observed in humans. For example, most genetically engineered mice do not exhibit the neuronal death in the SN that is the main hallmark of PD in human. The models based on delivery of virally-expressed genes into the SN do cause neurodegeneration but only locally in the SN, and they do not produce extra-nigral pathology, which is seen during progression of the disease in humans. Furthermore, many of these models lack Lewy bodies.

#### Alzheimer's disease

Alzheimer's disease (AD) was first described by Alois Alzheimer more than a century ago in Germany, and it constitutes one of the most common causes of senile dementia. According to a recent estimation, it is possible that almost 80% of individuals with dementia suffer from AD (Bi, 2010; Jellinger and Attems, 2010). AD refers to a clinical syndrome that occurs in the elderly and is severe enough to interfere with social and occupational activities. At least two clinical abnormalities are essential for diagnosis of the disease, namely, memory loss in an alert person and impairment of one or more of the following functions: language, attention, perception, judgment or problem solving (Förstl and Kurz, 1999).

AD is a severe progressive neurodegenerative brain disorder that affects approximately 5% of the population older than 65 years (Shah et al., 2008). According to the US Centers for Disease Control and Prevention (2003), the number of people in the world who are over the age of 65 will increase to around 1 billion by 2030. It has also been projected that by 2050 the number of dementia cases will reach around 14 million in Europe (Mura et al., 2010) and 13.2 million in the United States (Hebert et al., 2001). Furthermore, it has been estimated that the annual incidence of AD in the United States will increase from the 337,000 cases recorded in 1995 to 959,000 cases in 2050 (Hebert et al., 2001). At the level of individuals, AD decreases the quality of life and shortens life expectancy. At the societal level, the long-term care of AD patients in nursing homes is an economic challenge in Western countries, as illustrated by a report in which Olesen and colleagues (2012) showed that in Europe the annual cost for patients with dementia was EUR 105.2 billion in 2010. The mentioned date certainly indicate the tremendous impact of AD in terms of the enormous number of patients with this disease, the pressure on their relatives, and the negative socioeconomic consequences. In short, it can be said that AD is one of the major public health problems in the world.

#### Hallmarks of Alzheimer's disease

Amyloid beta (A $\beta$ ) plaques, neurofibrillary tangles (NFTs), hyperphosphorylated tau protein, and neuronal loss occurring in the brain tissue are considered to be the specific histopathological hallmarks of AD. The A $\beta$  plaques, also called senile plaques, are composed chiefly of extracellular deposits of the fibrillar form of A $\beta$  peptides, most comprising 38 to 43 amino acids (Glenner and Wong, 1984), along with NFTs that arise inside affected neurons and contain hyperphosphorylated tau protein filaments (Goedert et al., 1988; Haass and Selkoe, 2007).

In 1991, Hardy and Allsop suggested that the main event leading to development of AD involves altered expression of the transmembrane amyloid precursor protein (APP) leading to extracellular accumulation of A $\beta$ . This hypothesis, which was later named the amyloid cascade pathway (or amyloidogenic pathway), has served as the foremost explanation for how the disease develops (Kayed et al., 2003).

The A $\beta$  peptide was discovered by Glenner and Wong in (1984) and was later identified as the main component of senile plaques, arising as a product of proteolytic cleavage of APP (Wilquet and De Strooper, 2004). Two proteolytic routes called the amyloidogenic and nonamyloidogenic pathways have been suggested to be responsible for the cleavage of APP. This division occurs at six sites in the protein and is catalyzed by the enzymes  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -,  $\varepsilon$ -, and  $\zeta$ -secretase, of which the  $\alpha$ ,  $\beta$ , and  $\gamma$  forms are best known and have been studied extensively in relation to the pathogenesis of AD. Depending on the position of cleavage, A $\beta$  is usually designated A $\beta_x$  or A $\beta_{1-x}$ , where x represents the number of residues in the peptide (Lazo et al., 2008). The A $\beta$  plaques observed in the brain of AD patients consist predominantly of A $\beta_{1-40}$ and A $\beta_{1-42}$ , in which the C terminus ends with the 40<sup>th</sup> and the 42<sup>nd</sup> amino acid, respectively (Miller et al., 1993; Roher et al., 1993; Iwatsubo et al., 1994). In the brain, deposition of A $\beta_{1-40}$ 40 is observed primarily in the cerebral vasculature (Iwatsubo et al., 1994; Suzuki et al., 1994), whereas A $\beta_{1-42}$  is found predominantly in the parenchyma. Compared to A $\beta_{1-40}$ , A $\beta_{1-42}$ aggregates more easily (Jarrett et al., 1993) and also earlier in life (Iwatsubo et al., 1995; Lemere et al., 1996).

#### Amyloid beta

About 10% of the APP is processed via the **amyloidogenic pathway**, which results in formation of A $\beta$  plaques (Cohen and Kelly, 2003). Two types of  $\beta$ -secretase enzyme have been identified in the amyloidogenic cleavage process, and these are called APP-cleaving enzymes 1 and 2 (BACE-1 and BACE-2) (Jacobsen and Iverfeldt, 2009). BACE-1 initiates the cleavage of APP, which releases an extracellular soluble APP fragment (sAPP) and a 99amino-acid fragment (C99) that remains attached to the cell membrane. Thereafter, the C99 fragment is further processed by  $\gamma$ -secretase to yield A $\beta$  peptide and an intracellular domain of APP. The  $\gamma$ -secretase can act at two different positions in the C-terminal part of APP to produce peptides of different lengths (i.e.,  $A\beta_{1-40}$  and  $A\beta_{1-42}$ , respectively), as discussed above (Jacobsen and Iverfeldt, 2009). Although  $A\beta$  peptides containing 39 to 45 amino acids have also been found, those with 40 and 42 amino acids are most common (Lazo et al., 2008). A $\beta$  can aggregate in the extracellular space of the brain and forms amyloid plaques.

The A $\beta$  peptide occurs naturally in a monomeric form *in vivo*, and the monomers aggregate to form dimers, trimers, tetramers, dodecamers (Dwulet and Benson, 1986), and protofibrils. During incubation *in vitro* at 37 °C, A $\beta$  is initially found mainly as monomers (84%) and a very small portion of dimers; during further incubation, the proportion of oligomers increases, and, after two weeks, molecules with extremely high molecular weights are detected, which correspond to fibrils (Sarroukh et al., 2011). Over the last decades, many scientists have claimed that A $\beta$  oligomers are the most toxic form of the peptide. These oligomers can interact with neurons and glial cells, and activate mechanisms such as inflammation, phosphorylation of Tau protein (De Felice et al., 2008; Vanessa de Jesus et al., 2009), neuronal oxidative stress, long-term depression (LTD), inhibition of long-term potentiation (LTP) (De Felice et al., 2007; Lambert et al., 1998), spine loss, and finally cell death (Hardy and Selkoe, 2002; Lacor et al., 2007).

About 90% of the APP protein is cleaved by  $\alpha$ -secretase in the central region comprising the A $\beta$  peptide sequence. This is known as the **non-amyloidogenic** pathway, and it results in formation of an extracellular soluble N-terminal fragment (sAPP $\alpha$ ) and a long intracellular C-terminal fragment (C83) in neurons (Vanessa de Jesus et al., 2009). The intracellular domain of APP can be translocated to the nucleus and may function as a neuropeptide (Cao and Sudhof, 2001; Makin et al., 2005). In the healthy brain, APP is preferentially metabolized via this pathway.

The amyloidogenic pathway has long been considered to be the main mechanism behind development of AD, but this theory has recently been challenged by the results of new investigations emphasizing the involvement of other pathological factors in this context. These factors include synaptic alteration (Knobloch and Mansuy, 2008; Mitsuyama et al., 2009; Bi, 2010) and a deficit in synaptic mitochondria (Du et al., 2010), dystrophic neuritis, accumulation of abnormal endosomes/lysosomes and organelle turnover due to dysfunctional autophagy (Cataldo et al., 2000; Nixon, 2007), neuronal loss (Schliebs and Arendt, 2011), glia-mediated inflammation (Rodriguez et al., 2009; Bi, 2010), and impairment of adult

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neurogenesis in the hippocampus (Crews and Masliah, 2010). Lysosomes play a major role in degradation of old proteins and organelles, and recent studies have shown that lysosomal dysfunction and abnormal autophagic activity lead to altered generation of A $\beta$  and thus to AD pathogenesis (Bi, 2010).

#### Pathology

The loss of synapses and neurons leads to cognitive impairment and development of dementia. Neuronal loss and atrophy occur mainly in the neocortex, hippocampus, amygdala, and basal forebrain of AD patients (Pennanen et al., 2004; Devanand et al., 2007; Jauhiainen et al., 2009; Lain et al., 2010). Cholinergic neurons innervating the cerebral cortex, hippocampus, amygdala, and nucleus basalis of Meynert in the basal ganglia are affected early in AD (Coyle et al., 1983). Axonal abnormalities or degeneration of cholinergic neurons lead to decreased release of acetylcholine, which is believed to be the primary cause of cognitive deficits in aged individuals (Bartus et al., 1982). A study using an animal model of AD has shown that the neurotoxicity of A $\beta$  can be reduced by stimulating nicotinic receptors (Kawamata and Shimohama, 2011), and therefore attention has been focused on developing drugs that can inhibit acetylcholine esterase or stimulate acetylcholine receptors in order to restore cholinergic function.

In addition to the cholinergic system, other neurotransmitter systems can be affected in AD patients, including the monoaminergic, glutamatergic, and dopaminergic systems. In the mammalian nervous system, glutamate and GABA serve as the main excitatory and inhibitory transmitters, respectively, and dysfunction of these systems gives rise to various neurological and psychological disorders. Recently, Tiwari and Patel (2012) observed impaired glutamatergic and GABAergic function in the brain of transgenic (A $\beta$ PPswe-PS1dE9) mouse model of AD. Furthermore, Colom et al. (2011) injected A $\beta_{1-40}$  in the CA1 area of the hippocampus of rats and noted a 38% reduction in levels of choline acetyltransferase and a 26% decrease in the number of glutamate-immunoreactive neurons in the brain. Together, these data show that the functions of multiple neurotransmitter systems can be altered in the brain in AD. Today, the main drugs used to treat AD patients include cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists. The challenge is to develop a more multi-functional drug that can maintain neurotransmitter homeostasis.

The results of several surveys have suggested that high levels of oxidative stress and free radicals, or decreases in the antioxidant and/or free-radical-scavenging capacity play a role in the development of neurodegenerative diseases (Bilbul and Schipper, 2011). In AD, oxidative stress is manifested by, for example, increased protein oxidation, lipid peroxidation, and formation of reactive oxygen species (ROS) (Butterfield et al., 2006). In the presence of oxidative stress, proteins may modify their structure and function by cross-linking with other proteins, or through nitration or carbonylation, which generally leads to loss of function. Moreover, it is possible that the sporadic form of AD is initiated by mitochondrial dysfunction (Mancuso et al., 2010; Swerdlow et al., 2010). Together, the data currently available in this area illustrate the complexity of AD and the numerous factors and pathways that are involved in initiation of this disease, several of which should clearly be targeted in therapeutic approaches.

#### Astrogliosis in Alzheimer's disease

Astrocytes are a special type of glial cells that are present in the CNS and play important roles in the following (Sofroniew and Vinters, 2010): development, blood flow regulation, synaptic function, brain metabolism, formation of the blood brain barrier, and homeostasis of fluids, ions, pH, and neurotransmitters in healthy brains. Furthermore, astrocytes undergo cellular, functional, and morphological remodeling in response to all forms of brain injury, infection, ischemia, and neurodegenerative disease. These changes occur through a process called reactive astrogliosis (Sofroniew, 2009), which is reflected by upregulated expression of glial fibrillary acidic protein (GFAP) in the astrocytes (Sofroniew and Vinters, 2010). The modification of these cells varies with the severity of the injury or disease, and it includes progressive cellular hypertrophy, proliferation, and scar formation (Sofroniew, 2009). There is also evidence that dysfunction or side effects of reactive astrogliosis contribute to the development of AD (Sofroniew, 2009; Czlonkowska and Kurkowska-Jastrzebska, 2011; Li et al., 2011).

#### Risk factors

This section provides a short review of the risk factors for AD. Some of these are heritable and largely beyond our control, whereas others are associated with lifestyle or are environmental aspects that can potentially be changed. To facilitate the discussion, here these risk factors are assigned to genetic and non-genetic categories that are described briefly below.

#### Genetic factors

*APP, presenilin-1 (PS1), and presenilin-2 (PS2).* The early onset familial form of AD is linked to mutations in these genes: *APP* on chromosome 21, *PS1* on chromosome 14, and *PS2* on chromosome 1. Thirty-two different mutations in the *APP* gene have been found in 85 families, which together account for 10% to 15% of early onset familial AD (Bird, 2008; Raux et al., 2005). The Swedish and London mutations are examples of changes in the *APP* gene. Most of these mutations are located near the  $\gamma$ -secretase cleavage site of the gene and are associated with increased production of Aβ42 (Scheuner et al., 1996).

Over 176 different mutations have been found in the *PS1* gene in 390 families, and these account for 18% to 50% of early onset familial AD (Theuns et al., 2000). Rudzinski and colleagues (2008) reported that a *PS1* mutation designated N135S found in a Greek family was associated with memory loss in very young individuals (around 30 years of age).

Fourteen mutations have been detected in *PS2* in six families. Mutations in the *PS2* gene are associated with an increased ratio of A $\beta$ 42 to A $\beta$ 40, as also was noted for *PS1*, which is caused either by elevated production of A $\beta$ 42 and/or decreased production of A $\beta$ 40 (Citron et al., 1997; Scheuner et al., 1996). However, in contrast to *PS1*, mutations in *PS2* result in less efficient production of A $\beta$ 42 (Bentahir et al., 2006). Onset of AD generally occurs at an older age in individuals who have a mutation in *PS2* rather than in *PS1*.

*Apolipoprotein E (ApoE)*. The *ApoE* gene is located on chromosome 9, and it has been identified as the major risk factor for the sporadic form of AD with a late onset at around 60 years of age, which is more common than familial AD. This gene has several alleles that are designated *ApoE2, ApoE3*, and *ApoE4*. Having two *ApoE4* alleles is associated with a higher risk of developing the disease. *ApoE3* expresses the ApoE3 protein isoform, which is composed of 299 amino acids and has cysteine at position 112 and arginine at position 158. These positions are occupied by cysteine residues in the ApoE2 isoform and by arginine residues in ApoE4. These different amino acid substitutions affect the three-dimensional (3D) structures of the proteins and their lipid-binding abilities. The ApoE proteins play an important role in the metabolism of triglycerides and cholesterol (Bilbul and Schipper, 2011). In experiments conducted by Rapp and colleagues (2006), the uptake of cholesterol by neurons *in vitro* was lower when the cholesterol was bound to ApoE4 than when it was coupled to ApoE2 or ApoE3. Also, Michikawa et al. (2000) have reported that efflux of

cholesterol from neurons and astrocytes is less efficient when the cholesterol is bound to ApoE4. Moreover, in the Iranian population, Raygani et al. (2005) found a significantly high frequency of the *APOE*- $\epsilon$ 4 allele in patients suffering from AD.

*Down's syndrome.* Individuals with Down's syndrome are potentially at increased risk of AD after the age of 35 due to the presence of an additional chromosome 21 (trisomy 21) carrying the *APP* gene. The pathological picture shows the presence of A $\beta$  plaques and NFTs in the brain of these patients (Tagliavini et al., 1989).

*Other genes.* There are many reports concerning the association between increased risk of AD and polymorphism in different genes. Two of the polymorphisms that are discussed most often occur in the genes that encode anti-inflammatory interleukin (IL) and brain-derived neurotrophic factor (BDNF). Regarding the polymorphism in the IL genes, Qin and coworkers (2012) recently reviewed 32 case-control studies including 7,046 AD cases and 7,534 controls, and they concluded that an association exists between IL-1A -889C/T polymorphism and the risk of AD in Caucasian populations. Also, Lio et al. (2003) studied 132 AD patients in northern Italy and found that the single nucleotide polymorphism 1082A of IL-10 promoter was significantly more common in those patients compared to 213 healthy controls. Furthermore, Arosio et al. (2004) studied 65 AD patients and 65 controls and observed an association between an increased risk of AD and homozygosity for two polymorphisms: A allele of IL-10 (-1082 G/A) and C allele of IL-6 (-174 G/C).

Feher and collegues (2009) have suggested that Val66Met polymorphism of the gene encoding *BDNF* gene is associated with development of AD. Those researchers studied 160 AD patients and found a significantly higher frequency of the *BDNF* Val allele in those subjects than in controls. Kunugi et al. (2001) observed a significantly higher frequency of the C270T polymorphism of *BDNF* in 170 Japanese patients with sporadic AD, as compared to controls. This finding was confirmed by Riemenschneider et al. (2002) in a study of 210 German Alzheimer's patients, and these investigators also suggested that the *BDNF* C270T polymorphism is a risk factor for AD, particularly in individuals who lack the *ApoE*-ε4 allele.

#### Non-genetic factors

The following non-genetic factors can be related to AD:

*Hypertension.* According to epidemiological studies, individuals with hypertension (elevated systolic pressure) are at higher risk of developing AD late in life, and anti-hypertensive drugs may diminish the risk of dementia and cognitive decline (Tzourio et al., 2003).

*Cerebral ischemia/hypoxia* Individuals with stroke or transient ischemic attacks are also at greater risk of developing AD during old age (Kalaria, 2000). It is believed that this is due to overexpression of BACE1 in hypoxia, resulting in overproduction of A $\beta$  (Sun et al., 2006). *Lack of exercise*. Age-related cognitive deficits can be reduced by exercise. In animal models, voluntary wheel running has been found to decrease amyloid deposition and enhance A $\beta$  clearance, and there is evidence that treadmill exercise can ameliorate the accumulation of phosphorylated tau in rodents. Investigations of exercise-induced neuroprotection in both animal models and human populations have revealed the involvement of reduced inflammation in the CNS (Stranahan et al., 2012).

*Insulin/Glucose*. Several studies have discussed the association between diabetes, late-life dementia, and AD. Diabetes is usually characterized by obesity, heart disease, and high blood pressure, and those factors may increase the risk of developing AD.

*Increased lipids or cholesterol* High serum levels of these substances can raise the risk of AD during aging, regardless of the *ApoE* genes involved (Kivipelto et al., 2002).

*Estrogen deficiency*. It has been proposed that a deficit in this hormone is associated with an increased risk of AD, and that estrogen replacement therapy might improve cognitive function and decrease the risk of AD in women (Tang et al., 1996).

*High levels of glucocorticoids*. Such concentrations can be detected in the blood and saliva of AD patients, which suggests that glucocorticoids have adverse effects on hippocampal function and cognition in humans (Balldin et al., 1983).

*Melatonin*. This hormone is secreted by the pineal gland, and it is a powerful free radical scavenger and anti-inflammatory agent. Melatonin is also involved in inhibition of A $\beta$  aggregation and it can attenuate tau hyperphosphorylation (Reiter et al., 1997). According to Olcese et al. (2009), long-term oral administration of melatonin suppresses the A $\beta$  aggregation, decreased levels of cytokines such as tumor necrosis factor alpha (TNF $\alpha$ ) in hippocampus and reduced cortical expression of mRNA for three antioxidant enzymes (i.e., SOD-1, glutathione peroxidase, and catalase) in a transgenic mouse model of AD.

*Vitamin deficiencies* Inadequate levels of vitamins B1 (thiamin), B6 (pyridoxine), B12 (cobalamin), and B9 (folate) may augment the risk of AD (Bilbul and Schipper, 2011), and a diet of vitamin-D-free food has been found to intensify learning deficits in a rat model of AD (Taghizadeh et al., 2011). Vitamin A, which includes retinol, retinal, and retinoic acid, and  $\beta$ -carotene, has the potential to inhibit the formation of  $\beta$ -amyloid fibrils (Ono and Yamada, 2011). In addition, vitamin E has been shown to protect against neurodegeneration by lowering oxidative stress (Guan et al., 2012).

*Toxins*. The risk of AD is significantly increased by abuse of certain illicit drugs (e.g., methamphetamine) and by exposure to heavy metals such as copper, zinc, and particularly iron, or pesticides and herbicides (reviewed by Parron et al., 2011; Schrag et al., 2011). *Inflammatory factors*. Chemokines and cytokines, as well as reactive microglia, have been detected in and around A $\beta$  plaques in both animals and patients with AD (Bilbul and Schipper, 2011). Recently, Martin-Moreno et al. (2012) showed that prolonged oral administration of anti-inflammatory cannabinoids to transgenic (Tg) APP mice normalized the cognitive deficiency and reduced the density of Iba1-positive hippocampal microglia and expression of cyclooxygenase (COX) 2 protein and TNF $\alpha$  mRNA to the normal levels seen in wild-type mice.

*Other factors* It is also possible that the risk of AD late in life is increased by factors such as traumatic head injury, Parkinson disease, human immunodeficiency virus (HIV), stress, depression, and schizophrenia (Bilbul and Schipper, 2011).

#### Evaluation of drugs

Unfortunately, as already mentioned, no cure has yet been found for AD, because the exact pathogenesis of this disease is not known. As discussed above, many factors are involved in initiation and progression of AD. The treatments approved by the US Food and Drug Administration (FDA) consist of acetylcholinesterase (AChE) inhibitors and NMDA receptor antagonists that enhance cholinergic functions in the brain. Other treatment options include anti-inflammatory agents such as corticosteroids or NSAIDs (McGeer and Rogers, 1992), antioxidants, and estrogen replacement and anti-amyloid drugs.

*Cholinergic agents and NMDA receptor antagonists.* Tacrine is an AChE inhibitor used to treat AD patients. AChE and NMDA receptor antagonists can only alleviate symptoms and are effective mainly during the early stages of the disease. These drugs can have undesired side effects such as the following (reviewed by Wollen, 2010): for tacrine, liver problems and

loss of appetite or nausea; for NMDA receptor antagonists, hallucination, confusion, and mood swings.

*Anti-inflammatory agents*. Data from epidemiological studies have implied that NSAIDs help protect against AD, and there is evidence that the biological mechanisms of these drugs involve reduced production and aggregation of A $\beta$ , and inhibition of the activities of COX and  $\beta$ -secretase. It has also been suggested that allosteric modulation of  $\gamma$ -secretase activity constitutes a mechanism in this context, but clinical trials evaluating NSAIDs, including COX inhibitors and steroids, have failed to support the results of epidemiological studies (for reviews, see Cole and Frautschy, 2010; Imbimbo, 2009). Notably, alleviation of the microglial response to A $\beta$  has been observed in rodents given anti-inflammatory agents (Yamada and Nabeshima, 2000), and therefore researchers around the globe are testing numerous antiinflammatory drugs for treating patients with AD. This work is very difficult due to the large number of anti-inflammatory agents that have diverse and essentially unknown mechanisms of action. On the other hand, this diversity greatly improves the likelihood of success.

*Antioxidants*. As mentioned, there is evidence that oxidative stress plays an important role in the pathogenesis of AD, and hence antioxidants may be useful for preventing or delaying the onset of the disease (Yankner, 1996). Numerous antioxidants can be used in this context, and they act via different mechanisms. For example, acetyl-L-carnitine and R-alphalipoic acid inhibit factors that damage mitochondria (reviewed by Palacios et al., 2011), and idebenone exerts a dose-dependent anti-dementia effect in AD patients (Gutzmann and Hadler, 1998). The red-orange pigment beta-carotene found in carrots and other plants and fruits are also very effective in quenching oxygen radicals, and vitamins B, C, and E can shield against oxidative stress. Moreover, cellular components are protected by vitamin C in aqueous environments and by vitamin E in lipid environments. It has been recommended that multiple antioxidant agents can be used, because these substance differ regarding their mechanisms of action, and prolonged use of a single antioxidant is not suitable due to the risk of toxicity (Prasad et al., 2002).

*Estrogen*. Epidemiological investigations have indicated that the prevalence of AD is two to three times greater in women than men after the age of 65, and this gender difference has been linked to absence of the female hormone estrogen later in life. Yamada et al. (1999a) have demonstrated that  $17\beta$ -estradiol used as estrogen replacement therapy can partly prevent the

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memory deficit induced by  $A\beta_{1-42}$ . It has been proposed that estrogen delays the onset of AD by modulating cholinergic neuronal activity, monoamine metabolism, and expression of BDNF mRNA in the brain (Mateos et al., 2012). Estrogen is a nerve growth factor, and as such it promotes connections between synapses and survival of neurons. This hormone can also alleviate the excitotoxicity, oxidative injury, and neuronal degeneration induced by A $\beta$  (Simpkins et al., 1997). Amtul et al. (2010) studied transgenic mice treated with 17 $\beta$ -estradiol and found that a significantly higher level of APP was processed by  $\alpha$ -secretase in these animals, which resulted in increased levels of non-amyloidogenic sAPP $\alpha$  and a marked reduction in A $\beta$ 42 and A $\beta$  plaques. Enhanced A $\beta$ 42 degradation has also been observed in human neuroblastoma SH-SY5Y cells exposed to 17 $\beta$ -estradiol *in vitro* (Xiao et al., 2010). It should be pointed out that several clinical studies have failed to show any protective effect of estrogen against development of symptoms of AD, and the therapeutic use of estrogen may have undesirable effects such as inducing endometrial carcinoma and breast cancer (Green et al., 2012).

Together, the results of previous research suggest that prevention or treatment of AD will require drugs that contain one or several different active substances, each of which should be able to neutralize one or more risk factors for this disease.

#### Animal models of Alzheimer's disease

Age is the greatest risk factor for AD. Animals with a short lifespan age fairly quickly, and thus they can serve as suitable models for studying the mechanisms of normal aging and the pathological mechanisms of age-related diseases over a relative limited period of time. Another advantage of such animals is that they have a short gestation period, which is essential for investigating the effect of interventions over several generations. The fruit fly *Drosophila melanogaster* has a lifespan of only two to three months, and rabbits and rodents also have comparatively short lifespans and gestation periods. Accordingly, these animals constitute good general research models, and they can also provide opportunities to investigate specific diseases with relevant genetic backgrounds.

It was previously widely believed that only humans develop the entire spectrum of the pathological symptoms of familial and sporadic AD. However, this assumption was refuted when some features of AD neuropathology were also observed in non-human species. Amyloid deposits were found in aged bears, dogs, and primates, and NFTs were detected in

sheep, bears, and baboons. In one investigation (Woodruff-Pak, 2008), extensive Aβ accumulation, hyperphosphorylated tau, cholinergic neuronal loss, and massive brain atrophy were observed in one of five old mouse lemurs. Many studies have used numerous different species ranging from worms and flies to polar bears and genetically designed mice to clarify the mechanisms underlying the development of AD, and to find a suitable approach for treatment of patients with this disease (Woodruff-Pak, 2008). So far, however, no perfect animal model has been established that expresses all the pathological, behavioral, biochemical, and anatomical abnormalities associated with AD in humans. The animal models that are used today show partial neuropathological and behavioral deficits, which are induced by pharmacological and/or genetic manipulation of different species (reviewed by Yamada and Nabeshima, 2000). Examples of several types of animal models of AD are discussed briefly below.

*Neurotransmitter manipulation.* Cholinergic degeneration in the basal forebrain is related to cognitive dysfunction, and such degeneration is the earliest stage of AD pathology (Winkler et al., 1998). To study the role of this system in learning and memory deficit in dementia, scientists have developed various animal models (McDonald and Overmier, 1998). Cholinergic lesions have been induced by use of electrocoagulation, excitotoxins, fimbria/fornix transection, and cholinotoxin (reviewd by Yamada and Nabeshima, 2000). These animal models do not exhibit the neuropathological features of AD, such as amyloid plaques and NFTs, and they have been used primarily to evaluate the validity and effectiveness of therapeutic interventions with cholinergic drugs (Itoh et al., 1997).

*Transgenic animal models*. Different types of gene manipulations have been performed, particularly in mice and fruit flies, to generate animal models with Aβ-associated neuropathological features. The models can express human APP, C-terminal fragment of APP (Kammesheidt et al., 1997), Aβ, and familial AD mutations (Games et al., 1995). One line of transgenic mice contains double mutations that overexpress human APP695, which is widespread in Swedish families with early onset AD; this mouse model is designated Tg2576, and it displays cognitive impairment and AD-like neuropathy (Hsiao et al., 1996). Tg2576 mice characteristically exhibit abundant gliosis and neuritic dystrophy, which are accompanied by Aβ deposition, but not by neuronal loss in CA1 (Irizarry et al., 1997). In contrast to the Tg2576 animals, APP23 transgenic mice do show a significant decrease in the CA1 pyramidal population. Another example of a transgenic mouse model in this group carries the London mutation and is thus designated APP-London; increased levels of  $A\beta_{1-42}$  have been found in young APP-London animals, and neuritis plaques have been observed in old individuals (Calhoun et al., 1998).

Transgenic techniques have been used to generate animal models with NFT-like neuropathology. The first NFT-positive mouse model was called JNPL3. Cross-breeding of this model with Tg2576 animals created a new model exhibiting tau pathology but not A $\beta$ pathology (Lewis et al., 2001). Also, a triple transgenic mouse model was produced by crossing animals expressing the wild-type tau isoform with mice carrying the London and Swedish APP mutations and PS1 mutations; the new line in this case showed only cytoskeletal alteration and somatodendritic accumulation of tau (Boutajangout et al., 2004; Perl, 2010). Other efforts have led to transgenic mice that overexpressed human mutant PS1 and showed neurodegeneration without A $\beta$  deposits, and mice with PS1 mutations that displayed higher levels of A $\beta_{42}$  (4) in the brain (Duff et al., 1996).

*Non-transgenic animal models*. Many investigators have established that acute or chronic infusion of various A $\beta$  fragments into the brain of rodents can induce neurodegeneration in some parts of the brain and impair learning and memory (Pepeu et al., 1996; Flood et al., 1991). A $\beta_{1-40}$ , A $\beta_{1-42}$ , and A $\beta_{25-35}$  are the fragments used most extensively *in vivo*. In 1991, Kowall and colleagues observed neuronal loss in rats that had received an intracerebral injection of A $\beta_{1-40}$ , and in 1994 Nitta and coworkers found that rats given continuous cerebroventricular infusion of A $\beta_{1-40}$  at a dose of 300 pmol/day showed significant impairment of spatial reference memory in the water maze and passive avoidance tests. The rodent model using infusion of AB into the brain enables investigation of AB-associated pathology without any overexpression of genes. However, this method also has disadvantages, for example the biochemical form of A $\beta$  can be affected by the infusion time, and the temperature and the length of time the peptide is incubated in solution before the surgery can affect the toxicity of the peptide. It is also plausible that the site of needle insertion differs slightly between animals in the same experiment, because the injections are administered manually. Clear advantages of this model are that it is inexpensive and reproducible when carried out very carefully.

#### Phytoestrogens

Soy is the major source of phytoestrogens, and has long been used as traditional food. The name phytoestrogen comes from the Greek *phyto*, which means plant, and the word *estrogen*, which is the hormone that regulates fertility in female mammals. Phytoestrogens are found in seeds, fruits, and vegetables. These plant-derived compounds are structurally similar to estrogen and have estrogen-like activities. Indeed, phytoestrogens can act as estrogen agonists, showing synergic function with endogenous estrogen and thereby inducing estrogenic effects, or as estrogen antagonists that may block the estrogenic receptors or change their functional properties to prevent estrogenic activity (Brzezinski and Debi, 1999). There are a number of subtypes of phytoestrogens, including isoflavones, coumestans, lignans (Ososki and Kennelly, 2003), chalcones (Rafi et al., 2000), flavones (De Keukeleire et al., 1999), and prenylflavonoids (Kitaoka et al., 1998). There are two basic subgroups of isoflavones called aglycones and glycosides. Genistein (4',5,7-dihydroxyisoflavone) is the well-known aglycone form of isoflavones (Ososki and Kennelly, 2003). Both aglycones and glycosides are metabolized in the gastrointestinal tract after consumption (King et al., 1996).

#### Genistein metabolism

Genistein is the isoflavone that has received the most attention due to its estrogenic, neuroprotective, antioxidant, anti-inflammatory and anti-proliferative. The presence of OH at C-5 is necessary for the inhibitory effect of genistein, whereas OH groups at C-7 and C-4 are responsible for the greatest inhibitory action of this compound on tyrosine kinase activity (Ogawara et al., 1989). Genistein is present at concentrations of approximately 2.0 to 229  $\mu$ g/g in food, and, when within the gastrointestinal system, it is first metabolized to dihydrogenistein and then to 6'-hydroxy-O-DMA (Kurzer and Hu, 1997). The plasma level of genistein reaches a maximum after a single oral dose and declines with a half-life of approximately 9 h. Genistein can penetrate the blood-brain barrier and it has been detected in brain tissue in a dose-dependent manner (An et al., 2001; Robertson and Harrison, 2004) and in lower concentrations than in other tissues. An increased concentration of genistein in brain tissue has been observed two hours after gavage administration of the substance to rats (Chang et al., 2000). The low rate of penetration across the blood-brain barrier does not exclude the possibility that even a relatively low concentration of genistein can have a beneficial effect on brain function. The bioavailability and other properties of phytoestrogens

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and genistein can be affected by various factors, such as the method of administration, the dosage used, and the gender and metabolism of the recipient (Kelly et al., 1995).

#### Pharmacological properties of genistein

Phytoestrogens, including genistein, have stable structures and low molecular weight, which allows them to pass through cell membranes and interact with intracellular enzymes and receptors (Adlercreutz, 1999). Genistein can bind to three types of (ERs), designated ER $\alpha$ , ER $\beta$ , and ER $\gamma$  (Hawkins et al 2000), and it shows the greatest affinity for ER $\beta$ . The effects of estrogen are mediated through pathways that are either dependent (genomic) or not dependent (non-genomic) on the ERs in the nucleus. The genomic mechanisms involve binding of the estrogen-ER complex to the elements of the target gene promoter and to regulate nuclear gene transcription. By binding to the ERs, the phytoestrogens genistein may induce estrogenresponsive gene products that interfere with the metabolism or action of steroid hormones, and in that way affect transcription pathways (Santti et al., 1998). The non-genomic mechanisms involve ERs that do not bind to DNA but lead to rapid activation of signaling cascades such as mitogen-activated protein kinases (MAPKs) and protein kinase A (PKA), and C (PKC) (Singh et al., 1999; Qiu et al., 2003). Non-genomic effects include also inhibition of the activities of tyrosine kinase and DNA topoisomerase, suppression of angiogenesis, and exertion of antioxidant effects (Rusin et al., 2010). Oral administration of genistein can block COX2 expression via an ER-dependent mechanism and prevent inhibition of NF<sub>k</sub>B activity (Seibel et al., 2009).

#### Effects of phytoestrogens/genistein

It has been suggested that phytoestrogens can act as estrogen agonists to increase the levels of choline acetyltransferase and nerve growth factor messenger RNA in the frontal cortex and hippocampus (Pan et al., 1999). Moreover, there is evidence that these compounds can increase spine density and synapse formation in the hippocampus of adult animals and they may also interact with the transcription of neurotrophin genes (File et al., 2003). Phytoestrogens can improve cognitive function and memory as well (File et al., 2001). Both genistein and estrogen can be considered as antioxidants that scavenge free radicals (Moosmann and Behl, 1999), a task they achieve by donating hydrogen atoms from the phenolic hydroxyl group (Wright et al., 2001). The results of recent studies have shown that genistein has a neuroprotective effect against  $A\beta$  toxicity both *in vitro* and *in vivo* (Bang et al., 2004).

A soy-derived isoflavone such as genistein can protect low-density lipoprotein from oxidation (Badeau et al., 2005). Genistein at low concentrations stimulates cell proliferation, whereas at high concentrations it inhibits this function. The latter effect occurs at doses greater than 10  $\mu$ M due to the inhibition of the tyrosine kinase activity (Ososki and Kennelly, 2003). Genistein also exerts a protective effect on the cardiovascular system by causing the following: a drop in the plasma lipid concentration, decreased formation of thrombus (via inhibition of platelet action), and improvements in systemic arterial compliance and antioxidant activity (van der Schouw et al., 2000).

In humans, daily intake of a high concentration of genistein (i.e., approximately 10-fold above the average daily dose) can be mutagenic (Setchell et al., 1997). Furthermore, phytoestrogens such as coumestrol and genistein have been found to induce structural chromosomal aberrations in cultured human peripheral blood lymphocytes. Genistein can also inhibit the activity of topoisomerase II and exert a clastogenic effect on human chromosomes, which may result in the DNA strand breakage seen in human leukemia and gastric cancer (Sirtori et al., 2005). Moreover, Sassi-Messai et al. (2009) studied zebrafish and found that genistein induced apoptosis in those animals when administered at a dose of 20 mg/day, and this occurred in an ER-independent manner in the hind brain and the anterior spinal cord.

## AIMS OF THE RESEARCH

The general objective of the present research was to evaluate the effect of genistein on neurodegeneration.

The specific aims were as follows:

To study the effect of genistein on degeneration of neurons in the substantia nigra pars compacta in a rat model of Parkinson disease (*Paper I*).

To study the impact of genistein on learning and memory impairments, as well as the involvement of estrogen receptors and oxidative stress in relation to learning and memory deficit, in an animal model of Alzheimer's disease (*Paper II*).

To evaluate the toxic effect of  $A\beta_{1-40}$  injection into the rat brain and to ascertain whether genistein can protect neurons against  $A\beta$ -induced toxicity (*Paper III*).

To evaluate the morphological responses of astrocytes to the presence of  $A\beta_{1-40}$  in the brain before and after treatment with genistein *(Paper IV)*.

### METHODOLOGY

#### Animals (Papers I–IV)

The animals used in the studies were adult male Sprague Dawley or Wistar rats that weighed 250–300 g at the start of the experiments. The experiments were conducted in accordance with the policies stipulated in the *Guide for the Care and Use of Laboratory Animals* (NIH) and by the Research Council of Iran University of Medical Sciences (Tehran, Iran).

#### Genistein treatment (Papers I–IV)

The rats received 10 mg/kg genistein (Sigma Chemical Co.) one hour before surgery. Genistein was either dissolved in propylene glycol and administered by an intraperitoneal (i.p.) injection (*Paper I*) or dissolved in Cremophor-EL (Cr-EL) (BASF Corp.) and given orally by gavage (*Papers II–IV*). Cr-EL is a derivative of castor oil, and propylene glycol (also called 1-2propanediol) is a colorless, odorless, viscous liquid that is used as an emulsifier and a moisturizing agent.

#### Surgery (Papers I–IV)

In the study reported in *Paper I*, rats were anesthetized with an i.p. injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). Thereafter, the animals received a unilateral intrastriatal injection of 5  $\mu$ L of 0.9% saline containing 2.5  $\mu$ g/ $\mu$ L 6-hydroxydopamine-HCl (6-OHDA; Sigma Chemical CO.) and 0.2% ascorbic acid (w/v) at a rate of 1  $\mu$ L/min at the following coordinates: L –3 mm, AP +9.2 mm, V +4.5 mm from the center of the interaural line, according to the atlas of Paxinos and Watson (1998). The rats in the sham-operated group were given 5  $\mu$ L of 0.9% saline–0.2% ascorbic acid administered in a similar manner as the solution containing 6-OHDA.

The rats in the other three studies (*Papers II–IV*) were anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After that, the animals received 4  $\mu$ L of normal saline or A $\beta_{1-40}$  (0.5 nM/ $\mu$ L dissolved in 0.9% normal saline, pH 8.0; Sigma Chemical Co.) bilaterally in the hippocampus at coordinates of –3.5 mm posterior to bregma, 2 mm lateral to the sagittal suture, and 2.8 mm below the dura mater, according to the stereotaxic atlas (Paxinos and Watson, 1998).

In the second investigations (*Paper II*), the ER antagonist fulvestrant (10  $\mu$ g dissolved in 5  $\mu$ L of artificial CSF; Sigma Chemicals Co.) was given as an intracerebroventricular (i.c.v.) injection at a dose of 10  $\mu$ g/rat (5  $\mu$ L) at coordinates of –0.8 mm posterior and 1.4 mm lateral to bregma, and 4 mm below the dura (Paxinos and Watson, 1998) 30 min before injection of A $\beta$ . We used fulvestrant to block the estrogenic effect of genistein. Previous observations have indicated that i.c.v. injection of this drug reverses the inhibitory effect of estrogen on the pulse frequency of gonadotropin-releasing hormone (GhRH) (Steyn et al., 2007). Fulvestrant acts by disrupting shuttling of ER between the nucleus and the cytoplasm (Dauvois et al., 1993), and by decreasing cellular expression of ER (Wade et al., 1993).

#### Behavioral tests (Papers I and II)

#### Rotational behavior (Paper I)

The rats were tested for rotational behavior after injection of apomorphine hydrochloride (2 mg/kg, i.p.) given one week before (baseline) and two weeks after the surgery. Briefly, the animals were allowed to habituate to the cylindrical test chamber in a quiet isolated room for 10 min. Thereafter the drug was injected, and 1 min later full rotations were counted at 10-min intervals for 60 min.

The net number of rotations was defined as the positive scores (turns to the right) minus the negative scores (turns to the left). Since the toxin (6-OHDA) was injected in the left hemisphere, the animal showed rotations to the right.

#### Radial maze task (Paper II)

Spatial learning and memory can be tested using an eight-armed radial maze (RAM) that is made of black Plexiglas and has a recessed food cup at the end of each arm. In our experiments, rats were given free access to water, but the amount of food was restricted to keep them at around 80–85% of free-feeding body weight in order to induce food-searching behavior. The animals were allowed to move around freely in the maze and learned to visit each arm and not to re-enter an arm that had already been visited during the same test. Each entry into each arm with all four paws was scored during a period of 10 min. The number of correct choices or errors was recorded to assess the performance of the animals in each session. A re-entry in a visited arm was considered as an error. The rats were trained in the maze before the surgery, and those that made 6–7 correct choices out of 10 entries were selected for further use in the study.
#### *Y-maze (Paper II)*

The Y-maze test is used to evaluate the tendency of rodents to explore a new environment. The specific parts of the brain involved in performance of this task include the hippocampus, the basal forebrain, and the septal and prefrontal cortex. This test can be used to detect the presence of cognitive deficits and to assess the positive or negative effects of a toxin or treatment.

The maze we used was made of black Plexiglas, and the three arms (40L x 30H x 15W cm) converged in an equilateral triangular central area that was15 cm at its longest axis. In our experiments, a rat was first allowed to explore the maze for 8 min. Thereafter, the animal was left in the central triangle, and triple entries were recorded. An entry was considered to be complete when the base of the animal's tail was entirely within an arm. Alternation was defined as successive entries into the three arms in overlapping triplet sets. The maximum number of possible spontaneous alternations was determined as the total number of arms entered minus 2, and the percentage was calculated as the ratio of actual to possible alternations x 100.

#### Passive avoidance task (Paper II)

The passive avoidance task can be used to evaluate learning and memory in rodents. The apparatus in this test consists of one dark and one illuminated chamber separated by a guillotine door. On the training day, animals learn to avoid the dark chamber, because they were given a single inescapable foot shock (1 mA, 1 s) in that location on their first visit. If the animals remain a longer time in the illuminated chamber in a subsequent retention trial, it means that they have no problem remembering the foot shock previously delivered in the dark chamber. We evaluated the efficacy of A $\beta$  and genistein treatment by measuring what we called the initial latency on the training day and the step-through latency (STL) on the trial day. Latency was determined as the time interval between being placed in the starting chamber and entering the dark chamber.

#### **Biochemistry (Paper II)**

Rats were deeply anesthetized (ketamine, 150 mg/kg) and decapitated in a guillotine, and their brains were removed within 5 min. The hippocampi were isolated and blotted dry, and prepared as 5% tissue homogenates in ice-cold 0.9% saline solution. Each homogenate was

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centrifuged (1000g, 4 °C, 10 min), and an aliquot of the supernatant were stored at -80 °C until used.

*Hippocampal malondialdehyde (MDA) concentration* was used as a marker of lipid peroxidation index and was calculated by measuring thiobarbituric acid reactive substances (TBARS) in the hippocampal supernatants. Briefly, trichloroacetic acid and TBARS reagent were added to aliquots of supernatant and then mixed and incubated at 100 °C for 80 min. After cooling on ice, the samples were centrifuged at 1000g for 10 min, and the absorbance was read at 532 nm. The results of TBARS measurements were expressed as MDA equivalents, using tetraethoxypropane as standard.

*Hippocampal superoxide dismutase (SOD)* activity was measured by incubating supernatant with xanthine and xanthine oxidase in potassium phosphate buffer (PBS, pH 7.8; 37 °C) for 40 min. Next, nitroblue tetrazolium (NBT) was added to the samples, and formation of blue formazan was monitored spectrophotometrically at 550 nm. The amount of protein that inhibited NBT reduction to 50% of maximum was defined as 1 nitrite unit (NU) of SOD activity.

*Hippocampal nitrite (NO<sub>2</sub>) concentration* was assayed by the Griess method. The compound NO has a short half-life and is rapidly converted to the stable end products nitrate (NO<sub>3</sub>) and nitrite (NO<sub>2</sub>). In the assay used here, NO<sub>3</sub> was converted to NO<sub>2</sub> by cadmium, and then color development was done with Griess reagent (sulfanilamide and N-naphthyl ethylenediamine) in acidic medium. The absorbance was determined using a spectrophotometer at 540 nm.

*The protein content* of the supernatant was measured by the Bradford method, using bovine serum albumin (Sigma Chemical Co.) as the standard (Bradford, 1976).

#### Perfusion, nissl staining (Papers I, III, and IV)

Two *(paper I)* or three *(Paper III-IV)* weeks after surgery, animals were anesthetized with ketamine (150 mg/kg) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer solution. Thereafter, samples of brain tissue between 2.9 and 4.2 mm interaural *(Paper I)*, and between 5.04 and 3.00 mm caudal to the bregma *(Paper II)* were embedded and 50-µm *(Paper I)* or 20-µm *(Paper III-IV)* coronal sections were obtained and stained with cresyl violet (Nissl method).

#### Immunohistochemistry (Papers III and IV)

Sections were incubated at 4 °C overnight with antibodies against inducible and neuronal nitric oxide synthase (iNOS and nNOS), and A $\beta_{1-40}$  diluted in PBS containing 3.5% normal serum, 0.25% bovine serum albumin (BSA), and 0.25% Triton X-100. Thereafter, the sections were rinsed in PBS, incubated in 3% H<sub>2</sub>O<sub>2</sub>, washed in PBS, and then incubated for 1 h at room temperature with secondary antibodies followed by 3,3'-diaminobenzidine (DAB), and the nuclei were stained with Meyer's hematoxylin.

To detect glial fibrillary acidic protein (GFAP), all sections were incubated at 4 °C overnight with serum-free protein block and polyclonal antibodies against GFAP (1:1500; Dako, Denmark). Next, the sections were washed in PBS and incubated for 1 h at room temperature with alkaline phosphatase conjugated IgG antibodies (1:100; Dako, Denmark); this was achieved using liquid permanent red chromogen diluted with liquid permanent red substrate buffer. Some sections were also stained with 4,6-diamidino-2-phenylindole (DAPI) before mounting. The intensity of GFAP immunoreactivity was measured using the Zen program (Zen Software Inc., 2009) and 3D micrographs obtained in a confocal microscope.

#### Cell counting (Papers I and III)

The cell counting was performed to determine the relative changes in the number of neurons in the brains of the experimental rats compared to controls. Cells that were counted had a clear membrane and a visible nucleolus.

In the first (*Paper I*), we used every second Nissl-stained section for quantitative evaluation of cell survival. In a subsequent investigation (*Paper III*), the number of hippocampal neurons was counted in CA1 (four fields), CA3 (two fields), and the whole lateral blade (lb) of the dentate gyrus (DG) in a mediolateral direction in every sixth cresyl-violet-stained section. This was done using a light microscope (Zeiss, Germany; 400×, field diameter 440  $\mu$ m), and the iNOS- and nNOS-positive cells were counted in all hippocampal subfields in a total of five sections from each animal.

#### Morphometric study of astrocytes (Paper IV)

A confocal microscope (Olympus, Zeiss) was used to acquire images for morphometric analysis. We used two sections from each animal and captured 3D images (z-stack) of fifty astrocytes, each showing a clearly visible soma, a DAPI-stained nucleus, and no overlapping branches; these cells were located in an area between the hippocampal fissure and stratum granulare in the medial blade of the dentate gyrus (DGmb) in hippocampal formation. For the morphometric analysis, 3D images of 900 astrocytes were created from 14,000 consecutive 2D images taken at a uniform interval of 1.01 µm. The X, Y, and Z properties of the images were 0.132, 0.132, and 1 µm/pixel, respectively. All measurements were performed on coded slides. The length of branches was determined by drawing individual branches using Easy Image Analysis<sup>®</sup> 2000 software (Tekno Optic, Stockholm, Sweden). Volocity 5.5 (Perkin Elmer Inc., USA) was used to measure the following for each astrocyte: the surface area and volume of the DAPI-stained nucleus, the cell body, the entire cell (i.e., the cell body and branches), and the area and volume of the astrocyte territory was achieved by drawing a line connecting the tips of the branches.

## **RESULTS AND DISCUSSIONS**

The main findings and brief discussions of the four studies are presented below. For details, please see Papers I–IV.

#### Effects of genistein in a 6-OHDA rat model of Parkinson disease (Paper I)

*Rotational behavior.* The total net number of rotations in the tested rats showed that apomorphine i.p injection 2 weeks after surgery caused a very significant contralateral turning in the 6-OHDA lesion group (P < 0.001) and less significant rotations in the genistein-treated 6-OHDA lesion group (P < 0.005), as compared to the sham-operated group. The number of rotations in the 6-OHDA-genistein-treated group was significantly lower (P < 0.01) than in the 6-OHDA group not given genistein.

*Cell counting*. The results of histological analysis studies showed the following: no significant difference in the number of Nissl-stained neurons between the right and the left SNC in the sham group; a significant reduction on the left (injected) side only in the lesion group (only 6-OHDA injection); no such difference in the lesion-genistein group. Interestingly, the number of neurons in the SNC did not differ significantly between the sham and the lesion-genistein group.

In this study, we produced a rat model of PD by performing intrastriatal injection of 6-OHDA to cause unilateral damage to the nigrostriatal dopaminergic system. After being introduced in this location, the 6-OHDA is taken up by dopamine transporter and leads to permanent depletion of tyrosine hydroxylase (TH)-positive neurons. The toxic effect of 6-OHDA is related to production of intracellular free radicals (Schwarting and Huston, 1997) and mitochondrial dysfunction. The degeneration of neurons is followed by a reduction in the striatal dopamine level and upregulation of dopaminergic postsynaptic receptors on the damaged side (Schwarting and Huston, 1996). These changes generate motor asymmetry that can be evaluated using dopaminergic agonists such as apomorphine (Schwarting and Huston, 1996). This is done by recording the rotational behavior of animals in a test chamber, and the turns induced by apomorphine are considered to be particularly reliable as indicators of nigrostriatal dopamine depletion (Shapiro et al., 1987). The attenuation of rotational behavior we observed in the 6-OHDA-genistein-treated lesioned group in our study may have been due to a protective effect of genistein against nigral neurodegeneration, as well as maintenance of

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striatal dopamine at a level that is not accompanied by a marked turning behavior. Furthermore, it is plausible that genistein reduced damage and loss of neurons by counteracting oxidative stress (Goodman et al., 1996; Blum-Degen et al., 1998), and it may also have modulated dopaminergic activity (Cyr et al., 2002) and thereby decreased the apomorphine-induced rotations in the 6-OHDA-lesioned rats. In addition, there is growing evidence that estrogen provides its potent neuroprotective effects through mitochondrial mechanisms. Phytoestrogens such as genistein have a high binding affinity for ER $\beta$ , and this affinity is greater in the CNS regions than in the peripheral organs. The estrogenic effects may be exerted either directly or indirectly via signal transduction pathways that are induced not only by estrogens, but by other factors as well. Estrogens have been shown to influence the concentration and localization of anti-apoptotic proteins, and their protective actions may occur directly and synergistically with antioxidants such as glutathione. There is also evidence that estrogen prevents lipid peroxidation by sacrificing itself to oxidation (Singh et al., 2006).

In conclusion, the results of this study suggest that genistein can ameliorate 6-OHDA-induced neurodegeneration of dopaminergic neurons in rats.

## *Effects of genistein on learning and memory deficit in an* $A\beta_{1-40}$ rat model of AD (*Paper II*)

*Y-maze.* We used the Y-Maze test to measure the motivation of rats to explore new environments. In such a maze, rodents prefer to investigate a new arm rather than return to one they have already visited. Many parts of the brain, including the hippocampus, are involved in learning and remembering aspects of this behavior. Our results showed that the alternation score recorded at the end of the study was significantly lower for Aβ-injected rats (P < 0.01) compared to animals in a sham group. Moreover, the score was significantly higher (P < 0.05) for genistein-treated Aβ-injected animals compared to those that only received an Aβ injection. The alternation score was also higher for Aβ-injected rats given genistein and the estrogen receptor antagonist fully strait compared to the animals that only received an Aβ injection, although this difference was not statistically significant.

*Passive avoidance test.* Learning and memory can also be evaluated in rodents by use of the passive avoidance test. In short, the animals learn to avoid a part of a test chamber by remembering that they had previously experienced a harmful stimulus given in that

environment. When using this test, we found no significant difference in the initial latency between the groups. Regarding step-through latency (STL), we observed marked impairment of retention and recall capacity in the A $\beta$ -injected rats (p < 0.005) and the A $\beta$ -injected rats given genistein and fulvestrant (P < 0.01), as compared to the sham-operated animals. STL was significantly improved by genistein treatment compared to A $\beta$  injection (P < 0.05), but this difference was completely abolished by the presence of fulvestrant (P < 0.05).

*Ram task.* A $\beta$ -injected rats showed a significant deficit in spatial cognition and memory, as indicated by a lower number of correct choices (P < 0.01) and a higher number of errors (P < 0.01) compared to the sham-operated group. Administration of genistein resulted in a non-significant increase in the number of correct choices (35.6%) but led to a significantly lower number of errors (36.8%, P < 0.05). Genistein given together with fulvestrant did not affect the number of correct choices or errors in the A $\beta$ -injected animals.

*Oxidative stress*. Analyzing hippocampal tissue, we found that A $\beta$ -injected rats exhibited significantly elevated levels of MDA (P < 0.01) and nitrite (P < 0.005), and a significant reduction in SOD activity (P < 0.005) compared to the sham-operated group. Pretreatment with genistein significantly attenuated the increase in MDA (P < 0.05) but had no significant impact on the levels of nitrite and SOD.

In this study, we observed impaired memory in rats after injection of soluble  $A\beta_{1-40}$  into the dorsal hippocampus, which agrees with the results of previous investigations (Nitta et al., 1997; Tanaka et al., 1998). Furthermore, we noted that genistein pretreatment reduced, but did not completely prevented, the loss of memory caused by  $A\beta_{1-40}$ . The attenuation of memory loss by genistein may be related to the effect of this drug on certain mechanisms related to cognitive function in AD. For example, genistein has structure and activity similar to estrogen, and it functions as a relatively selective ER $\beta$  agonist (An et al., 2001), which may partly explain our findings. A part of attenuation of memory loss can be due to decreased cell loss of hippocampal neurons by genistein as suggested by the results of our paper III.

There is evidence that direct interaction of A $\beta$  with mitochondria induces production of free radicals, mitochondrial dysfunction, and cell death (Reddy, 2006), and antioxidants such as  $\alpha$ -tocopherol have been reported to protect against learning and memory deficits caused by A $\beta$  (Yamada et al., 1999b). It has also been suggested that the antioxidant activity of genistein is

due to the ability of this compound to decrease oxidant production by mitochondria (Borras et al., 2010). Activation of extracellular signal-regulated kinases (ERKs) is one of the pathways that can be influenced by estrogen (Singh et al., 1999). The highest levels of ERKs are found in the hippocampus and some other parts of the brain (Ortiz et al., 1995), and thus the effects of substances with affinity for ERs can be very pronounced in these areas. In addition, Borras and colleagues (2006) have shown that genistein can contribute to activation of MAP kinases and NF $\kappa$ B, and increase the antioxidant activity of manganese superoxide dismutase (MnSOD).

In conclusion, our results suggest that, in rat, pretreatment with genistein prevents  $A\beta_{1-40}$ induced impairment of short-term spatial recognition memory in a Y-maze and learning and memory in a passive avoidance test, and these effects occur via an estrogenic pathway and by attenuating oxidative stress.

#### Effects of genistein on the hippocampus in an $A\beta_{1-40}$ rat model of AD (Paper III)

*Nissl staining*. Cresyl violet staining of sections of hippocampus from sham-operated rats indicated normal morphology with no neuronal loss in the subfields of this region. However, in such sections from rats injected with  $A\beta_{1-40}$ , the lateral blade of dentate gyrus (DGlb) showed signs of extensive cell loss, and homogeneous extracellular pink material was observed close to the DGlb. Also, the numbers of neurons in CA1, CA3, and DGlb were significantly lower in the A $\beta$ -injected animals compared to the sham-operated group (P = 0.3, P = 0.002, and P < 0.0001, respectively). Finally, considering the six A $\beta$ -injected rats that also received genistein, the DGlb appeared completely normal in two, showed segmental degeneration in one, and had completely degenerated in three. Furthermore, the hippocampal sections from these rats did not contain the homogeneous extracellular pink material that we had observed in the animals received only A $\beta_{1-40}$  injection. The mean numbers of cells in the CA1, CA3, and DGlb were lower in the A $\beta$ -injected genistein-treated rats compared to the sham-operated genistein-treated rats (P = 0.03, P < 0.0001, and P < 0.0001, respectively). However, the rate of cell survival in the DGlb was significantly improved in the A $\beta$ -genistein-treated rats (P = 0.03, P < 0.0001, and P < 0.0001, respectively).

 $A\beta$  *immunoreactivity*. No immunoreactivity against anti-A $\beta$  antibody was observed in shamoperated and genistein-treated rats, whereas animals that received a hippocampal injection of  $A\beta_{1-40}$  showed positive extracellular immunoreactivity at the site of neurodegeneration in the DGlb.

*Congo red staining.* None of the brain sections from the rats included in this study showed any apple-green birefringence when studied in a polarizing microscope.

*iNOS and nNOS*. We found that intracellular iNOS<sup>+</sup> and nNOS<sup>+</sup> immunoreactivity in the hippocampus was more extensive in rats injected with  $A\beta_{1-40}$  compared to those that only underwent sham operation. Furthermore, in the hippocampus of the  $A\beta$ -injected rats, the mean number of iNOS<sup>+</sup> cells was significantly increased (P = 0.01), whereas there was only a statistically insignificant rise in the number of nNOS<sup>+</sup> cells. The  $A\beta$ -injected rats that were also given genistein displayed the same patterns of distribution of iNOS<sup>+</sup> and nNOS<sup>+</sup> cells as seen in the animals subjected only to  $A\beta$  injection. Genistein treatment alone raised the number of nNOS<sup>+</sup> cells (P = 0.0001), but not iNOS<sup>+</sup> cells, as compared to sham operation together with genistein treatment.

*GFAP immunoreactivity*. The A $\beta_{1-40}$ -injected animals exhibited extensive signs of astrogliosis in the hippocampus, seen as the presence GFAP<sup>+</sup> cells, and the reactive astrocytes contained dense networks with branches that in some cases overlapped. The mean intensity of GFAP immunoreactivity was significantly increased in the hippocampus of these rats compared to sham-operated animals (P = 0.0006). Genistein treatment of A $\beta_{1-40}$ -injected rats did not change the general pattern of GFAP immunoreactivity, although some minor differences were observed. For example, the A $\beta_{1-40}$  injected genistein-treated rats showed a sharp decline in the intensive GFAP immunoreactivity in locations where the DGlb appeared normal, and they also exhibited less astrogliosis in the polymorphic and granular cell layers of the DG compared to the animals exposed solely to A $\beta_{1-40}$ . Furthermore, the mean intensity of GFAP<sup>+</sup> immunoreactivity was decreased significantly in the animals given both A $\beta$  and genistein compared to those given A $\beta$  only (P < 0.02).

*Cremophor-EL (Cr-EL)*.  $A\beta_{1-40}$ -injected rats treated with Cr-EL showed diverse effects similar to those observed in the A $\beta$ -injected genistein-treated rats. In addition, compared to rats that received only  $A\beta_{1-40}$ , those given both  $A\beta_{1-40}$  and Cr-EL showed a similar number of Nissl-stained neurons in the CA1 and CA3, a larger number of such cells in the DGlb

(P = 0.02), an increased number of nNOS<sup>+</sup> cells (P = 0.01), and similar pattern and intensity of GFAP immunoreactivity.

Several methodological considerations of this study can be discussed, such as the variables that can affect the toxicity of the A $\beta$  peptide (Busciglio et al., 1992). A freshly prepared solution of A $\beta$  can be less toxic than a solution that has been incubated for hours at a temperature higher than 20 °C, because the A $\beta$  is in monomer form in the former but creates neurotoxic fibrils in the latter (Kim et al., 2003). We injected Aß solution within 30 min to 4 h of preparation, and hence it is likely that the peptide was primarily in monomer form. To elucidate the conformational forms of the  $A\beta_{1-40}$  used in our study, we sent a sample of the  $A\beta_{1-40}$  solution to Professor Per Hammarström at Linköping University for analysis by the thioflavin T fluorescence assay. The results showed that the solution we administered to rats contained both free and fibrillar forms of A $\beta_{1-40}$ . The relative fluorescence unit (RFU) for the fibrillar form was approximately 2% of the reference RFU for fully mature A $\beta$  fibrils, which indicated that the predominant proportion of the injected  $A\beta_{1-40}$  was in non-fibrillar form. On the other hand, the Congo red staining showed no apple-green birefringence in polarized microscopy, which is the standard method for detecting the fibrillar form of this peptide. It is plausible that the content of fibrils in the solution we used (i.e., 2%) was not large enough to induce apple-green birefringence. The lack of a large amount of fibrillar  $A\beta_{1-40}$  implies that even the non-fibrillar form of A $\beta$  has neurotoxic properties, as has also been suggested by other investigators (Resende et al, 2008).

Considering another methodological aspect of our study, Cuevas et al. (2011) have suggested that intrahippocampal injection of A $\beta$  increases expression of the receptor for advanced glycation end products (RAGE), which leads to events such as enhanced production of proapoptotic factors and NO. The pathological effects of A $\beta$  are associated with other events, including increased synaptic transmission (Cuevas et al., 2011), imbalance between elevated levels of inflammatory cytokines and decreased levels of neurotrophic factors in the brain tissue (Ji et al., 2011), and mitochondrial dysfunction (Ren et al., 2011; Tillement et al., 2011). Together, these findings show that A $\beta$  triggers a cascade of extra- and intracellular events, all of which may be involved in neuronal degeneration. NO has been found to induce neurotransmitter release from hippocampal slices (Lonart et al. 1992), and it is known to play a role in regulating hippocampal synaptic plasticity. The expression of NOS increases in various neurodegenerative diseases (Calabrese et al. 2007). Research has shown that expression of iNOS rises in both glial and nerve cells after exposure to A $\beta$  (Valles et al. 2010) or various inflammatory agents (Moncada et al. 1991; Yun et al. 1997), and it is prevented by treatment with genistein (Lu et al. 2009; Valles et al. 2010). In our study, genistein alleviated gliosis, and this was seen as decreased intensity of GFAP immunoreactivity. The mechanism of this effect of genistein is not known. However, previous studies have shown that inflammation-inducing agents such as  $A\beta$  can trigger NF- $\kappa$ B activation in astrocytes (Gonzalez-Velasquez et al. 2011) and thereby induce an inflammatory response, and this activation can be inhibited by genistein (Hsieh et al. 2011).

In conclusion, our findings suggest that genistein can inhibit the formation of  $A\beta$  deposits and the astrogliosis induced by injecting  $A\beta$  into the hippocampus.

# *Effects of genistein on astrocytes in an* $A\beta_{1-40}$ *rat model of* AD (*Paper IV*) *Qualitative observations*

*Sham-operated rats*. In the cortex, GFAP<sup>+</sup> astrocytes were small, sparsely distributed, and exhibited stellate morphology with multiple short branches, and the occurrence of these cells increased from layer 1 to layer 6. In the hippocampus, the CA1 subfield showed a few astrocytes with long branches, and the CA2 subfield displayed a dense network of small astrocytes with overlapping short branches. Both the DGmb and the DGlb exhibited weak GFAP immunoreactivity. In the area in focus in our morphometric analysis, the branches of each astrocyte either protruded symmetrically around the cell, thereby creating a stellate appearance, or they were asymmetrically arborized and pointed toward one side of the cell, with the nucleus located laterally in the cell body.

 $A\beta_{1\rightarrow 40}$ -injected rats. In these animals, the hippocampus from the CA1 subfield to the polymorph layer of the DG displayed extensive signs of GFAP immunoreactivity, particularly in the area of the DGlb that exhibited severe loss of neurons. The CA2 contained only a few GFAP<sup>+</sup> astrocytes, and the DGmb showed negative GFAP immunoreactivity. Overall, astrocytes in the hippocampus had multiple long branches that were either thin or thick. Most of the astrocytes were stellate in shape, and in some the nucleus was located laterally and the branches were directed towards one side of the cell.

 $A\beta_{1-40}$ -injected genistein-treated rats. In this group, the occurrence of GFAP<sup>+</sup> astrocytes in the cortex increased from layer 1 towards layer 6 in three of the rats, whereas such immunoreactivity was absent in the other two animals. The immunoreactivity was extremely

pronounced in the DGlb and the polymorphic layer of the hippocampus in the animals that exhibited neuronal degeneration, but it was weak in the rats with a normal DGlb. In three of the animals, the branches of the astrocytes in the hippocampus generally had short and thin branches, with a stellate form resembling that observed in the sham-operated rats. In the other two rats, the corresponding branches were long and thin, and many of the astrocytes had an atrophic appearance that included the lack of a distinct cell body and branches creating an irregular pattern with very little tertiary branching.

 $A\beta_{1-40}$ -injected rats treated with Cr-EL. The brains of these animals showed a pattern of gliosis that was very similar to that observed in the brains of the rats given only A $\beta$  injection (not discussed further here). Overall, astrocytes in the hippocampus of the four animals in this group had long branches of varying thickness, and many of them showed the atrophic pattern described for the genistein-treated rats.

#### Quantitative observations

All values obtained for the  $A\beta_{1-40}$ -injected rats given Cr-EL (with the exception of measurements of the surface area of both the soma and entire astrocyte) differed significantly from the corresponding values for the sham-operated rats, but showed the same pattern as the values for the  $A\beta_{1-40}$ -injected rats (not discussed further here).

*Astrocyte nucleus*. The mean volume of the nuclei in the astrocytes in the sham-operated rats was 663  $\mu$ m<sup>3</sup>. Injection of A $\beta_{1-40}$  led to a 37% increase in this parameter and a 27% increase in the surface area of the cells. Genistein treatment prevented the A $\beta_{1-40}$ -induced increase in nuclear volume and significantly decreased the increment of the surface area (P < 0.0001 vs. A $\beta$ -injected rats).

Astrocyte cell body (soma). Compared to astrocytes in the sham-operated group, those in the rats injected with  $A\beta_{1-40}$  showed a 23% increase in cell body volume and a 43% larger surface area (P < 0.0001). The  $A\beta_{1-40}$ -induced enlargement was significantly inhibited by treatment with genistein (volume P = 0.003 and surface area P < 0.0001 vs.  $A\beta$ -injected rats). Interestingly, genistein also reduced the enlargement of the cell body that was caused solely by insertion of the needle; this was indicated by the observation that, compared with astrocytes in the sham-operated animals, those in the genistein-treated rats had a 19% smaller mean cell body volume (P = 0.003) and a 6% smaller surface area (P < 0.0001).

*Total length of astrocyte branches*. Injection of  $A\beta_{1-40}$  caused a significant increase (15%; P = 0.004) in the total length of GFAP<sup>+</sup> branches, and this elongation was inhibited by genistein.

Astrocyte size (soma + branches). Injection of A $\beta_{1-40}$  caused an 11% increase in the volume (P = 0.03) and the surface area (P < 0.05) of the astrocytes, and both these increases were inhibited by genistein to a level that was even lower than that observed in the sham-operated rats (P < 0.0001 for volume; P < 0.001 for surface area).

Astrocyte territory. The functional territory of these cells was assessed by measuring the surface area and the volume of the tissue covered by individual astrocytes. Compared to the sham-operated rats, the animals that received an injection of  $A\beta_{1-40}$  showed increases of 22% in the mean territory volume (P < 0.0001) and 17% in the surface area (P < 0.004) of astrocytes, and genistein inhibited the effect of  $A\beta_{1-40}$  on the territory volume and also lessened the impact of the amyloid on territory surface area (P < 0.004).

*GFAP intensity*. Injection of A $\beta$  increased the presence of GFAP<sup>+</sup> astrocytes in the hippocampus by 135% (*P* = 0.0001), and this rise was inhibited by genistein.

Astrocytes are normally stellate in shape and have fine extending processes, but, depending on their location in the CNS, their morphology and size can be modified (Sullivan et al., 2010). This morphological transformation can occur fairly rapidly and requires redistribution of the cytoskeletal proteins (Safavi-Abbasi et al., 2001). In a diseased condition, such as the presence of large amount of A $\beta$  peptide in the brain, the astrocytic processes become convoluted and can exhibit swollen terminals. The results of our study (Paper IV) suggest that, when A $\beta_{1-40}$  is present in brain tissue, the astrocytes increase in 3D size so that can interact with a larger portion of the extracellular environment. The occurrence of reactive astrogliosis early after an injury is considered to be beneficial; this process can re-establish the chemical environment by removing harmful molecules and improve the physical environment by creating scar tissue that prevents spreading of harmful molecules to the healthy part of the tissue (Buffo et al. 2010).

In conclusion, the results of the current 3D confocal microscopy indicate that an astrocyte can enlarge the size of its nucleus, cell body, and branches in response to the presence of

 $A\beta_{1-40}$ . Furthermore, our findings suggest that genistein has anti-inflammatory properties and can inhibit  $A\beta_{1-40}$ -induced astrogliosis. Indeed, in our experiments, genistein ameliorated astrogliosis that was induced in the brains of rats by mechanical injury due to needle insertion.

## CONCLUSIONS

The findings of these studies show that:

- Genistein can ameliorate 6-OHDA-induced degeneration of dopaminergic neurons in a rat model of Parkinson's disease.

- Genistein can ameliorate  $A\beta_{1-40}$ -induced degeneration of hippocampal neurons in a rat model of Alzheimer's disease.

- Pretreatment with genistein prevents  $A\beta_{1-40}$ -induced impairments in aspects of learning and memory through a mechanism involving the estrogenic pathway and inhibition of oxidative stress.

- Genistein can inhibit the formation of  $A\beta$  deposits and astrogliosis induced by injecting  $A\beta$  into the hippocampus.

Collectively this shows that genistein has neuroprotective effects in animal models of Parkinson's and Alzheimer's disease.

## ACKNOWLEDGMENTS

The work of this thesis was carried out at the Department of Anatomy and Neuroscience, Iran University of Medical science, Tehran, Iran and at the Department of Clinical and Experimental Medicne, Linköping University, Sweden. I would like to express my sincere gratitude to both departments for their support. Also, I would like to express my gratitude to all the wonderful people who have helped and supported me during my time as a PhD student. In particular, thanks go to the following individuals:

Simin Mohseni, my supervisor and tutor. It has been a distinct honor to work with you. I thank you for providing me the opportunity to pursue my studies in your laboratory, for being so committed to my project, and for believing and trusting in me. You always manage to see the light when everything seems doomed to failure! I am also grateful to you for sharing with me your knowledge in the field of neuroscience and for giving me the freedom to develop my own ideas. Special thanks for the warm welcome into your family and for the very nice times you offered me, Sajjad, and Sepehr!

**Mehrdad Roghani**, for all the help that led to this project and for teaching me basic laboratory skills.

**Joghataei Mohammad-Taghi**, for excellent support in Tehran, good collaboration in organizing my PhD studies, and for sound advice in difficult moments.

**Jan Marcusson**, my former co-supervisor, and **David Engblom**, my current co-supervisor for your support.

**Per Hammarström** and, **Sofie Nyström** for your extensive knowledge about amyloid beta. Thank you for sharing your experience, your laboratory, and your time with me. I look forward to continue our collaboration in new project. Special thanks to you Sofie for always smiling and being so energetic.

**Arjang Rezakhani**, who joined us during a critical period, thank you for your extensive help in confocal microscopy—you brought positive energy to the lab and the office.

**Tourandokht Balouchnejad Mojarad**, for your scientific guidance and fruitful discussions during my master's work, as well as while I was pursuing my PhD. I also thank you for introducing me to the world of neuroscience.

**Aida**, for wonderful friendship and extremely useful support in confocal microscopy. You are a very good teacher!

Bengt-Arne Fredriksson, at the core facility, for friendly guidance and helpful assistance.

To all my friends on the 11th floor—Nina, Anna N, Anna E, Sara, Ulrika, Sofie, Namik, Johan R, Ana Maria, and Jakob—for creating a wonderful and enjoyable environment in which to work. Also Johan B, Björn, Ludmila, Camilla and Unn, for providing friendly and pleasant discussions during coffee breaks, and Fredrik and Anders for creating scientific environment, I will never forget the 11th floor!

**Mitra** and **Vahid**, my dear friends, I will never fail to remember your enormous help, and I am looking forward to having our weekly party again.

**My parents**, for your endless love and support. You are the best! Thank you for never hesitating to help me. I am sure you will always be there for me when I need you!

My dear sister **Mahsa**, for making things so pleasant at my apartment and for entertaining Sepehr while I was busy with my thesis.

And finally, for the most important person in my life, **Sajjad**. I cannot find the words to thank you enough. You always stand by my side. I never lose hope when I am with you, and I am so grateful that I get to be with you for the rest of my life.

#### REFERENCES

Adlercreutz H. 1999. Phytoestrogens. State of the art. Environ Toxicol Pharmacol 7:201-7.

- Amtul Z, Wang L, Westaway D, Rozmahel RF. 2010. Neuroprotective mechanism conferred by 17beta-estradiol on the biochemical basis of Alzheimer's disease. Neuroscience 169:781-6.
- An J, Tzagarakis-Foster C, Scharschmidt TC, Lomri N, Leitman DC. 2001. Estrogen receptor beta-selective transcriptional activity and recruitment of coregulators by phytoestrogens. J Biol Chem 276:17808-14.
- Antonini A, Poewe W. 2007. Fibrotic heart-valve reactions to dopamine-agonist treatment in Parkinson's disease. Lancet Neurol 6:826-9.
- Arosio B, Trabattoni D, Galimberti L, Bucciarelli P, Fasano F, Calabresi C, Cazzullo CL, Vergani C, Annoni G, Clerici M. 2004. Interleukin-10 and interleukin-6 gene polymorphisms as risk factors for Alzheimer's disease. Neurobiol Aging 25:1009-15.
- Badeau M, Tikkanen MJ, Appt SE, Adlercreutz H, Clarkson TB, Hoikkala A, Wahala K, Mikkola TS. 2005. Determination of plasma genistein fatty acid esters following administration of genistein or genistein 4'7-O-dioleate in monkeys. Biochim Biophys Acta 1738:115-20.
- Balldin J, Gottfries CG, Karlsson I, Lindstedt G, Langstrom G, Walinder J. 1983. Dexamethasone suppression test and serum prolactin in dementia disorders. Br J Psychiatry 143:277-81.
- Bang OY, Hong HS, Kim DH, Kim H, Boo JH, Huh K, Mook-Jung I. 2004. Neuroprotective effect of genistein against beta amyloid-induced neurotoxicity. Neurobiol Dis 16:21-8.
- Bartus RT, Dean RL, 3rd, Beer B, Lippa AS. 1982. The cholinergic hypothesis of geriatric memory dysfunction. Science 217:408-14.
- Ben Gedalya T, Loeb V, Israeli E, Altschuler Y, Selkoe DJ, Sharon R. 2009. Alpha-synuclein and polyunsaturated fatty acids promote clathrin-mediated endocytosis and synaptic vesicle recycling. Traffic 10:218-34.
- Bentahir M, Nyabi O, Verhamme J, Tolia A, Horre K, Wiltfang J, Esselmann H, De Strooper
  B. 2006. Presenilin clinical mutations can affect gamma-secretase activity by different mechanisms. J Neurochem 96:732-42.
- Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. 2000. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 3:1301-6.

- Bi X. 2010. Alzheimer disease: update on basic mechanisms. The Journal of the American Osteopathic Association 110:S3-9.
- Bilbul M, Schipper HM. 2011. Risk profiles of Alzheimer disease. Can J Neurol Sci 38:580-92.
- Bird TD. 2008. Genetic aspects of Alzheimer disease. Genet Med 10:231-9.
- Blum-Degen D, Haas M, Pohli S, Harth R, Romer W, Oettel M, Riederer P, Gotz ME. 1998. Scavestrogens protect IMR 32 cells from oxidative stress-induced cell death. Toxicol Appl Pharmacol 152:49-55.
- Borras C, Gambini J, Gomez-Cabrera MC, Sastre J, Pallardo FV, Mann GE, Vina J. 2006. Genistein, a soy isoflavone, up-regulates expression of antioxidant genes: involvement of estrogen receptors, ERK1/2, and NFkappaB. FASEB J 20:2136-8.
- Bourque M, Dluzen DE, Di Paolo T. 2009. Neuroprotective actions of sex steroids in Parkinson's disease. Front Neuroendocrinol 30:142-57.
- Boutajangout A, Authelet M, Blanchard V, Touchet N, Tremp G, Pradier L, Brion JP. 2004. Characterisation of cytoskeletal abnormalities in mice transgenic for wild-type human tau and familial Alzheimer's disease mutants of APP and presenilin-1. Neurobiol Dis 15:47-60.
- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-54.
- Brundin P, Barker RA, Parmar M. 2010. Neural grafting in Parkinson's disease Problems and possibilities. Prog Brain Res 184:265-94.
- Brzezinski A, Debi A. 1999. Phytoestrogens: the "natural" selective estrogen receptor modulators? Eur J Obstet Gynecol Reprod Biol 85:47-51.
- Buffo A, Rolando C, Ceruti S. 2010. Astrocytes in the damaged brain: molecular and cellular insights into their reactive response and healing potential. Biochem Pharmacol 79:77-89.
- Busciglio J, Lorenzo A, Yankner BA. 1992. Methodological variables in the assessment of beta amyloid neurotoxicity. Neurobiol Aging 13:609-12.
- Butterfield DA, Gnjec A, Poon HF, Castegna A, Pierce WM, Klein JB, Martins RN. 2006. Redox proteomics identification of oxidatively modified brain proteins in inherited Alzheimer's disease: an initial assessment. J Alzheimers Dis 10:391-7.

- Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA, Stella AM. 2007. Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. Nat Rev Neurosci 8:766-75.
- Calhoun ME, Kurth D, Phinney AL, Long JM, Hengemihle J, Mouton PR, Ingram DK, Jucker M. 1998. Hippocampal neuron and synaptophysin-positive bouton number in aging C57BL/6 mice. Neurobiol Aging 19:599-606.
- Cao X, Sudhof TC. 2001. A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. Science 293:115-20.
- Cataldo AM, Peterhoff CM, Troncoso JC, Gomez-Isla T, Hyman BT, Nixon RA. 2000. Endocytic pathway abnormalities precede amyloid beta deposition in sporadic Alzheimer's disease and Down syndrome: differential effects of APOE genotype and presenilin mutations. Am J Pathol 157:277-86.
- Chang HC, Churchwell MI, Delclos KB, Newbold RR, Doerge DR. 2000. Mass spectrometric determination of Genistein tissue distribution in diet-exposed Sprague-Dawley rats. J Nutr 130:1963-70.
- Chaudhuri KR, Schapira AH. 2009. Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. Lancet Neurol 8:464-74.
- Centers for Disease Control and Prevention. 2003. Public health and aging: trends in aging--United States and worldwide. JAMA-J Am Med Assoc 289:1371-3.
- Citron M, Westaway D, Xia W, Carlson G, Diehl T, Levesque G, Johnson-Wood K, Lee M, Seubert P, Davis A and others. 1997. Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice. Nat Med 3:67-72.
- Cohen FE, Kelly JW. 2003. Therapeutic approaches to protein-misfolding diseases. Nature 426:905-9.
- Cole GM, Frautschy SA. 2010. Mechanisms of action of non-steroidal anti-inflammatory drugs for the prevention of Alzheimer's disease. CNS Neurol Disord Drug Targets 9:140-8.
- Colom LV, Castaneda MT, Banuelos C, Puras G, Garcia-Hernandez A, Hernandez S, Mounsey S, Benavidez J, Lehker C. 2010. Medial septal beta-amyloid 1-40 injections alter septo-hippocampal anatomy and function. Neurobiol Aging 31:46-57.
- Colom LV, Castaneda MT, Hernandez S, Perry G, Jaime S, Touhami A. 2011. Intrahippocampal amyloid-beta (1-40) injections injure medial septal neurons in rats. Curr Alzheimer Res 8:832-40.

- Corti O, Lesage S, Brice A. 2011. What genetics tells us about the causes and mechanisms of Parkinson's disease. Physiol Rev 91:1161-218.
- Coyle JT, Price DL, DeLong MR. 1983. Alzheimer's disease: a disorder of cortical cholinergic innervation. Science 219:1184-90.
- Crews L, Masliah E. 2010. Molecular mechanisms of neurodegeneration in Alzheimer's disease. Hum Mol Genet 19:R12-20.
- Cuevas E, Lantz SM, Tobon-Velasco JC, Newport GD, Wu Q, Virmani A, Ali SF, Santamaria A. 2011. On the in vivo early toxic properties of A-beta 25-35 peptide in the rat hippocampus: involvement of the Receptor-for-Advanced Glycation-End-Products and changes in gene expression. Neurotoxicol Teratol 33:288-96.
- Cyr M, Calon F, Morissette M, Di Paolo T. 2002. Estrogenic modulation of brain activity: implications for schizophrenia and Parkinson's disease. J Psychiatry Neurosci 27:12-27.
- Czlonkowska A, Kurkowska-Jastrzebska I. 2011. Inflammation and gliosis in neurological diseases--clinical implications. J Neuroimmunol 231:78-85.
- Dauvois S, White R, Parker MG. 1993. The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. J Cell Sci 106 (Pt 4):1377-88.
- De Felice FG, Velasco PT, Lambert MP, Viola K, Fernandez SJ, Ferreira ST, Klein WL. 2007. Abeta oligomers induce neuronal oxidative stress through an N-methyl-Daspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine. J Biol Chem 282:11590-601.
- De Felice FG, Wu D, Lambert MP, Fernandez SJ, Velasco PT, Lacor PN, Bigio EH, Jerecic J, Acton PJ, Shughrue PJ and others. 2008. Alzheimer's disease-type neuronal tau hyperphosphorylation induced by A beta oligomers. Neurobiol Aging 29:1334-47.
- De Keukeleire D, De Cooman L, Rong H, Heyerick A, Kalita J, Milligan SR. 1999. Functional properties of hop polyphenols. Basic Life Sci 66:739-60.
- de Lau LM, Bornebroek M, Witteman JC, Hofman A, Koudstaal PJ, Breteler MM. Dietary fatty acids and the risk of Parkinson disease: the Rotterdam study. Neurology 64: 2040–45.
- de Lau LM, Breteler MM. 2006. Epidemiology of Parkinson's disease. Lancet Neurol 5: 525-35.
- Dehay B, Bezard E. 2011. New animal models of Parkinson's disease. Mov Disord 26:1198-1205.

- Devanand DP, Pradhaban G, Liu X, Khandji A, De Santi S, Segal S, Rusinek H, Pelton GH, Honig LS, Mayeux R and others. 2007. Hippocampal and entorhinal atrophy in mild cognitive impairment: prediction of Alzheimer disease. Neurology 68:828-36.
- Devine MJ, Plun-Favreau H, Wood NW. 2011. Parkinson's disease and cancer: two wars, one front. Nat Rev Cancer 11:812-23.
- Devore EE, Grodstein F, van Rooij FJ, Hofman A, Stampfer MJ, Witteman JC, Breteler MM. 2010. Dietary antioxidants and long-term risk of dementia. Arch Neurol 67:819-25.
- Driver JA, Logroscino G, Lu L, Gaziano JM, Kurth T. 2011. Use of non-steroidal antiinflammatory drugs and risk of Parkinson's disease: nested case-control study. BMJ 342:d198.
- Du H, Guo L, Yan S, Sosunov AA, McKhann GM, Yan SS. 2010. Early deficits in synaptic mitochondria in an Alzheimer's disease mouse model. P Natl Acad Sci USA 107:18670-5.
- Duff K, Eckman C, Zehr C, Yu X, Prada CM, Perez-tur J, Hutton M, Buee L, Harigaya Y, Yager D and others. 1996. Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1. Nature 383:710-3.
- Dwulet FE, Benson MD. 1986. Characterization of a transthyretin (prealbumin) variant associated with familial amyloidotic polyneuropathy type II (Indiana/Swiss). J Clin Invest 78:880-6.
- Ehrt U, Broich K, Larsen JP, Ballard C, Aarsland D. 2010. Use of drugs with anticholinergic effect and impact on cognition in Parkinson's disease: a cohort study. J Neurol Neurosurg Psychiatry 81:160-5.
- Feher A, Juhasz A, Rimanoczy A, Kalman J, Janka Z. 2009. Association between BDNF Val66Met polymorphism and Alzheimer disease, dementia with Lewy bodies, and Pick disease. Alz Dis Assoc Dis 23:224-8.
- File SE, Hartley DE, Alom N, Rattray M. 2003. Soya phytoestrogens change cortical and hippocampal expression of BDNF mRNA in male rats. Neurosci Lett 338:135-8.
- File SE, Jarrett N, Fluck E, Duffy R, Casey K, Wiseman H. 2001. Eating soya improves human memory. Psychopharmacology (Berl) 157:430-6.
- Flood JF, Morley JE, Roberts E. 1991. Amnestic effects in mice of four synthetic peptides homologous to amyloid beta protein from patients with Alzheimer disease. P Natl Acad Sci USA 88:3363-6.
- Forstl H, Kurz A. 1999. Clinical features of Alzheimer's disease. Eur Arch Psychiatry Clin Neurosci 249:288-90.

- Games D, Adams D, Alessandrini R, Barbour R, Berthelette P, Blackwell C, Carr T, Clemens J, Donaldson T, Gillespie F and others. 1995. Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. Nature 373:523-7.
- Glenner GG, Wong CW. 1984. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Bioph Res Co 120:885-90.
- Goedert M, Wischik CM, Crowther RA, Walker JE, Klug A. 1988. Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated protein tau. P Natl Acad Sci USA 85:4051-5.
- Gonzalez-Velasquez FJ, Reed JW, Fuseler JW, Matherly EE, Kotarek JA, Soto-Ortega DD, Moss MA. 2011. Activation of brain endothelium by soluble aggregates of the amyloid-beta protein involves nuclear factor-kappaB. Curr Alzheimer Res 8:81-94.
- Goodman Y, Bruce AJ, Cheng B, Mattson MP. 1996. Estrogens attenuate and corticosterone exacerbates excitotoxicity, oxidative injury, and amyloid beta-peptide toxicity in hippocampal neurons. J Neurochem 66:1836-44.
- Green LE, Dinh TA, Smith RA. 2012. An estrogen model: the relationship between body mass index, menopausal status, estrogen replacement therapy, and breast cancer risk. Comput Math Methods Med 2012:792375.
- Guan JZ, Guan WP, Maeda T, Makino N. 2012. Effect of vitamin E administration on the elevated oxygen stress and the telomeric and subtelomeric status in Alzheimer's disease. Gerontology 58:62-9.
- Gutzmann H, Hadler D. 1998. Sustained efficacy and safety of idebenone in the treatment of Alzheimer's disease: update on a 2-year double-blind multicentre study. J Neural Transm Suppl 54:301-10.
- Haass C, Selkoe DJ. 2007. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Bio 8:101-12.
- Hardy J, Allsop D. 1991. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Pharmacol Sci 12:383-8.
- Hardy J, Selkoe DJ. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353-6.

- Hawkins MB, Thornton JW, Crews D, Skipper JK, Dotte A, Thomas P. 2000. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc Natl Acad Sci U S A 97:10751-6.
- Hebert LE, Beckett LA, Scherr PA, Evans DA. 2001. Annual incidence of Alzheimer disease in the United States projected to the years 2000 through 2050. Alz Dis Assoc Dis 15:169-73.
- Heman MA, Zhang SM, Rueda-deCastro AM, Colditz GA, Speizer FE, Ascherio A. 2001. Cigarette smoking and the incidence of Parkinson's disease in two prospective studies. Ann Neurol 50: 780–86.
- Henchcliffe C, Beal MF. 2008. Mitochondrial biology and oxidative stress in Parkinson disease pathogenesis. Nat Clin Pract Neurol 4:600-9.
- Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G. 1996. Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. Science 274:99-102.
- Hsieh HM, Wu WM, Hu ML. 2011. Genistein attenuates D-galactose-induced oxidative damage through decreased reactive oxygen species and NF-kappaB binding activity in neuronal PC12 cells. Life Sci 88:82-8.
- Imbimbo BP. 2009. An update on the efficacy of non-steroidal anti-inflammatory drugs in Alzheimer's disease. Expert Opin Investig Drugs 18:1147-68.
- Investigators PSGP. 2011. A randomized pilot trial of estrogen replacement therapy in postmenopausal women with Parkinson's disease. Parkinsonism Relat Disord 17:757-60.
- Irizarry MC, McNamara M, Fedorchak K, Hsiao K, Hyman BT. 1997. APPSw transgenic mice develop age-related A beta deposits and neuropil abnormalities, but no neuronal loss in CA1. J Neuropathol Exp Neurol 56:965-73.
- Itoh A, Nitta A, Katono Y, Usui M, Naruhashi K, Iida R, Hasegawa T, Nabeshima T. 1997. Effects of metrifonate on memory impairment and cholinergic dysfunction in rats. Eur J Pharmacol 322:11-9.
- Iwatsubo T, Mann DM, Odaka A, Suzuki N, Ihara Y. 1995. Amyloid beta protein (A beta) deposition: A beta 42(43) precedes A beta 40 in Down syndrome. Annals of neurology 37:294-9.
- Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y. 1994. Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43). Neuron 13:45-53.

- Jacobsen KT, Iverfeldt K. 2009. Amyloid precursor protein and its homologues: a family of proteolysis-dependent receptors. Cellular and molecular life sciences : CMLS 66:2299-318.
- Jarrett JT, Berger EP, Lansbury PT, Jr. 1993. The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. Biochemistry 32:4693-7.
- Jauhiainen AM, Pihlajamaki M, Tervo S, Niskanen E, Tanila H, Hanninen T, Vanninen RL, Soininen H. 2009. Discriminating accuracy of medial temporal lobe volumetry and fMRI in mild cognitive impairment. Hippocampus 19:166-75.
- Javoy F, Sotelo C, Herbet A, Agid Y. 1976. Specificity of dopaminergic neuronal degeneration induced by intracerebral injection of 6-hydroxydopamine in the nigrostriatal dopamine system. Brain Res 102:201-15.
- Jellinger KA, Attems J. 2010. Prevalence of dementia disorders in the oldest-old: an autopsy study. Acta neuropathologica 119:421-33.
- Ji C, Song C, Zuo P. 2011. The mechanism of memory impairment induced by Abeta chronic administration involves imbalance between cytokines and neurotrophins in the rat hippocampus. Curr Alzheimer Res 8:410-20.
- Kalaria RN. 2000. The role of cerebral ischemia in Alzheimer's disease. Neurobiol Aging 21:321-30.
- Kalinderi K, Fidani L, Katsarou Z, Bostantjopoulou S. 2011. Pharmacological treatment and the prospect of pharmacogenetics in Parkinson's disease. Int J Clin Pract 65:1289-94.
- Kammesheidt A, Kato K, Ito K, Sumikawa K. 1997. Adenovirus-mediated NMDA receptor knockouts in the rat hippocampal CA1 region. Neuroreport 8:635-8.
- Kawamata J, Shimohama S. 2011. Stimulating nicotinic receptors trigger multiple pathways attenuating cytotoxicity in models of Alzheimer's and Parkinson's diseases. J Alzheimers Dis 24 Suppl 2:95-109.
- Kayed R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, Glabe CG. 2003. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science 300:486-9.
- Kelly GE, Joannou GE, Reeder AY, Nelson C, Waring MA. 1995. The variable metabolic response to dietary isoflavones in humans. Proc Soc Exp Biol Med 208:40-3.
- Kim HJ, Chae SC, Lee DK, Chromy B, Lee SC, Park YC, Klein WL, Krafft GA, Hong ST. 2003. Selective neuronal degeneration induced by soluble oligomeric amyloid beta protein. FASEB J 17:118-20.

- King RA, Broadbent JL, Head RJ. 1996. Absorption and excretion of the soy isoflavone genistein in rats. J Nutr 126:176-82.
- Kitaoka M, Kadokawa H, Sugano M, Ichikawa K, Taki M, Takaishi S, Iijima Y, Tsutsumi S, Boriboon M, Akiyama T. 1998. Prenylflavonoids: a new class of non-steroidal phytoestrogen (Part 1). Isolation of 8-isopentenylnaringenin and an initial study on its structure-activity relationship. Planta Med 64:511-5.
- Kivipelto M, Helkala EL, Laakso MP, Hanninen T, Hallikainen M, Alhainen K, Iivonen S, Mannermaa A, Tuomilehto J, Nissinen A and others. 2002. Apolipoprotein E epsilon4 allele, elevated midlife total cholesterol level, and high midlife systolic blood pressure are independent risk factors for late-life Alzheimer disease. Ann Intern Med 137:149-55.
- Klivenyi P, Vecsei L. 2011. Pharmacological models of Parkinson's disease in rodents. Methods Mol Biol 793:211-27.
- Knobloch M, Mansuy IM. 2008. Dendritic spine loss and synaptic alterations in Alzheimer's disease. Mol Neurobiol 37:73-82.
- Koprich JB, Johnston TH, Huot P, Reyes MG, Espinosa M, Brotchie JM. 2011. Progressive neurodegeneration or endogenous compensation in an animal model of Parkinson's disease produced by decreasing doses of alpha-synuclein. PLoS One 6:e17698.
- Kowall NW, Beal MF, Busciglio J, Duffy LK, Yankner BA. 1991. An in vivo model for the neurodegenerative effects of beta amyloid and protection by substance P. P Natl Acad Sci USA 88:7247-51.
- Kunugi H, Ueki A, Otsuka M, Isse K, Hirasawa H, Kato N, Nabika T, Kobayashi S, Nanko S. 2001. A novel polymorphism of the brain-derived neurotrophic factor (BDNF) gene associated with late-onset Alzheimer's disease. Mol Psychiatry 6:83-6.
- Kurzer MS, Xu X.1997. Dietary phytoestrogens. Annu Rev Nutr 17:353-81.
- Lacor PN, Buniel MC, Furlow PW, Clemente AS, Velasco PT, Wood M, Viola KL, Klein WL. 2007. Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:796-807.
- Lai BC, Marion SA, Teschke K, Tsui JK. 2002. Occupational and environmental risk factors for Parkinson's disease. Parkinsonism Relat Disord 8:297-309.
- Lain AH, Lieberman AP, Pfannl R, Hedley-Whyte ET. 2010. Nodular bilateral amygdala degeneration in demented individuals. Acta neuropathologica 120:683-8.

- Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL and others. 1998. Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. P Natl Acad Sci USA 95:6448-53.
- Lazo ND, Maji SK, Fradinger EA, Bitan G, Teplow DB. 2008. The Amyloid β Protein. Amyloid Proteins: Wiley-VCH Verlag GmbH. p 384-491.
- Lemere CA, Blusztajn JK, Yamaguchi H, Wisniewski T, Saido TC, Selkoe DJ. 1996. Sequence of deposition of heterogeneous amyloid beta-peptides and APO E in Down syndrome: implications for initial events in amyloid plaque formation. Neurobiol Dis 3:16-32.
- Lewis J, Dickson DW, Lin WL, Chisholm L, Corral A, Jones G, Yen SH, Sahara N, Skipper L, Yager D and others. 2001. Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. Science 293:1487-91.
- Li C, Zhao R, Gao K, Wei Z, Yin MY, Lau LT, Chui D, Hoi Yu AC. 2011. Astrocytes: implications for neuroinflammatory pathogenesis of Alzheimer's disease. Curr Alzheimer Res 8:67-80.
- Lio D, Licastro F, Scola L, Chiappelli M, Grimaldi LM, Crivello A, Colonna-Romano G, Candore G, Franceschi C, Caruso C. 2003. Interleukin-10 promoter polymorphism in sporadic Alzheimer's disease. Genes Immun 4:234-8.
- Lonart G, Wang J, Johnson KM. 1992. Nitric oxide induces neurotransmitter release from hippocampal slices. Eur J Pharmacol 220:271-2.
- Lu H, Shi JX, Zhang DM, Wang HD, Hang CH, Chen HL, Yin HX. 2009. Inhibition of hemolysate-induced iNOS and COX-2 expression by genistein through suppression of NF-small ka, CyrillicB activation in primary astrocytes. J Neurol Sci 278:91-5.
- Makin OS, Atkins E, Sikorski P, Johansson J, Serpell LC. 2005. Molecular basis for amyloid fibril formation and stability. P Natl Acad Sci USA 102:315-20.
- Mancuso M, Orsucci D, LoGerfo A, Calsolaro V, Siciliano G. 2010. Clinical features and pathogenesis of Alzheimer's disease: involvement of mitochondria and mitochondrial DNA. Adv Exp Med Biol 685:34-44.
- Martin-Moreno AM, Brera B, Spuch C, Carro E, Garcia-Garcia L, Delgado M, Pozo MA, Innamorato NG, Cuadrado A, de Ceballos ML. 2012. Prolonged oral cannabinoid administration prevents neuroinflammation, lowers beta-amyloid levels and improves cognitive performance in Tg APP 2576 mice. J Neuroinflammation 9:8.

- Mateos L, Persson T, Kathozi S, Gil-Bea FJ, Cedazo-Minguez A. 2012. Estrogen protects against amyloid-beta toxicity by estrogen receptor alpha-mediated inhibition of Daxx translocation. Neurosci Lett 506:245-50.
- McDonald MP, Overmier JB. 1998. Present imperfect: a critical review of animal models of the mnemonic impairments in Alzheimer's disease. Neurosci Biobehav Rev 22:99-120.
- McGeer PL, Rogers J. 1992. Anti-inflammatory agents as a therapeutic approach to Alzheimer's disease. Neurology 42:447-9.
- Meredith GE, Sonsalla PK, Chesselet MF. 2008. Animal models of Parkinson's disease progression. Acta neuropathologica 115:385-98.
- Michikawa M, Fan QW, Isobe I, Yanagisawa K. 2000. Apolipoprotein E exhibits isoformspecific promotion of lipid efflux from astrocytes and neurons in culture. J Neurochem 74:1008-16.
- Miller DL, Papayannopoulos IA, Styles J, Bobin SA, Lin YY, Biemann K, Iqbal K. 1993. Peptide compositions of the cerebrovascular and senile plaque core amyloid deposits of Alzheimer's disease. Arch Biochem Biophys 301:41-52.
- Mitsuyama F, Futatsugi Y, Okuya M, Karagiozov K, Peev N, Kato Y, Kanno T, Sano H, Koide T. 2009. Amyloid beta: a putative intra-spinal microtubule-depolymerizer to induce synapse-loss or dentritic spine shortening in Alzheimer's disease. Italian journal of anatomy and embryology = Archivio italiano di anatomia ed embriologia 114:109-20.
- Moncada S, Palmer RM, Higgs EA. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 43:109-42.
- Moosmann B, Behl C. 1999. The antioxidant neuroprotective effects of estrogens and phenolic compounds are independent from their estrogenic properties. P Natl Acad Sci USA 96:8867-72.
- Morano A, Jimenez-Jimenez FJ, Molina JA, Antolin MA. 1994. Risk-factors for Parkinson's disease: case-control study in the province of Caceres, Spain. Acta Neurol Scand 89:164-70.
- Mura T, Dartigues JF, Berr C. 2010. How many dementia cases in France and Europe? Alternative projections and scenarios 2010-2050. European journal of neurology : the official journal of the European Federation of Neurological Societies 17:252-9.
- Murakami K, Miyake Y, Sasaki S, Tanaka K, Fukushima W, Kiyohara C, Tsuboi Y, Yamada T, Oeda T, Miki T and others. 2010. Dietary intake of folate, vitamin B6, vitamin B12

and riboflavin and risk of Parkinson's disease: a case-control study in Japan. Br J Nutr 104:757-64.

- Nitta A, Fukuta T, Hasegawa T, Nabeshima T. 1997. Continuous infusion of beta-amyloid protein into the rat cerebral ventricle induces learning impairment and neuronal and morphological degeneration. Jpn J Pharmacol 73:51-7.
- Nitta A, Itoh A, Hasegawa T, Nabeshima T. 1994. beta-Amyloid protein-induced Alzheimer's disease animal model. Neurosci Lett 170:63-6.
- Nixon RA. 2007. Autophagy, amyloidogenesis and Alzheimer disease. J Cell Sci 120:4081-91.
- Ogawara H, Akiyama T, Watanabe S, Ito N, Kobori M, Seoda Y. 1989. Inhibition of tyrosine protein kinase activity by synthetic isoflavones and flavones. J Antibiot (Tokyo) 42:340-3.
- Olcese JM, Cao C, Mori T, Mamcarz MB, Maxwell A, Runfeldt MJ, Wang L, Zhang C, Lin X, Zhang G and others. 2009. Protection against cognitive deficits and markers of neurodegeneration by long-term oral administration of melatonin in a transgenic model of Alzheimer disease. J Pineal Res 47:82-96.
- Olesen J, Gustavsson A, Svensson M, Wittchen HU, Jonsson B. 2012. The economic cost of brain disorders in Europe. European journal of neurology : the official journal of the European Federation of Neurological Societies 19:155-162.
- Ono K, Yamada M. 2011. Vitamin A and Alzheimer's disease. Geriatr Gerontol Int.
- Ortiz J, Harris HW, Guitart X, Terwilliger RZ, Haycock JW, Nestler EJ. 1995. Extracellular signal-regulated protein kinases (ERKs) and ERK kinase (MEK) in brain: regional distribution and regulation by chronic morphine. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:1285-97.
- Ososki AL, Kennelly EJ. 2003. Phytoestrogens: a review of the present state of research. Phytother Res 17:845-69.
- Palacios HH, Yendluri BB, Parvathaneni K, Shadlinski VB, Obrenovich ME, Leszek J, Gokhman D, Gasiorowski K, Bragin V, Aliev G. 2011. Mitochondrion-specific antioxidants as drug treatments for Alzheimer disease. CNS Neurol Disord Drug Targets 10:149-62.
- Pan Y, Anthony M, Clarkson TB. 1999. Effect of estradiol and soy phytoestrogens on choline acetyltransferase and nerve growth factor mRNAs in the frontal cortex and hippocampus of female rats. Proc Soc Exp Biol Med 221:118-25.

- Parkinson J. 1817. An essay on the shaking palsy: Printed by Whittingham and Rowland for Sherwood, Neely, and Jones.
- Parron T, Requena M, Hernandez AF, Alarcon R. 2011. Association between environmental exposure to pesticides and neurodegenerative diseases. Toxicol Appl Pharmacol 256:379-85.
- Paxinos G, Watson C. 1998. The rat atlas in stereotaxic coordinates. New York: Academic.
- Pennanen C, Kivipelto M, Tuomainen S, Hartikainen P, Hanninen T, Laakso MP, Hallikainen M, Vanhanen M, Nissinen A, Helkala EL and others. 2004. Hippocampus and entorhinal cortex in mild cognitive impairment and early AD. Neurobiol Aging25:303-10.
- Pepeu G, Giovannelli L, Casamenti F, Scali C, Bartolini L. 1996. Amyloid beta-peptides injection into the cholinergic nuclei: morphological, neurochemical and behavioral effects. Prog Brain Res 109:273-82.
- Perez RG, Waymire JC, Lin E, Liu JJ, Guo F, Zigmond MJ. 2002. A role for alpha-synuclein in the regulation of dopamine biosynthesis. J Neurosci 22:3090-9.
- Perl DP. 2010. Neuropathology of Alzheimer's disease. Mt Sinai J Med 77:32-42.
- Pickrell AM, Pinto M, Hida A, Moraes CT. 2011. Striatal dysfunctions associated with mitochondrial DNA damage in dopaminergic neurons in a mouse model of Parkinson's disease. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:17649-58.
- Prasad KN, Cole WC, Prasad KC. 2002. Risk factors for Alzheimer's disease: role of multiple antioxidants, non-steroidal anti-inflammatory and cholinergic agents alone or in combination in prevention and treatment. J Am Coll Nutr 21:506-22.
- Qin X, Peng Q, Zeng Z, Chen Z, Lin L, Deng Y, Huang X, Xu J, Wu H, Huang S and others. 2012. Interleukin-1A -889C/T polymorphism and risk of Alzheimer's disease: a metaanalysis based on 32 case-control studies. J Neurol. (Epub ahead of print).
- Qiu J, Bosch MA, Tobias SC, Grandy DK, Scanlan TS, Ronnekleiv OK, Kelly MJ. 2003.
   Rapid signaling of estrogen in hypothalamic neurons involves a novel G-proteincoupled estrogen receptor that activates protein kinase C. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:9529-40.
- Rafi MM, Rosen RT, Vassil A, Ho CT, Zhang H, Ghai G, Lambert G, DiPaola RS. 2000. Modulation of bcl-2 and cytotoxicity by licochalcone-A, a novel estrogenic flavonoid. Anticancer Res 20:2653-8.

- Rapp A, Gmeiner B, Huttinger M. 2006. Implication of apoE isoforms in cholesterol metabolism by primary rat hippocampal neurons and astrocytes. Biochimie 88:473-83.
- Raux G, Guyant-Marechal L, Martin C, Bou J, Penet C, Brice A, Hannequin D, Frebourg T, Campion D. 2005. Molecular diagnosis of autosomal dominant early onset Alzheimer's disease: an update. J Med Genet 42:793-5.
- Raygani AV, Zahrai M, Doosti M, Javadi E, Rezaei M, Pourmotabbed T. 2005. Association between apolipoprotein E polymorphism and Alzheimer disease in Tehran, Iran. Neurosci Lett 375:1-6.
- Reddy PH. 2006. Amyloid precursor protein-mediated free radicals and oxidative damage: implications for the development and progression of Alzheimer's disease. J Neurochem 96:1-13.
- Reiter RJ, Carneiro RC, Oh CS. 1997. Melatonin in relation to cellular antioxidative defense mechanisms. Horm Metab Res 29:363-72.
- Ren R, Zhang Y, Li B, Wu Y. 2011. Effect of beta-amyloid (25-35) on mitochondrial function and expression of mitochondrial permeability transition pore proteins in rat hippocampal neurons. J Cell Biochem 112:1450-7.
- Resende R, Pereira C, Agostinho P, Vieira AP, Malva JO, Oliveira CR. 2007. Susceptibility of hippocampal neurons to Abeta peptide toxicity is associated with perturbation of Ca2+ homeostasis. Brain Res 1143:11-21.
- Riemenschneider M, Schwarz S, Wagenpfeil S, Diehl J, Muller U, Forstl H, Kurz A. 2002. A polymorphism of the brain-derived neurotrophic factor (BDNF) is associated with Alzheimer's disease in patients lacking the Apolipoprotein E epsilon4 allele. Mol Psychiatry 7:782-5.
- Robertson JF, Harrison M. 2004. Fulvestrant: pharmacokinetics and pharmacology. Br J Cancer 90 Suppl 1:S7-10.
- Rodriguez JJ, Olabarria M, Chvatal A, Verkhratsky A. 2009. Astroglia in dementia and Alzheimer's disease. Cell Death Differ 16:378-85.
- Roher AE, Lowenson JD, Clarke S, Woods AS, Cotter RJ, Gowing E, Ball MJ. 1993. beta-Amyloid-(1-42) is a major component of cerebrovascular amyloid deposits: implications for the pathology of Alzheimer disease. P Natl Acad Sci USA 90:10836-40.
- Ross GW, Abbott RD, Petrovitch H, Morens DM, Grandinetti A, Tung KH, Tanner CM, Masaki KH, Blanchette PL, Curb JD and others. 2000. Association of coffee and

caffeine intake with the risk of Parkinson disease. JAMA-J Am Med Assoc 283:2674-9.

- Rudzinski LA, Fletcher RM, Dickson DW, Crook R, Hutton ML, Adamson J, Graff-Radford NR. 2008. Early onset familial Alzheimer Disease with spastic paraparesis, dysarthria, and seizures and N135S mutation in PSEN1. Alzheimer disease and associated disorders 22:299-307.
- Rusin A, Krawczyk Z, Grynkiewicz G, Gogler A, Zawisza-Puchalka J, Szeja W. 2010. Synthetic derivatives of genistein, their properties and possible applications. Acta Biochim Pol 57:23-34.
- Safavi-Abbasi S, Wolff JR, Missler M. 2001. Rapid morphological changes in astrocytes are accompanied by redistribution but not by quantitative changes of cytoskeletal proteins. Glia 36:102-15.
- Santti R, Makela S, Strauss L, Korkman J, Kostian ML. 1998. Phytoestrogens: potential endocrine disruptors in males. Toxicol Ind Health 14:223-37.
- Sarroukh R, Cerf E, Derclaye S, Dufrene YF, Goormaghtigh E, Ruysschaert JM, Raussens V. 2011. Transformation of amyloid beta(1-40) oligomers into fibrils is characterized by a major change in secondary structure. Cellular and molecular life sciences : CMLS 68:1429-38.
- Sassi-Messai S, Gibert Y, Bernard L, Nishio S, Ferri Lagneau KF, Molina J, Andersson-Lendahl M, Benoit G, Balaguer P, Laudet V. 2009. The phytoestrogen genistein affects zebrafish development through two different pathways. PLoS One 4:e4935.
- Scheuner D, Eckman C, Jensen M, Song X, Citron M, Suzuki N, Bird TD, Hardy J, Hutton M, Kukull W and others. 1996. Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. Nat Med 2:864-70.
- Schliebs R, Arendt T. 2011. The cholinergic system in aging and neuronal degeneration. Behav Brain Res 221:555-63.
- Schrag M, Mueller C, Oyoyo U, Smith MA, Kirsch WM. 2011. Iron, zinc and copper in the Alzheimer's disease brain: a quantitative meta-analysis. Some insight on the influence of citation bias on scientific opinion. Prog Neurobiol 94:296-306.
- Schwarting RK, Huston JP. 1996. The unilateral 6-hydroxydopamine lesion model in behavioral brain research. Analysis of functional deficits, recovery and treatments. Prog Neurobiol 50:275-331.

- Schwarting RK, Huston JP. 1997. Behavioral and neurochemical dynamics of neurotoxic meso-striatal dopamine lesions. Neurotoxicology 18:689-708.
- Seibel J, Molzberger AF, Hertrampf T, Laudenbach-Leschowski U, Diel P. 2009. Oral treatment with genistein reduces the expression of molecular and biochemical markers of inflammation in a rat model of chronic TNBS-induced colitis. Eur J Nutr 48:213-20.
- Setchell KD, Zimmer-Nechemias L, Cai J, Heubi JE. 1997. Exposure of infants to phytooestrogens from soy-based infant formula. Lancet 350:23-7.
- Shah RS, Lee HG, Xiongwei Z, Perry G, Smith MA, Castellani RJ. 2008. Current approaches in the treatment of Alzheimer's disease. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 62:199-207.
- Shapiro RM, Glick SD, Camarota NA. 1987. A two-population model of rat rotational behavior: effects of unilateral nigrostriatal 6-hydroxydopamine on striatal neurochemistry and amphetamine-induced rotation. Brain Res 426:323-31.
- Simpkins JW, Green PS, Gridley KE, Singh M, de Fiebre NC, Rajakumar G. 1997. Role of estrogen replacement therapy in memory enhancement and the prevention of neuronal loss associated with Alzheimer's disease. Am J Med 103:19S-25S.
- Singh M, Dykens JA, Simpkins JW. 2006. Novel mechanisms for estrogen-induced neuroprotection. Exp Biol Med (Maywood) 231:514-21.
- Singh M, Setalo G, Jr., Guan X, Warren M, Toran-Allerand CD. 1999. Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:1179-88.
- Sirtori CR, Arnoldi A, Johnson SK. 2005. Phytoestrogens: end of a tale? Ann Med 37:423-38.
- Sofroniew MV. 2009. Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci 32:638-47.
- Sofroniew MV, Vinters HV. 2010. Astrocytes: biology and pathology. Acta neuropathologica 119:7-35.
- Steyn FJ, Anderson GM, Grattan DR. 2007. Differential effects of centrally-administered oestrogen antagonist ICI-182,780 on oestrogen-sensitive functions in the hypothalamus. J Neuroendocrinol 19:26-33.
- Stranahan AM, Martin B, Maudsley S. 2012. Anti-inflammatory effects of physical activity in relationship to improved cognitive status in humans and mouse models of Alzheimer's disease. Curr Alzheimer Res 9:86-92.

- Sullivan SM, Bjorkman ST, Miller SM, Colditz PB, Pow DV. 2010. Structural remodeling of gray matter astrocytes in the neonatal pig brain after hypoxia/ischemia. Glia 58:181-94.
- Sun X, He G, Qing H, Zhou W, Dobie F, Cai F, Staufenbiel M, Huang LE, Song W. 2006. Hypoxia facilitates Alzheimer's disease pathogenesis by up-regulating BACE1 gene expression. P Natl Acad Sci USA 103:18727-32.
- Suzuki N, Iwatsubo T, Odaka A, Ishibashi Y, Kitada C, Ihara Y. 1994. High tissue content of soluble beta 1-40 is linked to cerebral amyloid angiopathy. Am J Pathol 145:452-60.
- Swerdlow RH, Burns JM, Khan SM. 2010. The Alzheimer's disease mitochondrial cascade hypothesis. J Alzheimers Dis 20 Suppl 2:S265-79.
- Taghizadeh M, Djazayery A, Salami M, Eshraghian MR, Zavareh SA. 2011. Vitamin-D-free regimen intensifies the spatial learning deficit in Alzheimer's disease. Int J Neurosci 121:16-24.
- Tagliavini F, Giaccone G, Linoli G, Frangione B, Bugiani O. 1989. Cerebral extracellular preamyloid deposits in Alzheimer's disease, Down syndrome and nondemented elderly individuals. Prog Clin Biol Res 317:1001-5.
- Tanaka T, Yamada K, Senzaki K, Narimatsu H, Nishimura K, Kameyama T, Nabeshima T. 1998. NC-1900, an active fragment analog of arginine vasopressin, improves learning and memory deficits induced by beta-amyloid protein in rats. Eur J Pharmacol 352:135-42.
- Tanaka K., Miyake Y., Fukushima W., Sasaki S, Kiyohara C., Tsuboi Y., Yamada T., Oeda T., Miki T., Kawamura N., Sakae N., Fukuyama H., Hirota Y., Nagai M., Fukuoka Kinki Parkinson's Disease Study Group., 2011. Occupational risk factors for Parkinson's disease: a case-control study in Japan. BMC Neurol 11: 83.
- Tang MX, Jacobs D, Stern Y, Marder K, Schofield P, Gurland B, Andrews H, Mayeux R. 1996. Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. Lancet 348:429-32.
- Theuns J, Del-Favero J, Dermaut B, van Duijn CM, Backhovens H, Van den Broeck MV, Serneels S, Corsmit E, Van Broeckhoven CV, Cruts M. 2000. Genetic variability in the regulatory region of presenilin 1 associated with risk for Alzheimer's disease and variable expression. Hum Mol Genet 9:325-31.
- Tillement L, Lecanu L, Papadopoulos V. 2011. Alzheimer's disease: effects of beta-amyloid on mitochondria. Mitochondrion 11:13-21.

- Tiwari V, Patel AB. 2012. Impaired Glutamatergic and GABAergic Function at Early Age in AbetaPPswe-PS1dE9 Mice: Implications for Alzheimer's Disease. J Alzheimers Dis 28:765-9.
- Ton TG, Heckbert SR, Longstreth WT, Jr., Rossing MA, Kukull WA, Franklin GM, Swanson PD, Smith-Weller T, Checkoway H. 2006. Nonsteroidal anti-inflammatory drugs and risk of Parkinson's disease. Mov Disord 21:964-9.
- Tzourio C, Anderson C, Chapman N, Woodward M, Neal B, MacMahon S, Chalmers J. 2003. Effects of blood pressure lowering with perindopril and indapamide therapy on dementia and cognitive decline in patients with cerebrovascular disease. Arch Intern Med 163:1069-75.
- Valles SL, Dolz-Gaiton P, Gambini J, Borras C, Lloret A, Pallardo FV, Vina J. 2010. Estradiol or genistein prevent Alzheimer's disease-associated inflammation correlating with an increase PPAR gamma expression in cultured astrocytes. Brain Res 1312:138-44.
- van der Schouw YT, de Kleijn MJ, Peeters PH, Grobbee DE. 2000. Phyto-oestrogens and cardiovascular disease risk. Nutr Metab Cardiovasc Dis 10:154-67.
- Vanessa de Jesus R, Guimarães FM, Diniz BS, Forlenza OV. 2009. Neurobiological pathways to Alzheimer's disease: amyloid-beta, Tau protein or both? Dement Neuropsychol 3: 188-194
- Wade GN, Blaustein JD, Gray JM, Meredith JM. 1993. ICI 182,780: a pure antiestrogen that affects behaviors and energy balance in rats without acting in the brain. Am J Physiol 265:R1392-8.
- Wahner AD, Bronstein JM, Bordelon YM, Ritz B. 2007. Nonsteroidal anti-inflammatory drugs may protect against Parkinson disease. Neurology 69:1836-42.
- Wilquet V, De Strooper B. 2004. Amyloid-beta precursor protein processing in neurodegeneration. Curr Opin Neurobiol 14:582-8.
- Winkler J, Thal LJ, Gage FH, Fisher LJ. 1998. Cholinergic strategies for Alzheimer's disease. J Mol Med (Berl) 76:555-67.
- Wollen KA. 2010. Alzheimer's disease: the pros and cons of pharmaceutical, nutritional, botanical, and stimulatory therapies, with a discussion of treatment strategies from the perspective of patients and practitioners. Altern Med Rev 15:223-44.
- Woodruff-Pak DS. 2008. Animal models of Alzheimer's disease: therapeutic implications. J Alzheimers Dis 15:507-21.

- Wright JS, Johnson ER, DiLabio GA. 2001. Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. J Am Chem Soc 123:1173-83.
- Xiao M, Cao GL, Marshall C, Hu G. 2010. Hypothesis: multiple factors are associated with the lack of any beneficial effects of oestrogen-replacement therapy in the late postmenopausal stage. Clin Exp Pharmacol Physiol 37:873-6.
- Yamada K, Nabeshima T. 2000. Animal models of Alzheimer's disease and evaluation of anti-dementia drugs. Pharmacol Ther 88:93-113.
- Yamada K, Ren X, Nabeshima T. 1999a. Perspectives of pharmacotherapy in Alzheimer's disease. Jpn J Pharmacol 80:9-14.
- Yamada K, Tanaka T, Han D, Senzaki K, Kameyama T, Nabeshima T. 1999b. Protective effects of idebenone and alpha-tocopherol on beta-amyloid-(1-42)-induced learning and memory deficits in rats: implication of oxidative stress in beta-amyloid-induced neurotoxicity in vivo. Eur J Neurosci 11:83-90.
- Yankner BA. 1996. Mechanisms of neuronal degeneration in Alzheimer's disease. Neuron 16:921-32.
- Yun HY, Dawson VL, Dawson TM. 1997. Nitric oxide in health and disease of the nervous system. Mol Psychiatry 2:300-10.