Molecular and cellular mechanisms of Ginkgo biloba extract [EGb 761®] in improving age-related and ß-amyloid induced neuronal dysfunctions

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Reham Mahmoud Abdel-Kader
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Dekan: Prof. Dr. Steinhilber

1. Gutachter: Prof.Dr. W.E. Mueller

2. Gutachter: Prof. Dr. M. Schubert-Zsilavecz

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Table of contents

Abbreviations

3APS, Ramiprosate,Alzhemed	3-amino-1- propane-o-sulfonic acid
6-OHDA	6-hydroxydopamine
Ach	Acetylcholine
AchE	Acetyl cholinesterase
AD	Alzheimer's Disease
ADAM10	ADAM metallopeptidase domain 10
ADAM-17,TACE	ADAM metallopeptidase domain 17, Tumor necrosis factor alpha converting enzyme
ADAS	Alzheimer's disease assessment scale
ADDL	Aβ derived diffusible ligand
AICD	APP intracellular domain
APOE	Apolipoprotein E
APP	Amyloid precursor protein
Aß	Amyloid beta
ATP	adenosine triphosphate
BACE-1	beta site APP cleavage enzyme 1
BACE-2	beta site APP cleavage enzyme 2
BB	Bilobalide
BSA	Bovine Serum Albumin
BuChE	Butyryl cholinesterase
ChEI	Cholinesterase inhibitor
COX	Cytochrome-c-oxidase
DBC	Dissociated brain cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPH	1,6-Diphenyl-1,3,5-hexatriene
ECE	Endothelin-converting enzyme
EU	European Union
FAD	familial AD
FCS	Fetal calf serum
FDG-PET	18-F-deoxy-glucose positron emission tomography
FTDP-17	Fronto-temporal-dementia and parkinsonism linked to chromosome 17
GA	Ginkgolide A
GB	Ginkgolide B
GC	Ginkgolide C
GCS	Glutamyl-cysteinyl synthetase
GDS	Global deterioration scale
GJ	Ginkgolide J
GPx	Glutathione peroxidase
HNE	4-hydroxy-2-nonenal
HS	Horse Serum
Hu	Human
IDE	Insulin degrading enzyme
KGDHC	Alpha -ketoglutarate dehydrogenase complex
LRP	Low-density lipoprotein receptor-related protein

Abbreviations

MMP	Mitochondrial membrane potential
MMSE	Mini-mental status examination
NEP	Neprilysin
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NOS	Nitric oxide synthese
NSAID	Non-steroidal anti-inflammatory drug
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PDHC	Pyruvate dehydogenese
PPAR- γ	Proxisome proliferated activated receptor- γ
PS	Presenilin
PTP	Permeability transition pore
RAGE	Receptor for advanced glycation end products
Rh-123	Rhodamine 123
ROS	Reactive oxygen species
SAMR	Senescence accelerated resistant mice
SAMP	Senescence accelerated prone mice
SDH	succinate dehydrogenase
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TCA	Tricarboxylic acid cycle
Tg	Transgenic
TMA-DPH	Trimethylammonium 1,6-Diphenyl-1,3,5-hexatriene
TNF- alpha	Tumor necrosis factor alpha
WHO	World health organization
Wt	Wild type

1 Introduction

1.1 Alzheimer's disease

1.1.1 A century of Alzheimer's disease

Alzheimer's disease [AD] is a brain disorder named after the German physician "Dr. Alios Alzheimer". In November 1906, Alois Alzheimer presented the case of his patient "Frau Auguste D.," a 51-year-old woman brought to see him in 1901 by her family. Auguste had developed memory disorder, hallucinations, delusions and language deficits. Her case deteriorated, and within a few years she was bed-ridden. After Auguste's death in 1906, Dr. Alzheimer performed a brain autopsy and observed histo-pathological changes which are recognized till today as typical characteristic features of AD.

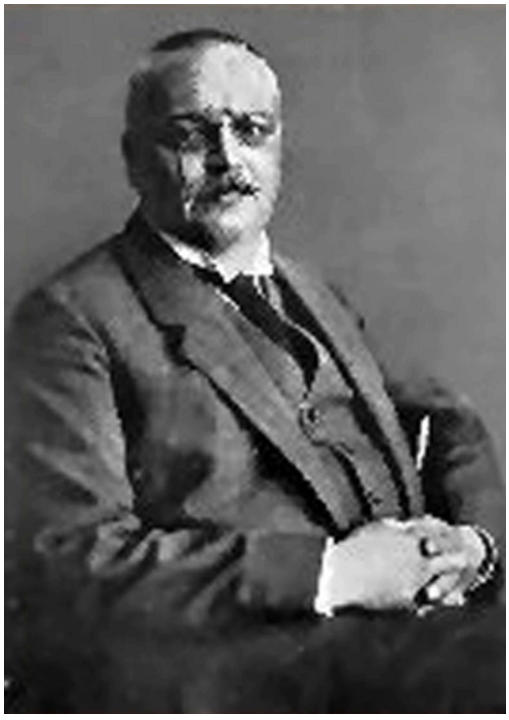


Figure 1-1

Dr. Alois Alzheimer [1864-1915]

Within 6 months Dr. Alzheimer presented his findings at the 37th reunion of Southwest German psychiatrists meeting in Tuebingen. Sarcastic as it may sound, due to its "lower importance" only the title of Dr. Alzheimer's

presentation was announced, with a statement between brackets declaring that the lecture "was inappropriate for a short presentation".

> *11. Herr Alzheimer (München): Über einen eigenartigen schweren Erkrankungsprozeß der Hirnrinde (zu kurzem Referat nicht geeignet).*

Figure 1-2

The 11th contribution in the Southwest German psychiatrist meeting in Tuebingen

Dr. Alzheimer's contribution in the Tuebingen meeting was briefly announced and was regarded unsuitable for an oral presentation.

Although the disease entered in 1907 the medical literature, the term "Alzheimer's disease" was coined by Emil Kraepelin in 1910. The importance of AD has increased since then and has become a major concern in the last decades due to its high incidence.

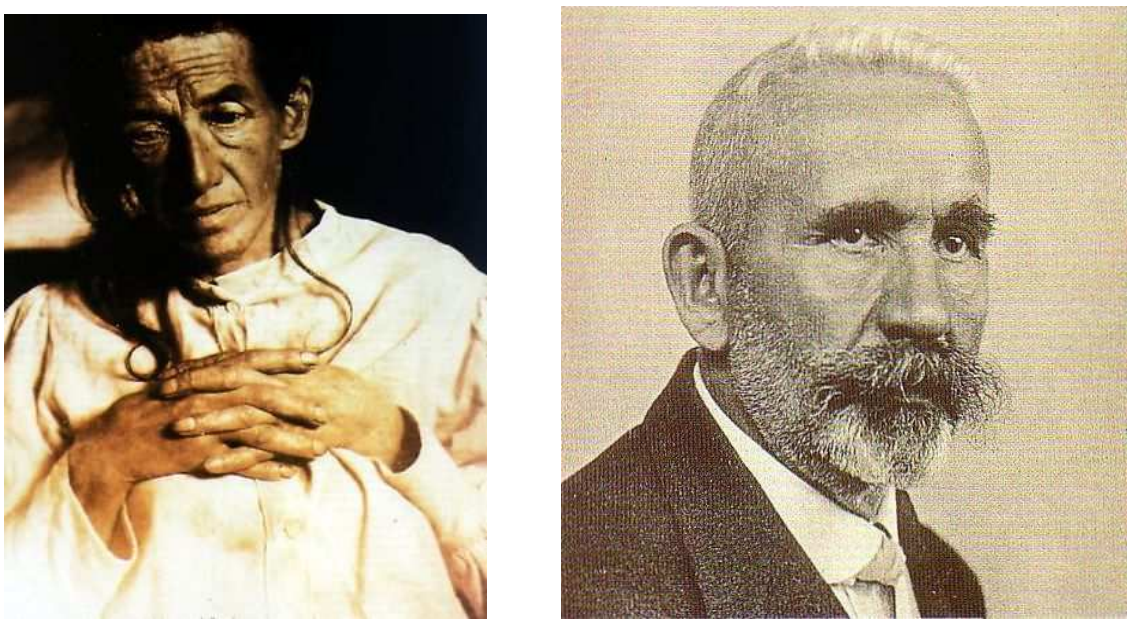


Figure 1-3

Auguste D [left] and Dr. Emil Kraepelin [right].

1.1.2 Prevalence

Alzheimer's disease is the most common cause of dementia which accounts for 60 % to 80 % of all cases.

Dementia is a clinical syndrome of loss or decline in memory and other cognitive abilities. In 2005, it was estimated that there are 24 million people with dementia worldwide (Ferri *et al.* 2005). By 2040, it is anticipated that this figure will have increased to 81 million.

According to the latest studies in 2005 it can be calculated that the estimated number of people with dementia living in the European Union is approximately 5.3 million. The estimated number of people with dementia in Germany in 2005 was 1,010,245. This represents 1.22 % of the total population, which is slightly higher than the EU average of 1.14 % (Ferri *et al.* 2005). Moreover, one must take into consideration that these figures under-estimate the number of people with dementia in Germany, as it is impossible to obtain sufficiently detailed population statistics of the number of people in Germany over the age of 94.

A very recent report [2008] about AD in the USA shows alerting figures concerning this disease. Around 5.2 million people have AD in the USA and statistically calculated every 71 seconds someone in America develops AD.

Women are more likely to develop AD than men. The reason behind this is most probably because on average basis women live longer than men, therefore their longer life expectancy increases the time during which they could develop AD.

Despite the striving of researchers in finding answers to diagnosis and treatment of AD, one has to face the facts that the number of patients with AD are unfortunately growing rapidly. The good news is that the number of

people developing AD is increasing because their life span is increasing, thanks to medicine, social and environmental conditions!

1.1.3 Diagnosis

Diagnosis of AD is complex and can't be easily distinguished from other forms of dementia. The National Institute of Neurological and Communicative Disorders and Stroke, and the Alzheimer's Disease and Related Disorders Association proposed the NINCDS-ADRDA Criteria for clinical diagnosis of AD in 1984. Lately, after the emergence of distinctive and reliable biomarkers of AD, revised diagnostic criteria for AD have been suggested in order to update the current criteria (Dubois *et al.* 2007).

The degree of cognitive impairment can be quantified by means of cognitive tests. The mini-mental status examination [MMSE], the global deterioration scale [GDS] and the Alzheimer's disease assessment scale [ADAS] are such examples of cognitive tests.

Macroscopically AD is characterized by reduced brain weight, hippocampal and cortical atrophy and enlarged ventricles. A succession of neuropathologic diagnostic criteria for AD has been established over the past 20 years. In 1997 a committee formed under the auspices of the national institute of aging and the Reagen institute, published new diagnostic criteria that included cortical densities of both neuritic plaques and neurofibrillary tangles (Hyman and Trojanowski 1997). The spreading of AD pathology begins in the [trans] entorhinal area of the basal mediotemporal cortex, progressing to the hippocampus and finally to the entire neocortex with relative preservation of the occipital area striata (Braak and Braak 1991). Hence, the definitive diagnosis of AD requires a microscopic examination of the cerebral cortex. Cortical biopsy can provide an ultimate diagnosis but it's not a routine procedure, therefore a definite AD diagnosis is obtained only post-mortem.

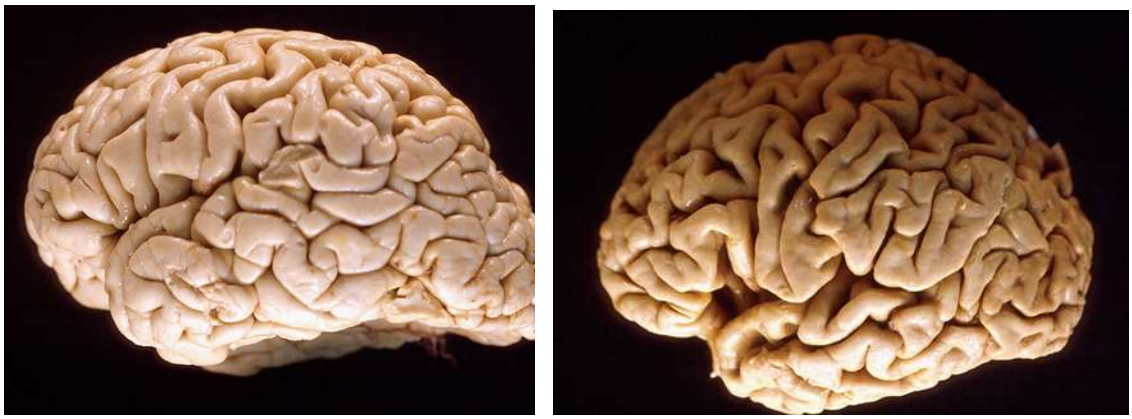


Figure 1-4

Normal brain [left] vs. Alzheimer brain [right]

Brain from patient with AD [right] demonstrating significant cortical atrophy, widening of the sulci and narrowing of the gyri. Frontal and temporal lobes are more affected than the occipital lobe.

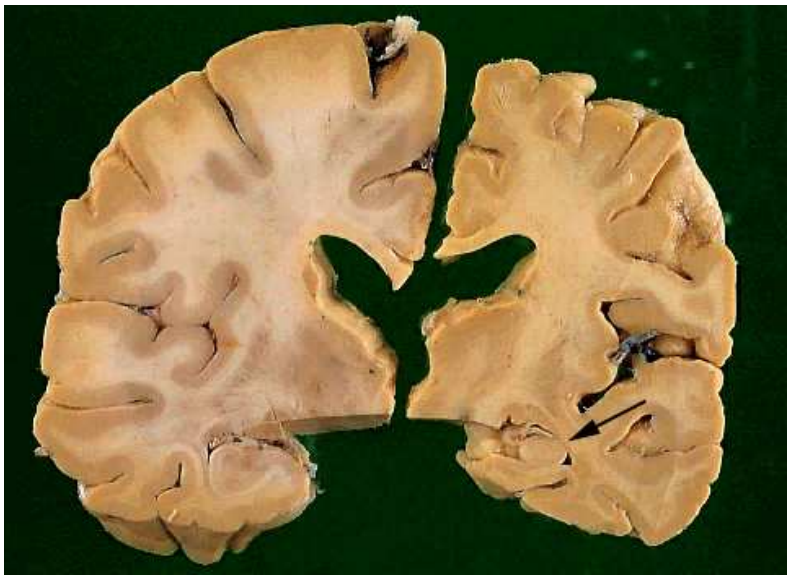


Figure 1-5

A section from normal brain [left] and one from Alzheimer's patient [right]

Note how much smaller is the brain on the right. The hippocampus is noticeably smaller [arrow].

However, it would be much more useful to be able to diagnose AD as early as possible [definitely not post-mortem]!

Present diagnostic measures have become more accurate and one can nowadays diagnose AD, but unfortunately not before symptoms start appearing. This is not really beneficial if treatment is pathologically targeted

and not symptomatic treatment, since it is well known that the pathological processes in AD begin decades before the first symptoms appear.

Therefore, researches in the field of AD diagnosis are extensive and still on going. Measuring biomarkers like amyloid beta [Aß] peptide and phosphorylated tau in CSF, using MRI and CT scans to determine atrophy, or 18-F-deoxy-glucose positron emission tomography [FDG-PET] for measuring the impaired metabolism in AD, are all helpful methods in distinguishing AD from other types of dementia. Moreover, the use of Pittsburgh compound B in PET scans is useful in detecting regions with Aß. But unfortunately none of these diagnostic tools is utterly 100% reliable, for example measuring atrophy alone is not really helpful because it can occur during aging or due to another form of dementia. FDG-PET scans are quite promising but still a qualitative method and the cost and limited availability of PET scanners will constrain their widespread use.

Maybe trying to combine 2 or more of the diagnostic methods, for example measuring both biomarkers in CSF and performing simultaneously MRI scans can lead to a more sensitive or accurate judgment.

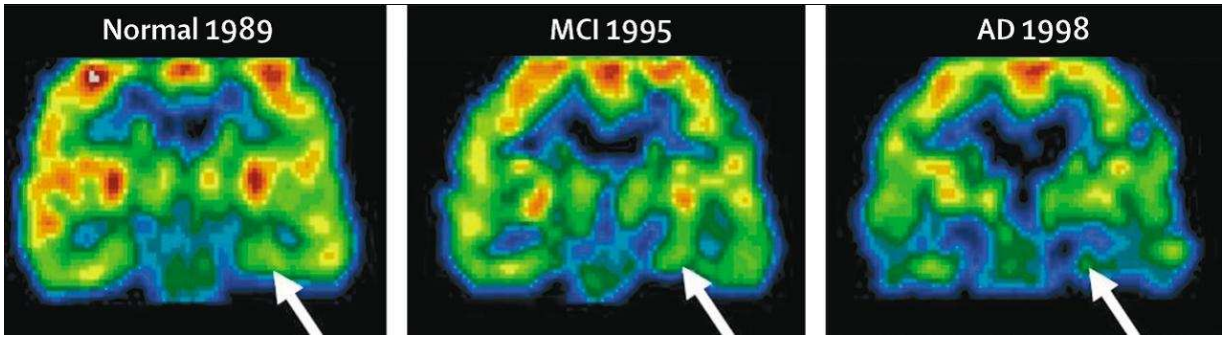


Figure 1-6

Metabolic reductions on FDG-PET

FDG-PET scans in a 71-year-old cognitively normal woman at baseline (1989) and over 9 years. During this period the patient declined to MCI and later was diagnosed with AD, confirmed at autopsy. For each observation a coronal PET scan is depicted at the level of the entorhinal cortex and anterior hippocampus. Arrows point to the inferior surface of the entorhinal cortex with progressively darker colors on the PET scans, which indicates progressive reductions in glucose metabolism.

1.1.4 Risk Factors

Early onset AD is not very common and occurs below the age of 60. The majority of early onset AD is caused by rare genetic variations found in a small number of families worldwide recognized as familial AD and can develop sometimes in individuals as young as 30. However, the most common form is the sporadic form, which represents approximately 90 % of AD cases. Unfortunately, the causes of the sporadic form of AD are not yet known. The risk factors which seem to play a major role in both types are discussed below.

1.1.4.1 Sporadic AD

For the widespread late onset sporadic AD, age is by far the most common risk factor (Gao *et al.* 1998). During aging, cells in the human brain, like cells in other organ systems, experience cellular changes such as oxidative stress, mitochondrial dysfunction, metabolic impairment, DNA damage and apoptosis (Mattson 2006).

Changes in neurotransmitter and neurotrophic factor signaling pathways which are amplified in neurodegenerative diseases are also a consequence of aging. Although the abovementioned mechanisms seem to connect aging to AD, the exact means and the order of events are still obscure.

Other non-genetic risk factors besides aging are environmental factors, including aspects of diet and lifestyle. For instance patients with more varied activities, including intellectual, physical, recreational and social activities, are less likely to develop AD (Friedland *et al.* 2001). Also low calorie diets and increased consumption of omega 3 fatty acids have been linked to a decreased risk of developing AD. The role of other diseases such as hypertension, hyperlipidemia, type 2 diabetes mellitus and hyperhomocysteinemia in increasing the risk for developing AD is still debatable. Other medical risk factors include head trauma, clinical depression,

and some medications such as vitamin E or NSAIDs may reduce the risk of acquiring dementia. (Ownby *et al.* 2006; Patterson *et al.* 2008; Mattson 2006).

The only well established genetic factor that can increase a person's susceptibility for sporadic AD is Apolipoprotein E [APOE]. APOE belongs to the low density lipoprotein receptor gene family, and it is suggested that it could provide a system for lipid transport and cholesterol homeostasis in the brain (Pitas *et al.* 1987). There are three different isoforms of APOE gene [APOE-ε2, APOE-ε3 and APOE-ε4]. Increased risk is linked with inheritance of the APOE-ε4 allele has helped explain some of the variations in age of onset of Alzheimer's disease based on whether people have inherited zero, one, or two copies of the ε 4 allele from their parents. The more APOE-ε4 alleles inherited, the lower the age of disease onset (Corder *et al.* 1993;Saunders *et al.* 1993). The exact mechanism is unidentified, but Aß deposits are more abundant in ε 4-positive than in ε 4-negative cases (Schmechel *et al.* 1993). In addition, APOE4 is associated with a number of other factors that may contribute to AD pathology, including low glucose usage, mitochondrial abnormalities, and cytoskeletal dysfunction (Mahley *et al.* 2006).

1.1.4.2 Familial AD

Although familial AD [FAD] is very rare its importance should not be underestimated. Fact is without the familial cases we would have been far behind in our knowledge about AD. FAD is associated with an autosomal dominant pattern of inheritance, with three major genes whose mutations are known to contribute to the disease. In the mid 80’s, as Mann *et al* observed that individuals with Down’s syndrome develop the clinical and neuropathological features of AD, studies on the amyloid precursor protein [APP] as a genetic determinant of AD began (Mann *et al.* 1985). Since Down’s syndrome is characterized by the presence of an extra copy of genetic material on chromosome 21, the involvement of this chromosome in AD was

then considered. A few years later the gene encoding for APP was identified and its mutations as a leading cause of hereditary AD (Kang *et al.* 1987). The different APP mutations were given their names according to the families where they were discovered, for example the Swedish mutation was first discovered in 2 Swedish families by Lannfelt and his coworkers (Mullan *et al.* 1992). The APP mutations are clustered near the α-, β-, or γ-secretase cleavage sites, having a direct effect on APP processing [see figure 1-7]. The discovery of these mutations led to the development of transgenic cell and animal models that were essential for many thriving findings in the field of AD.

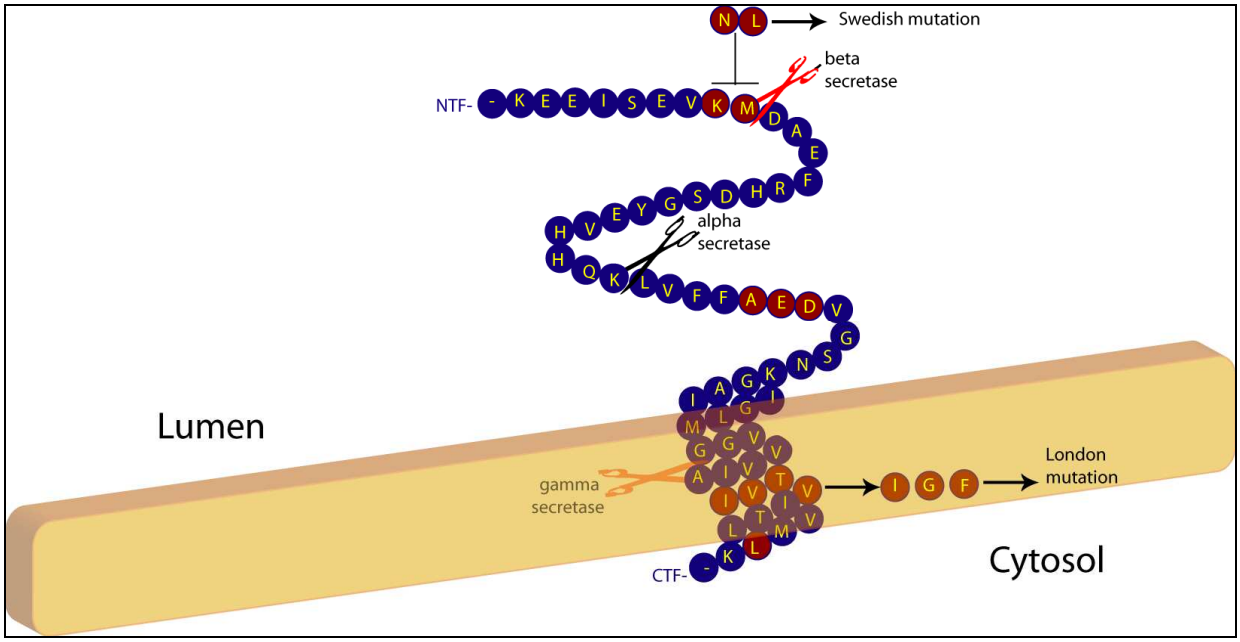


Figure 1-7

Amyloid precursor protein

Amyloid precursor protein showing the sites where FAD mutations occur [illustrated in red]. The Swedish and London mutations are clustered near the ß-secretase and γ-secretase respectively.

In the current study a cell model over expressing the Swedish double mutation and a transgenic animal model expressing both the Swedish double and the London mutations were utilized. The Swedish APP double mutation [KM670/671NL] promotes the processing of APP by the ß-secretase causing a massive increase in total Aß levels (Citron *et al.* 1992). In the London mutation the total amount of Aß doesn't change , however the proportion of

Aß 42 increases by 50–90 % (Suzuki *et al.* 1994). Thus the London mutation shifts the balance of γ-secretase cleavage slightly toward the 42 over the 40 cleavage site.

Although the APP mutations were the first identified in FAD, it is believed that they are responsible for only 5-20 % of all FAD cases. On the other hand mutations in presenilin 1 [PS1] gene found on chromosome 14 are assumed to be accountable for approximately 85 % FAD. Homologue to PS1 are mutations in presenilin 2 [PS2] localized on chromosome 1, and they are very rare. Research conducted on PS mutations revealed that they specifically promote Aß 42 generation from APP (Turner 2006).

1.1.5 Neuropathology

After the death of Auguste D in April 1906 [refer to section 1.1.1], her brain was sent to Munich for analysis. Alzheimer's use of the silver staining method helped him identify the neuropathalogical characteristics of AD, the neuritic plaques and neurofibrillary tangles. Whereas plaques have been reported before in an elderly patient with epilepsy, Alzheimer was the first to describe the tangle pathology. He also attempted to illustrate the tangles as he saw them during his investigation [see figure 1-8]. Interestingly, after 100 years these 2 hallmarks remain the only pathological evidence for the postmortem definite diagnosis of AD. Of course the molecular composition of the plaques and tangles were at that time unidentified. After the recognition of the cross-ß structure of the extracellular plaques and the paired helical structure of the tangles in the 60's (KIDD 1963;Terry *et al.* 1964), it took another 20 years of research before their major components were revealed. Ever since tau was identified as the major tangle component and Aβ as the major plaque constituent a modern era of research began in AD.

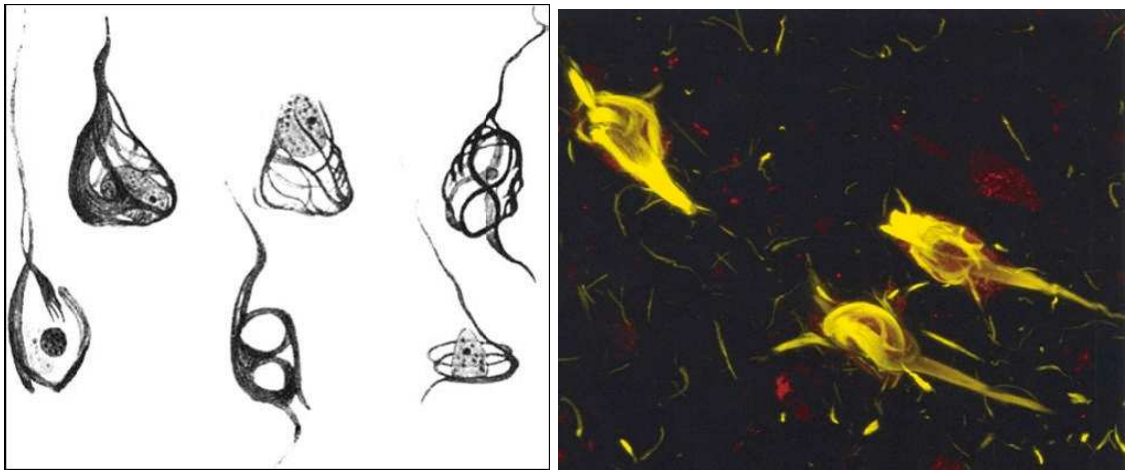


Figure 1-8

Neurofibrillary tangles

The similarity between Dr. Alzheimer's drawings of the neurofibrillary tangles [left] 100 years ago, and a recent confocal microscopy picture of the tangles (Helbecque *et al.* 2003) [right] is demonstrated above.

1.1.5.1 Neurofibrillary tangles

The intracellular tangles arranged in paired helical filaments are made of full length, hyperphosphorylated tau, a protein involved in microtubule assembly and stabilization. Hyperphosphorylation of tau is common to all diseases with tau filaments and may be required for toxicity. Since filamentous tau deposits are found in a number of other neurodegenerative diseases, including progressive supranuclear palsy, corticobasal degeneration and Pick's disease, its importance in AD was always doubted. However, the findings that mutations in the tau gene leads to inherited "fronto-temporal-dementia and parkinsonism linked to chromosome 17" [FTDP-17] enlightened the importance of tau and its role in AD.

In the human brain, six tau isoforms are produced from a single gene through alternative mRNA splicing (Goedert *et al.* 1989). They fall into two groups on the basis of numbers of microtubule-binding repeats, with three isoforms having three repeats each and three isoforms having four repeats each. In the normal brain, a correct ratio of three-repeat to four-repeat tau isoforms is essential for preventing neurodegeneration and dementia. Most missense mutations in tau reduce the ability of tau to interact with microtubules

(Hasegawa *et al.* 1998), and some also promote aggregation into filaments. Mutations lead to the relative overproduction of four-repeat tau, (Hutton *et al.* 1998;Spillantini *et al.* 1998;D'Souza *et al.* 1999) therefore altering its ratio to the three-repeat form.

It appears likely that a reduced ability to interact with microtubules and/ or alteration of the ratios of the different tau isoforms is necessary for initiating toxic function that can cause neurodegeneration.

Although the amyloid cascade hypothesis which accentuates the importance of Aß as the initiator of AD is highly supported, one should not undervalue tau and its role in AD.

1.1.5.2 Amyloid plaques

The Plaque filaments found in AD and other diseases are extracellular and have the molecular fine structure of amyloid. This term refers to filaments with a diameter of around 10 nm that have a cross-beta structure and characteristic binding properties to the congo-red dye. The emphasis on amyloidal research directly led in 1984 to the discovery of Aß peptides, the 39- 43 residue peptides now known to be the major protein constituent of amyloid plaques in AD and cerebrovascular amyloid deposits (Glenner and Wong 1984).

A few years later amyloid precursor protein gene was identified (Kang *et al.* 1987) and Yanker *et al* demonstrated that Aß could be toxic to cultured neurons (Yankner *et al.* 1989). Progressed research in this field emphasized the role and toxicity of Aß peptide leading to the proposal of the amyloid cascade hypothesis. This hypothesis briefly states that accumulation of Aß aggregates causes synaptic dysfunction and biochemical changes. These changes modify the tau protein into insoluble paired helical filaments. Together these events cause progressive neuronal loss associated with multiple neurotransmitter deficiencies and cognitive failure.

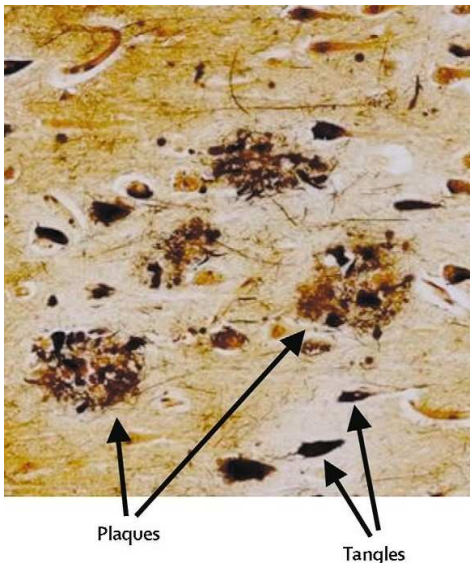


Figure 1-9
Plaques and tangles in AD tissue

1.1.6 Amyloid beta: first the making…

Shortly after the elucidation of the amino acid sequence of the Aß peptide, the neuronal isoform of its precursor protein APP695 was cloned (Kang *et al.* 1987). The other 2 isoforms 770 and 751,which are mainly expressed in non-neuronal cells, were characterized a year later (Kitaguchi *et al.* 1988;Ponte *et al.* 1988;Tanzi *et al.* 1988). According to the isoform the molecular weight of APP ranges between 110 and 140 kDa. Full-length APP contains a large extracellular domain [corresponding to approximately 88% of the total protein mass for the main neuronal isoform], a single transmembrane region and a small cytoplasmic tail. The physiological function of APP is still not clear, however it is suggested that APP acts as a contact receptor due to its role in adhesion of neurons to glia cells and tissue maintenance (Gralle and Ferreira 2007).

APP can be cleaved by 2 alternative pathways, either the non-amyloidogenic pathway by the action of α-secretase or the amyloidogenic pathways by β-secretase yielding Aß [figure 1-10].The α-secretase pathway forms an extracellular soluble N-terminus [APPα] (Hooper *et al.* 1997), and a membrane bound c- terminal fragment [C-83], and the ß-secretase leads to the formation of extracellular soluble APPß and C-99. According to the position where the γ-secretase cleaves the C-99 fragment either the Aß 40 is formed or the longer form Aß 42.

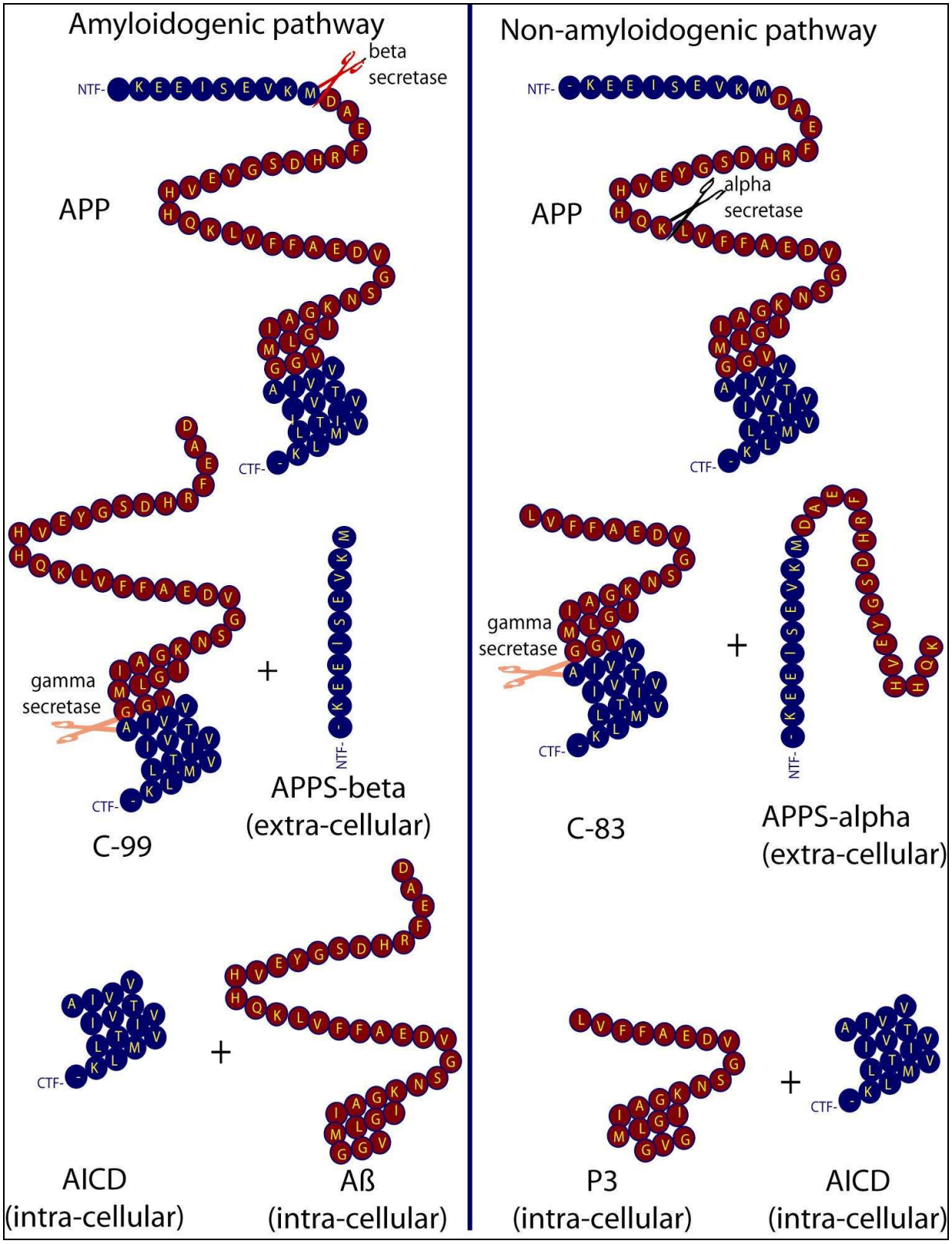


Figure 1-10

APP cleavage pathways

Amyloidogenic splicing of APP by beta and gamma secretases leading to the formation of Aß [left], and the non-amyloidogenic pathway cleaving the APP in the Aß region by alpha-secretase and gamma-secretase [right]. The Aß region is illustrated in red.

1.1.6.1 Alpha secretase

Alpha secretase [α-secretase] cleavage occurs at the lys 16-leu 17 bond within the Aß domain, preventing the formation and deposition of Aß. To date, mainly 2 proteins have been identified to have α-secretase activity, known as ADAM 17 and ADAM 10. Both ADAM 17 [also known as tumor necrosis factor alpha converting enzyme = TACE] and ADAM 10 are membrane bound metalloprotienases belonging to the adamlysin family of proteins (Blobel 1997;Hooper and Turner 2002;Slack *et al.* 2001). Although the main function of TACE is to cleave the TNF- alpha precursor to release TNF-alpha from cells, its α-secretase activities have been seen in cultured cells *in vitro* but its *ex vivo* role is still questionable. For example, disruption of the TACE gene abolished the augmented secretion of sAPP in mouse fibroblasts in response to phorbol ester (Buxbaum *et al.* 1998). However basal formation and secretion of sAPPα was unaffected in the cells derived from TACE knockout mice (Parvathy *et al.* 1998).

The second enzyme, ADAM 10 has shown α-secretase activity in different cell systems *in vitro* (Lammich *et al.* 1999) and also *in vivo*. In a transgenic mouse model for human APP, over-expression of ADAM 10 enhanced cognitive functions, reduced the formation of Aß 40 and Aß 42, and prevented their deposition in plaques. Whereas the catalytically inactive ADAM 10 led to an enhancement of the number and size of amyloid plaques in the brains of double-transgenic mice (Postina *et al.* 2004). Interestingly, a significant decrease of platelet ADAM 10 levels has been observed in AD patients together with a similar decrease in sAPPα in both thrombin-activated platelets and CSF (Colciaghi *et al.* 2002).

Although both ADAM 10 and ADAM 17 have shown in several cell models their α-secretase activities, the evidence present seems to be in favor of ADAM 10 more than ADAM 17. Another study highlighting the effect of ADAM 10 showed that in situ hybridization revealed overlapping expression

of APP and ADAM 10 but not ADAM 17 in both mouse and human brains (Marcinkiewicz and Seidah 2000).

As mentioned above the α-secretase pathway leads to formation of sAPPα and a C-terminal stub [C-83]. Further processing of the C-83 fragment by γ-secretase yields truncated fragments of about 3 kDa [p3], preventing the formation of Aß. sAPPα was reported to modulate synaptic transmission and is neuroprotective against ischemic and excitotoxic injury (Mattson *et al.* 1993;Morimoto *et al.* 1998;Smith-Swintosky and Mattson 1994). It is quite remarkable that the α-secretase pathway not only prevents formation of the toxic Aß, but leads to the formation of neuroprotective products.

1.1.6.2 Beta secretase

The beta secretase [ß-secretase] cleavage of APP occurs less frequent than α-secretase splicing, but it is much more critical. Similar to the α-secretases, 2 enzymes having ß-secretase activity are identified till now. These are known as BACE-1 and BACE-2 [beta site APP cleavage enzyme (Huse and Doms 2000;Nunan and Small 2000).

BACE-1 is expressed at very high levels in pancreas, at moderate levels in brain, and at low levels in most peripheral tissues (Vassar *et al.* 1999;Yan *et al.* 1999). Surprisingly the ß-secretase activity is high in brain only, and almost undetectable in pancreas (Sinha *et al.* 1999). BACE-1 exists mainly at the Trans golgi network and endosomal system and to a lower extent in the ER and on the cell surface (Capell *et al.* 2000;Haniu *et al.* 2000;Huse *et al.* 2000). BACE-1 over-expression in cell models led to the increase of the ß-secretase cleavage products including Aß 40 and Aß 42 (Vassar *et al.* 1999). Also in a transgenic mouse line expressing human BACE-1, the amyloidogenic processing of APP is increased and levels of Aß 40/42 are elevated (Bodendorf *et al.* 2002). Knocking out the BACE-1 gene completely impairs the ß-secretase cleavage of APP and abolishes the generation of Aß

(Cai *et al.* 2001) showing no phenotypic alterations in the mice (Luo *et al.* 2001).

In humans several studies indicated the importance of ß-secretase in AD. In the cortex from AD patients a 2.7-fold increase in protein expression of BACE was observed and also the C-99 fragment was shown to be double that present in non-demented controls (Holsinger *et al.* 2002). Moreover Fukumoto *et al* were able to show that the activity of ß-secretase increases in the temporal cortex by aging in non-demented humans (Fukumoto *et al.* 2003). It seems from the above evidence that BACE-1 is involved in the amyloidogenic processing of APP and plays a major role in AD.

Despite the fact that BACE-2 is 55% identical to BACE-1 and shows similar substrate specificity (Farzan *et al.* 2000), it is not highly expressed in the brain (Bennett *et al.* 2000) and on the contrary to BACE-1 there is no direct evidence linking it to AD pathology.

1.1.6.3 Gamma secretase

Due to the complexity of the gamma secretase [γ-secretase] complex it was identified step-wise, one component after the other. Starting with the evidence that γ-secretase is an aspartyl protease and that presenilin is critical for γ -secretase activity, led to the discovery that presenilin itself was an aspartyl protease, and therefore a part of the γ -secretase complex. Further studies revealed nicastrin, a presenilin-interacting protein found by co-isolation upon immunoaffinity purification (Yu *et al.* 2000). Since over-expression of presenilin and nicastrin still did not result in increased γ-secretase activity, it was clear that other associated proteins were yet to be discovered. Finally Aph-1 and Pen-2, which encode proteins of seven and two predicted transmembrane domains respectively (Francis *et al.* 2002;Goutte *et al.* 2002), were discovered and the picture of γ-secretase was complete!

The c-fragments C-83 and C-99 which are the products of α- and ß-secretases respectively can be further cleaved by the γ-secretase. The scission of the C-83 stub is harmless and yields p3 fragment and APP intracellular domain [AICD].The exact role of AICD remains ambiguous, some studies report that it can alter gene expression and lead to increased apoptosis (Kim *et al.* 2003) and other studies indicate that AICD is important for mitochondrial function (Hamid *et al.* 2007). Moreover, Ma *et al* reported that AICD correlates with enhanced memory and synaptic plasticity (Ma *et al.* 2007).

On the other hand the cleavage of the C-99 fragment leads to the formation of AICD in addition to variable Aß fragments [Aß 38, 40 or 42] according to the position of the cut. The precise site of scission has an important influence on the self-aggregating potential and resulting pathogenicity of Aß, as only the Aß 42 peptide has a strong propensity to oligomerize *in vivo*.

1.1.6.4 Amyloid beta…"the peptide from Hell"

In 1907, shortly after Alzheimer's report, Oskar Fischer published his observations of plaques and tangles in more typical late onset "senile" dementia. He speculated that the plaques found in 12 of 16 post-mortem brains from elderly subjects with dementia which were absent in age-matched non demented subjects, resulted from deposition of a foreign, presumably infectious, agent (Gouras *et al.* 2005). Demandingly, Fischer studied these depositions extensively until he concluded that the plaques are not a result of infection and most probably they are derived from degenerating neuronal processes. In 1911, Alzheimer described in a more detailed report the phenomenon of AD, and acknowledged Fischer for his efforts in characterizing the plaques by describing them as "Fischer's Plaques". Interestingly Alzheimer summarized the different views of their origin without providing his interpretation, probably due to his knowledge that the tools to answer this question are still not available.

73 years later these tools became available, and a breakthrough occurred in this field of research as Aß was identified, isolated and characterized as the constituent of the amyloid plaques. Glenner *et al* were able to isolate and characterize Aß in the vasculature of AD brains in 1984 (Glenner and Wong 1984). Numerous investigations kept on proving the toxicity of Aß and led to the well known "amyloid hypothesis" stating that Aß aggregation is the cause and not an effect of AD (Hardy and Selkoe 2002). Until then it was assumed that secreted Aß gradually increase in the extracellular space, aggregating into amyloid plaques which causes neuronal toxicity (Yankner *et al.* 1989).

Intraneuronal or intracellular Aß was first discovered in 1994 (Wertkin *et al.* 1993;Lee *et al.* 1998), here again this was also due to the development of immunohistochemical equipment. Cell biological studies reported that Aß is generated in the ER (Cook *et al.* 1997), Golgi apparatus (Xu *et al.* 1997) and endosomal-lysosomal system (Koo and Squazzo 1994). Present evidence correlates the toxicity of intracellular Aß to AD and cytotoxicity rather than extracellular Aß. Since initially it was thought that extracellular Aß present in plaques is toxic, primarily studies focused on Aß fibrils (Lorenzo and Yankner 1994). However nowadays lower order Aß assemblies such as oligomers are believed to be the more toxic form and are highly linked to AD (Walsh *et al.* 2002;Cleary *et al.* 2005) .

Aß consists of 39-43 amino acids, the most common forms are Aß 40 and Aß 42, which is known to self-assemble and form different aggregates, from monomers, to oligomers, protofibrils and finally fibrils. The mechanism behind this aggregation is still vague. Most researchers tend to lean towards the theory that it is a seeded process that requires a nucleus rather than a linear one (Finder and Glockshuber 2007). The formation of the nucleus which is the rate limiting step is followed by assimilation of more Aß oligomers leading to the formation of the fibrils. For the explanation of the transition of Aß monomers to oligomers, different assumptions exist. What seems to be

definite is that high-order insoluble oligomers acquire a ß-sheet structure, which appears to be responsible for initiating aggregate formation. The question which arises is in which stage Aß reconfigures to the ß-sheet structure? One model considers that Aß monomers exist in equilibrium between α-helical and ß-sheet conformation, and that only the ß-sheet fraction is capable of self-aggregating and thereby shifting the equilibrium (Finder and Glockshuber 2007). This theory is supported by the finding that dimers, tetramers and octomers of Aß with ß-sheet structures occur as early-assembly intermediates (Mastrangelo *et al.* 2006). The second proposal is that the alpha-helical monomers aggregate to oligomers, and then the oligomers transform their structure to ß-sheet conformation. This is based on the finding that an α-helical, oligomeric intermediate accumulates during fibrillization (Kirkitadze *et al.* 2001), and that soluble low molecular weight oligomers have been identified (Walsh *et al.* 1999).

Leaving aside how the ß-sheet structure oligomers are formed, they seem to be the nucleus that grows by seeding forming protofibrils and finally fibrils.

According to the latest findings the lower order Aß oligomers seem to be the most neurotoxic species. Cognitive impairment and synaptic loss have been shown to correlate with the amount of soluble oligomers in AD patients and the formation of soluble oligomers is elevated in AD brain tissue (Finder and Glockshuber 2007).

In vitro and *in vivo* experiments have shown that Aß can cause several cellular and synaptic dysfunctions, such as oxidative stress, mitochondrial impairment, apoptosis and inflammation (Haass and Selkoe 2007;Nakagawa *et al.* 2000). From the 2 most common forms Aß 40 and Aß 42, Aß 42 is especially apparent within the neurons, and more prone to aggregation (Finder and Glockshuber 2007). Oligomeric Aβ 42 is far more toxic than monomeric Aβ 42 and, in carefully controlled experiments, is more toxic than fibrillar Aβ 42 (Dahlgren *et al.* 2002).

In cerebrospinal fluid Aß 42 is increased in the first stages in sporadic AD but then declines with disease progression (Jensen *et al.* 1999). This can indicate that initially elevated Aß 42 leads to its aggregation and deposition, and this deposition of Aß 42 results later in a decline of its levels in CSF and plasma. Fact is, Aß is not only present in AD patients but also accumulation of Aß is observed in non-demented controls especially with aging (Fukumoto *et al.* 2003) .Thus Aß is generated constitutively and this raises the possibility that Aß has a normal physiological function. The exact role of Aß is still not known but neuronal excitation increased with Aß (Gouras *et al.* 2005). Both Aβ 40 and Aβ 42 modulate potassium channels in neurons. However, Aβ 40 but not Aβ 42, is able to counteract the effects of secretase inhibitors, drawing further distinction between the two forms (Plant *et al.* 2003).

Person to person variability in absolute levels of Aß [40 or 42] makes it difficult to draw conclusions about disease progression. However, recently the importance of the ratio Aß 42/40 has gained more and more attention, and a reduction of the ratio correlates with the onset and progression of AD. This was shown in CSF and plasma levels of AD patients (Findeis 2007). Another study showed also the importance of this ratio in a transgenic mice model; by demonstrating that over expressing Aß 40 was able to prevent the Aß 42 linked amyloid deposition and premature death. These results seem to indicate that a shift towards the shorter form of Aß, or a decrease in Aß 42/40 ratio seems to be protective and beneficial.

Concluding, Aß appears to be present in physiological amounts and most probably has a potential normal function. However increase in the longer form of Aß aggregates, mainly oligomers, or the ratio Aß 42/40 seems to be deleterious, and can cause neurotoxicity. Still to be answered is the question whether this non-physiological toxic Aß is due to an increase in the production of Aß, decrease in the degradation of Aß, an increase of self-

assembly and formation of ß-sheet structures or a combination of more than one?

1.1.7 Amyloid beta…first the making…then the breaking…

Although the pathway of Aß production has been extensively studied since the discovery of Aß, only recently the catabolism of Aß has started to gain attention. Understanding the mechanisms behind Aß degradation and the enzymes involved, could be an important therapeutic tool in the future for eliminating Aß levels in brains of AD patients.

A few enzymes have been reported with the capability to break down Aß, the first to be identified was the "insulin degrading enzyme" [IDE]. A year later neprilysin [NEP] was reported to metabolize Aß *in vitro*, and later on *in vivo*. Other members of the NEP family such as endothelin-converting enzyme [ECE] were examined for their ability to degrade Aß and proved their efficacy.

The biological features of proteases known to cleave Aß are summarized in the table below

Common Name	Other names	class	Sub-cellular location	Other substrates
Neprilysin	CD10, CALLA, EC 3.4.24.15 NEP	M	Cellular & intracellular membrane including presynaptic membrane	Enkephalin, cholecystokinin, neuropeptide Y, substance P, opiod peptides, atrial natriuretic peptides, bombesin-like peptides, chemotatic peptides, adrenocorticotropin hormone (ACTH)
Insulin degrading enzyme	EC 3.4.24.56 Insulysin, IDE	M	Cytosol, cellular, and intracellular membrane extracellular space	Insulin, glucagon, atrial natriuretic factor, β-endorphin amylin, APP intracellular domain TGFα
Endothelin-converting enzyme	EC 3.4.24.71 ECE	M	Trans-Golgi network Cell surface	Big endothelin, substance P, bradykinin, oxidized insulin B chain
Angiotensin-converting enzyme	EC 3.4.15.1; ACE; dipeptidyl carboxypeptida-se	M	plasma membranes perinuclear region	Angitensin-I, enkaphalins, bradykinin

Table 1.1

Biological features of Aβ degrading enzymes

Summary of the major enzymes known to cleave Aβ peptide (Wang *et al.* 2006)

1.1.7.1 Neprilysin [NEP]

NEP gene is located on chromosome 3q21-q27, and is composed of 750 amino acids with an approximate molecular weight of 86 kDa (Malfroy *et al.* 1988). It consists of a short N-terminal cytoplasmic tail, a membrane spanning domain, and a large c-terminal extracellular catalytic domain. NEP is expressed in a variety of tissues including the brain. In the brain NEP is mainly expressed in areas susceptible to Aß deposition such as the hippocampus.

In 1995, NEP was linked to Aß degradation by Howell *et al* (Howell *et al.* 1995). Further investigations proved the *in vivo* capability of Aß cleavage by NEP. Worth mentioning was the study by Iwata *et al* showing that Aß 42 is

degraded by NEP in the hippocampus of rats, this process was blocked by NEP inhibitor leading to accumulation of Aß and plaque formation (Iwata *et al.* 2000). Subsequently, the same authors reported that the levels of Aß 40 and Aß 42 were elevated in NEP knockout mice (Iwata *et al.* 2001).

Others were able to demonstrate the ability of NEP to degrade not only monomeric form of Aß but also the oligomeric form (Kanemitsu *et al.* 2003). Quantitative analysis showed that NEP mRNA was significantly lower in AD (Caccamo *et al.* 2005;Yasojima *et al.* 2001), and an inverse relationship was observed with both Aß plaques and Aß levels (Wang *et al.* 2006).

From the aforementioned evidence NEP appears to contribute to the normal metabolism and accumulation of Aß in AD.

1.1.7.2 Insulin degrading enzyme IDE

IDE gene was mapped to chromosome 10q23-q25, consisting of 1019 amino acids. The Aß degrading property of IDE was first described in 1994 by Kurochekin *et al* (Kurochkin and Goto 1994). IDE is expressed in several tissues, such as liver, skeletal muscles and brain, and is primarily located in the cytosol (Wang *et al.* 2006). Anatomical data was first provided in suggesting that IDE is associated with neuropathlogical hallmarks of AD (Bernstein *et al.* 1999). Immunostaining revealed the presence of IDE in cortical and sub cortical neurons, senile plaques and microvessels (Morelli *et al.* 2004). Reduced mRNA levels and activity of IDE in the hippocampus of cases at high risk of developing AD and in APOE4 carriers (Zhao *et al.* 2007;Cook *et al.* 2003) supported the hypothesis that IDE activity may contribute to Aß accumulation in AD patients.

Additionally, animal studies suggest a role of IDE in Aß degradation. Transgenic mice over expressing IDE demonstrated reduced levels of Aß accumulation and prevented amyloid plaque formation (Leissring *et al.* 2003). On the other hand IDE knockout mice demonstrated increased Aß load and

the AICD (Miller *et al.* 2003;Farris *et al.* 2003). Another study also reported the ability of IDE to degrade AICD, suggesting that IDE is not specific to insulin and Aß only (Edbauer *et al.* 2002).

Although the above evidence suggests the involvement of IDE in Aß metabolism, however the proof for a genetic association remains controversial. As an example, one of the most recent studies concluded that there is no association of IDE haplotypes with the risk of developing dementia (Marlowe *et al.* 2006). Nevertheless, one can't overlook the strong *in vivo* data present linking IDE activity and Aß, and from a therapeutic point of view up-regulation or increasing activity of IDE remains a viable prospect.

1.1.7.3 Endothelin converting enzyme [ECE]

ECE is a transmembrane metalloprotease that catalyzes the conversion of the inactive precursor pro-endothelin to its potent vasoactive peptide endothelin. The most abundant form, ECE-1 is encoded by the gene located on the chromosome 1p36, and consists of 758 amino acids. In addition to pro-endothelin, ECE has been reported to hydrolyze other peptides such as bradykinin, substance P and neurotensin *in vitro* [see table 1.1]. ECE-1 is a member of the NEP family, and has shown 37 % homology to NEP (Sansom *et al.* 1998). Given this information and the fact that ECE-1 is non-specific in its substrate repertoire as mentioned above, it is not surprising that ECE-1 was examined as a potential Aß-degrading enzyme. The ability of ECE-1 to cleave Aß was first noticed by Eckmann *et al*, when they observed that phosphoramidon caused an increase of Aß accumulation in a cell line that expressed ECE, but not in another cell model devoid of ECE (Eckman *et al.* 2001). Recombinant soluble ECE-1 was demonstrated to hydrolyze Aß 40 and Aß 42 *in vitro* at multiple cleavage sites [see diagram 1-11]. Since ECE-1 devoid mice do not survive, heterozygous mice showing a 27 % decrease in ECE-1 activity were investigated for their Aß levels and they exhibited higher levels of Aß (Eckman *et al.* 2003).

ECE-2 which is relatively less studied isoform, has recently gained attention as an Aß degrading enzyme. ECE-2 is mainly localized in the brain; however its overall expression is only 1-2 % as much as the more abundant form ECE-1 (Wang *et al.* 2006). ECE-2 knockout mice develop increased amounts of Aß 40 and Aß 42 (Eckman *et al.* 2006). Interestingly, a recent microarray study of gene expression patterns demonstrated that ECE-2 was down-regulated in AD patients (Weeraratna *et al.* 2007). Therefore, it seems that ECEs play a role in Aß metabolism, ECE-2 role is less apparent, but this could be due to the fact that it was not extensively studied like ECE-1, owing to its scarcity.

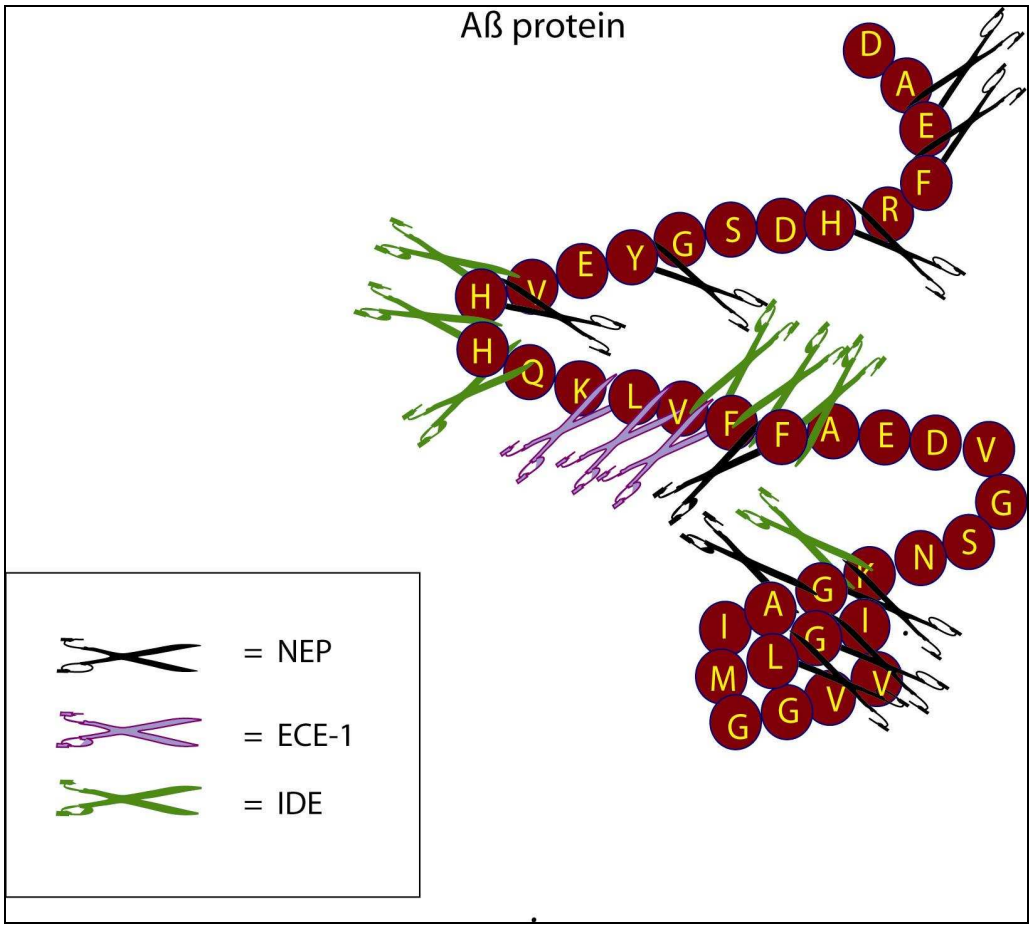


Figure 1-11

Aß cleavage sites by NEP, ECE-1 and IDE

1.1.8 Role of oxidative and nitrosative stress in AD

Reactive oxygen species [ROS] include both radicals such as oxygen ions and non-radicals such as peroxides. Under physiological conditions ROS are produced as natural byproducts of aerobic mitochondrial respiration. They play a physiological role in enzymatic reactions, cell signaling and neurotransmission. But unfortunately ROS can also cause deleterious effects on tissues when they exceed physiological amounts. This phenomenon known as oxidative stress, describes a situation where there is an imbalance between production and detoxification of ROS. Various cellular defense mechanisms such as anti-oxidant enzymes and vitamins serve to prevent the accumulation of ROS. However, in many cases such defense mechanisms are overburdened and oxidative stress leads to lipid peroxidation, DNA damage and finally cell death.

Aging and many aging-related diseases which include neurodegenerative diseases and Alzheimer's disease have been associated with excessive formation of ROS. The high vulnerability of the brain to oxidative stress could be explained by high metabolic rate [consumes about 20 % of oxygen uptake] its high unsaturated lipid to volume ratio and its reduced capacity for cellular regeneration compared with other organs.

The importance of net mitochondrial ROS production and its link to aging can be supported by the observations that enhancing mitochondrial antioxidant defenses can increase longevity. It has been recently shown that over-expression of catalase experimentally targeted to mitochondria increased lifespan in an already long-lived mouse strain (Schriner *et al.* 2005). Moreover, a study of gene expression in the human brain suggested that oxidative damage plays a major role in the cognitive decline that accompanies aging (Lu *et al.* 2004).

Since oxidative stress has been associated with aging, and the fact that aging is by far the greatest risk factor for sporadic AD, it was assumed that oxidative stress could be connected to AD. In light of this postulation many researchers directed their work towards this hypothesis. Nowadays, there is extensive literature supporting a role for mitochondrial dysfunction and oxidative damage in the pathogenesis of AD (Nunomura *et al.* 2001). Deficiencies in mitochondrial proteins that could contribute to ROS production have been detected in brains from AD patients (Kish *et al.* 1999;Maurer *et al.* 2000;Chandrasekaran *et al.* 1997). Electrophilic aldehydes such as malondialdehyde and free 4-hydroxy-2-nonenal [HNE] are elevated in AD brain tissue (Markesbery and Lovell 1998;Sayre *et al.* 1997;Williams *et al.* 2006). These aldehydes react with cellular nucleophiles such as DNA, proteins and lipids. Supporting this finding, glutathione transferase which is the enzyme responsible for clearance of HNE is decreased in several regions in AD brain including the hippocampus (Lovell *et al.* 1998). Another anti-oxidant enzymes, thioredoxin is also decreased in AD amygdala and hippocampus/ parahippocampal gyrus (Lovell *et al.* 2000). On the contrary SOD displays elevated expression levels in AD brains compared to age matched controls (Marcus *et al.* 2006;Schuessel *et al.* 2006). This could be explained as a compensatory mechanism against free radical damage in AD patients.

So the fact is oxidative stress and AD are related, the question that arises is how? The exact mechanisms are not known but here again Aß seems to play a role in this matter. Studies reported that Aß can increase ROS levels (Butterfield *et al.* 1999) and that oxidative stress leads to intracellular accumulation of Aß (Misonou *et al.* 2000). Further on, transgenic PC12 cells with APP mutations, that lead to increased Aß production, show increased oxidative stress (Marques *et al.* 2003a) and in transgenic mice models

oxidative damage and lipid peroxidation was shown to precede Aß deposition (Pratico *et al.* 2001).

Not only oxidative stress but also nitrosative stress is implicated in AD. Physiologically Nitric oxide synthese [NOS] in neurons [nNOS, type INOS] and endothelial cells (eNOS, type III NOS) produce nanomolar amounts of NO for short periods in response to transient increases in intracellular Ca^{+2}, which is essential for neurotransmission. The brain produces more Nitric oxide [NO] for signal transduction than the rest of the body combined, and its synthesis is induced by excitatory stimuli. Under various pathological conditions large amounts of NO are produced in the brain as a result of the induced expression of iNOS, excessive NO can exert various deleterious roles. It appears that both neuronal and glial NOS may play a role in the pathogenesis of AD and peroxynitrite formation. Increased expression of nNOS was reported in neurons with neurofibrillary tangles in the hippocampus and enthorinal cortex of AD patients as well as in reactive astrocytes near amyloid plaques (Simic *et al.* 2000;Thorns *et al.* 1998). Moreover, increased nitrotyrosine levels were demonstrated in hippocampus, neocortical regions, ventricular fluid and CSF of AD patients (Hensley *et al.* 1998;Smith *et al.* 1997). Importantly, the high levels of 3 nitrotyrosine in cerebrospinal fluid correlated with the decrease in cognitive function in AD patients (Tohgi *et al.* 1999).The role of Aß on NO levels has also been tested. Chronic intra-cerebroventricular infusion of Aß can also cause ROS and peroxynitrite formation and subsequent tyrosine nitration of proteins (Sarchielli *et al.* 2003). Also stimulation of cortical neurons with Aß 42 increased NO release and decrease cellular viability (Law *et al.* 2001).

Therefore multiple evidence suggest that both oxidative and nitrosative stress play a role in neurodegenerative diseases including AD. Although the exact mechanisms behind their role are still debatable, Aß seems to be involved somehow.

1.1.9 Mitochondrial dysfunction and AD

The role of mitochondria in aging and neurodegenerative diseases has received much more attention in the last decade. Due to the present evidence nowadays, mitochondrial dysfunction is believed to play a pivotal role in aging and AD.

Simply, mitochondria are double-membranous, self-replicating organelles with a circular genome of 16.5 kb DNA (Chen and Chan 2005). They are essential for cell viability and functioning. Mitochondria contain their own mitochondrial DNA and machinery for transcription, translation, and protein assembly. The mitochondrial DNA codes for 13 polypeptides that are part of the mitochondrial electron-transport chain, which is involved in the oxidative phosphorylation that generates adenosine triphosphate [ATP]. The production of ATP [energy] is the major function of the mitochondria and this is why they are known as power house of the cell. Oxidative phosphorylation operates through five protein complexes embedded in the inner membrane of the mitochondria known as respiratory chain complexes [see figure 1-12]. Since neuronal functions and synaptic neurotransmission require vast amounts of energy, mitochondria occupy an essential task by generating ATP and maintaining calcium homeostasis (Nicholls and Budd 2000;Kann and Kovacs 2007). Therefore, impairment in mitochondrial vital functions may have serious and deleterious consequences on neuronal physiology.

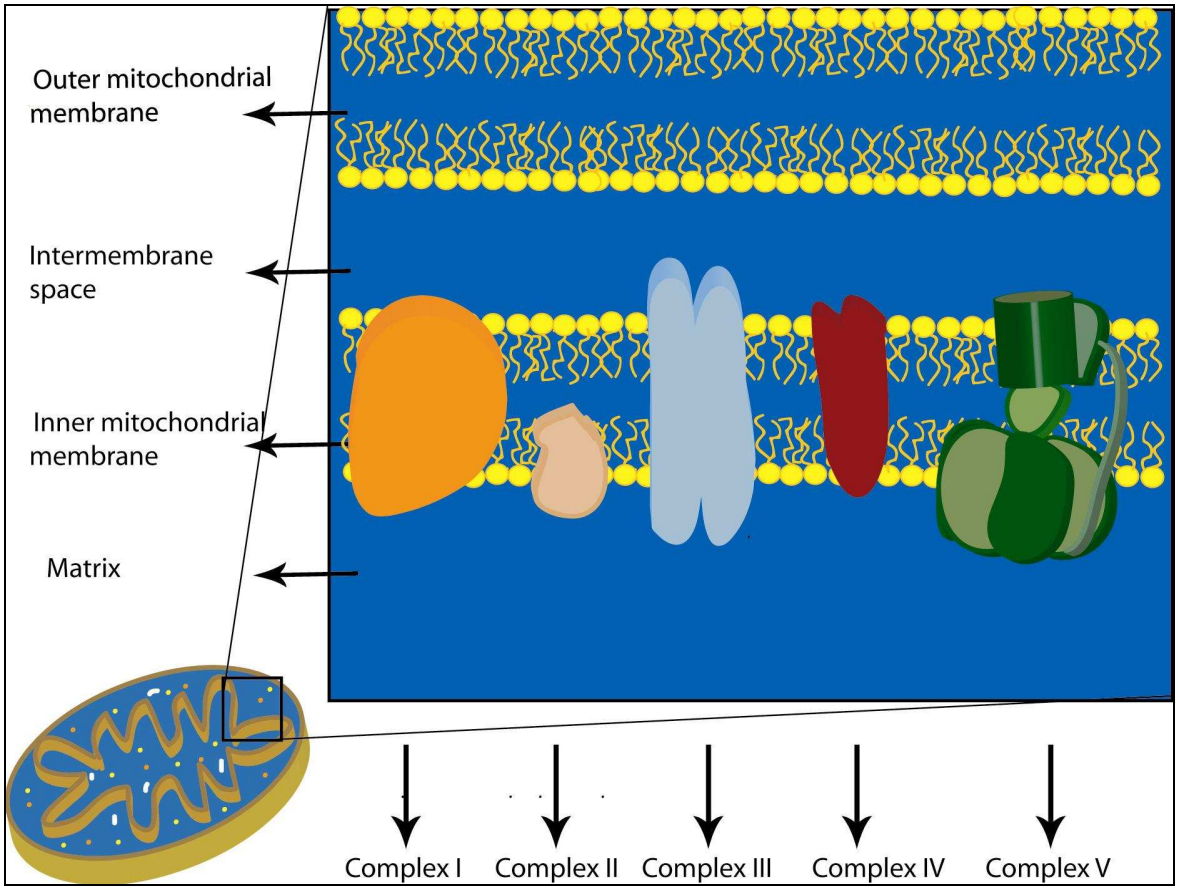


Figure 1-12

Diagram showing mitochondria and the respiratory chain

Researchers have in the last few years found many evidences linking mitochondrial dysfunction to AD. Baloyannis reviewed the morphologic alterations of the mitochondria in 22 brains of AD patients, and reported disruption of the cristae and/or osmiophilic inclusions (Baloyannis 2006).

Morphometric studies of the mitochondria in AD revealed a significant reduction in mitochondria density in endothelial cells as well as in fibroblasts obtained from patients with AD (Stewart *et al.* 1992).This observation was also seen in mitochondria from Frontal and temporal cortex of AD patients (Hirai *et al.* 2001).

Not only morphological changes in AD mitochondria were noticed but more importantly functional changes. Positron emission tomography [PET] showed decrease energy metabolism in AD brains (Azari *et al.* 1993;Grady *et al.* 1988). Tricarboxylic acid cycle [TCA], which takes place in the mitochondria,

is the main pathway for oxidation of glucose in the brain. Deficiency in the two key enzymes of the rate-limiting step of the TCA cycle, Pyruvate dehydrogenese [PDHC] and alpha -ketoglutarate dehydrogenase complex, [KGDHC] has been documented in AD cases by multiple groups, suggesting defects in glucose metabolism in the AD brains (Gibson *et al.* 2000;Sorbi *et al.* 1983). Normally, electrons from the TCA are transported across the respiratory chain in order to produce ATP. Deficiency of cytochrome-c-oxidase [COX] [complex IV of the respiratory chain] in different AD brain regions has been reported (Bosetti *et al.* 2002;Mutisya *et al.* 1994). So it seems that COX and most probably PDHC and KGDHC activities are decreased in AD patients, the exact mechanism of their deactivation is not known but interestingly it has been reported that Aß inhibits both COX and KGDHC in isolated brain mitochondria (Casley *et al.* 2002).

Aß seems also to have other toxic effects on the mitochondria in cell and animal models. Previous studies showed that in the presence of Ca^{+2} Aß 40 and $Aß_{25–35}$ induce the opening of Permeability transition pore [PTP] (Mancuso *et al.* 2006;Mancuso *et al.* 2003). PTP induction, a phenomenon characterized by a sudden increase in the permeability of the inner mitochondrial membrane, plays a key role in apoptotic cell death by facilitating the release of apoptogenic factors.

The toxic effects of Aß on the mitochondria were observed in transgenic animal models as well, for example Keil *et al* demonstrated a decrease in mitochondrial membrane potential and ATP levels in APP-transgenic mouse when compared to littermate non-transgenic mice. Accumulation of Aß in Tg2576 AD transgenic mice and mouse neuroblastoma cells expressing human APP correlated with high levels of H_2O_2, impaired cytochrome-oxidase activity, and increased carbonylation of mitochondrial proteins (Manczak *et al.* 2006).

Anandatheerthavarada *et al* linked amyloid to the mitochondrion. These authors showed, for the first time, that APP is targeted to neuronal mitochondria. They showed in a transgenic mouse model of AD [Tg2576] that over-expresses Swedish APP, accumulation of incompletely translocated full-length APP in the mitochondrial compartment of the cortex and hippocampus known to be affected in AD (Anandatheerthavarada *et al.* 2003). The same group then extended their results in human AD brains. They found that non-glycosylated full-length and C-terminally–truncated APP was associated with mitochondria in samples from the brains of individuals with AD, but not with mitochondria in samples from non-demented subjects (Devi *et al.* 2006). However, the frontal cortex, hippocampus, and amygdala showed the highest accumulation of APP in the mitochondria of all three categories of AD brains. Triple-labeling immuno-histochemistry of AD brains revealed the accumulation of APP in the mitochondria of cholinergic neurons of all stages of AD brains.

In order to study more extensively the effect of mitochondria in AD the cytoplasmic hybrid ("cybrid") technique, first described in 1989 (King and Attardi 1989), has been applied. In this technique, mitochondria/ mtDNA from human AD and control platelets is transferred to cultivable cells depleted of endogenous mtDNA. Interestingly the AD cybrids showed elevated secretion of Aß, they also confirmed the COX deficiency found in the mitochondria of AD patients. AD cybrids also show elevated spontaneous death with apoptotic nuclear morphology and decrease in mitochondrial membrane potential (Khan *et al.* 2000). Moreover, increased caspase-3 activity and elevated cleavage of caspase substrate were previously reported (Khan *et al.* 2000;Onyango *et al.* 2005).

Wrapping up this section, mitochondrial dysfunction is always involved in AD, whether present in post-mortem AD tissue, transgenic cell or animal models or AD cybrids. Additionally, Aβ the major pathologic hallmark for

AD plays a key role in mitochondrial impairment. However which starts first, Aβ aggregation or mitochondrial dysfunction in this viscous cycle is still an unanswered question.

1.1.10 Therapeutic interventions

After 100 years of AD discovery the absence of treatment with a major impact is quite disappointing. The drugs approved are mainly for symptomatic treatments, and this is related to the fact that the pathways that cause AD are still not fully declared. Although multiple drugs have now been approved, their expected benefits are marginal. However, the causes behind AD are being slowly identified and new treatments in the pipeline which are aimed at these mechanisms are being developed.

In this section a short overview of the current approved drugs for AD is given, followed by the new therapeutic interventions under development or in clinical trials.

1.1.10.1 Acetylcholinesterase inhibitors

Post-mortem findings in the early 1970s such as reduced acetylcholine [Ach] release and presynaptic cholinergic deficits in brains of AD patients led to the development of the cholinergic hypothesis of AD. The 'cholinergic deficit hypothesis' was the dominant theory in AD in the early 1980s. This hypothesis states that degeneration of cholinergic neurons in the basal forebrain nuclei causes disturbances in presynaptic cholinergic terminals in the hippocampus and neocortex, which is important for memory disturbances and other cognitive symptoms (Terry, Jr. and Buccafusco 2003). It was anticipated that restoring the cholinergic balance by inhibition of Ach breakdown would improve cognitive functions and retard the progression of AD. The 2 Cholinesterases, namely, acetyl cholinesterase [AchE] and butyryl cholinesterase [BuChE] control the availability of Ach in the synapses.

In 1993, the FDA approved the first drug for the treatment of AD, the cholinesterase inhibitor [ChEI] tacrine, which was soon followed by other ChEIs: donepezil [1996], rivastigmine [2000] and galantamine [2001]. All ChEIs interfere with the degrading of Ach by blocking AchE. Donepezil and

galantamine are selective AchE inhibitors while rivastigmine inhibits AchE and BuChE with similar affinity. Theoretically, this dual action could be valuable because in AD, BuChE levels tend to be higher compared with AchE, which decreases in the course of the disease process (Lane *et al.* 2006). In addition to inhibiting AchE, galantamine allosterically modulates presynaptic nicotinic receptors.

The efficiency of these drugs has been studied in more than 30 randomized double-blind clinical trials, most of which indicate the symptomatic treatment influence of AchEI. In a 2006 Cochrane review, it was concluded that the acetyl cholinesterase inhibitors donepezil, rivastigmine, and galantamine are efficacious in mild to moderate AD (Birks 2006). Overall, they are safe drugs, but exhibiting predominant gastrointestinal side-effects including nausea, vomiting, and diarrhea. The incidence of side-effects can usually be reduced by starting treatment with a low dose, which is escalated slowly. However caution should be taken in prescribing AchEI to patients with cardiovascular diseases, GIT problems, or Asthma.

1.1.10.2 Memantine

Memantine, a low to moderate affinity, uncompetitive NMDA [N-methyl-D-aspartate] receptor antagonist, was approved in 2003 for AD treatment. Glutamatergic dysfunction can lead to an excessive influx of calcium ions through NMDA receptors, leading to neuronal death. Such “excitotoxicity” has been implicated in AD (Danysz *et al.* 2000). Memantine is thought to block selectively the effects associated with abnormal transmission of the neurotransmitter glutamate, without preventing the physiological transmission associated with normal functioning (Wilcock 2003).

Memantine treatment of patients with moderate-to-severe AD has been shown to confer significant benefits on cognition whether alone or when administered concomitantly with other ChEI (Gauthier *et al.* 2005;Reisberg *et*

al. 2003;Tariot *et al.* 2004). The efficacy of memantine in patients with mild- to moderate AD is debatable, and the latest Cochrane review concluded that there are no data to lend support to the notion that memantine has any beneficial effect in mild stages (Areosa and Sherriff 2003). Due to the scarcity of trials, the assessment of memantine as a disease modifying drug is difficult. However, memantine is well tolerated, with few adverse events, and may be a useful therapeutic adjunct in patients with moderate to severe disease, typically defined as an MMSE less than 15 points (Areosa and Sherriff 2003).

1.1.10.3 Piracetam

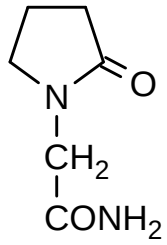


Figure 1-13

Chemical structure of the compound piracetam

Piracetam [2-oxo-pyrrolidine carboxylic acid] was first synthesized in 1964 at the pharmaceutical company UCB. Piracetam is popularly referred to as a "smart drug", or scientifically rephrased a Nootropic ["mind-related"] drug. This name was first assigned to Piracetam due to its unique pharmacological actions which include facilitating inter-hemispheric transfer and enhancing cerebral resistance to cognitive impairments induced by hypoxia and aging (Giurgea *et al.* 1983). It is prescribed for the treatment of cognitive impairments, cerebral insufficiencies and cortical myoclonus.

Several clinical trials have shown the efficacy of piracetam for cognitive impairments. In 2001 Tsolaki *et al* reported that the efficacy of piracetam is comparable with AchE inhibitors (Tsolaki *et al.* 2001). A meta-analysis

including 19 trials concluded that piracetam was effective in patients with cognitive impairment (Waegemans *et al.* 2002).

The mechanisms lying behind the actions of piracetam are not fully elucidated. It has been reported that piracetam can restore changes that occur in brain membrane fluidity during aging, and elevates both muscarinic cholinergic and NMDA receptor density in rodents (Cohen and Muller 1993;Pilch and Muller 1988). Moreover, mitochondrial protective properties have been also described for piracetam (Keil *et al.* 2006).

1.1.10.4 Ginkgo Biloba extract

An extract of Ginkgo biloba leaves was first registered as a medication by Dr. Willmar Schwabe GmbH & Co in France under the trademark Tanakan by IPSEN. Several different Ginkgo extracts exist nowadays in the European market as well as in the US as dietary supplement. In Germany it's a highly prescribed medication and since the early 1990s, Ginkgo biloba leaf extracts are becoming one of the most popularly used supplements for memory enhancement in the US.

The fact that Ginkgo extracts constitute several active compounds such as flavonoids and terpenoids, gives it its unique toti-potent properties. A standardized leaf extract [EGb 761®] was reported as an anti-oxidant, anti-apoptotic, gene regulator and comprises other mechanisms of action, unlike synthetic drugs, which provide a single target for a single receptor as the mechanism of action.

Concerning its clinical efficacy a vast number of clinical trials and meta-analysis are present, but unfortunately major differences in the experimental parameters makes it quite challenging trying to reach a reasonable conclusion. In light of the present studies Ginkgo has been reported as a safe drug, and shows potential in enhancing cognition and function in patients with dementia [for further details on Ginkgo extract refer to section 1.2].

1.1.11 Novel therapeutic strategies

Due to the modest benefits acquired from currently existing AD therapies, scientists are striving to develop more potent AD drugs. A number of new compounds with different targets are being tested for their safety and efficacy in clinical trials. Reducing Aß production is one goal and under development are several drugs targeting secretases.

1.1.11.1 Targeting secretases: Gamma secretase

As already mentioned, γ-secretase is involved in the last step of APP cleavage to yield either Aß 40 or Aß 42 [see section 1.1.4.5]. Unfortunately, besides APP, γ-secretase has other substrates including the transmembrane protein notch receptor-1. Notch is necessary for growth and development and notch related side effects of γ-secretase inhibition [e.g. severe gastrointestinal and haemopoetic side effects, neurodegeneration] have been hindering the development of clinically valuable γ-secretase inhibitors so far.

Nevertheless, γ-secretase inhibitors have been developed that do not affect notch signaling and have shown good tolerability in phase I studies (Petit *et al.* 2001;Siemers *et al.* 2005). A well tolerated compound, LY450139, reduced the amount of Aß in the plasma, but not in the CSF (Siemers *et al.* 2006). Designing new-generation γ-secretase inhibitors that selectively decrease APP cleavage without affecting other targets is a cumbersome process. One approach targets the substrate-docking site of γ-secretase to selectively interfere with APP binding (Wolfe 2006). Another approach is modulating γ-secretase instead of inhibiting it, for example shifting the cleavage towards more production of Aß 40 in preference to Aß 42. Some NSAIDS [non-steriodal anti-inflammatory drugs] have this property by allosterically modulating γ-secretase and are now in phase III clinical trials (Eriksen *et al.* 2003;Lleo *et al.* 2004). Tarenflurbil, the most promising candidate, was not very successful in a phase II clinical trial. The primary

analysis failed where there was no overall effect on the primary outcomes. But planned analyses suggested that there was evidence of a favourable effect of treatment on activities of daily living and global function in the subgroup of patients with mild AD (Wilcock *et al.* 2008).

1.1.11.2 Targeting secretases: Beta secretase inhibitors

Another method of decreasing Aß generation involves inhibiting ß-secretase [BACE], which is essential for the cleavage of APP to produce Aß. Keeping in mind that BACE has fewer substrates compared to γ-secretase and that knockout mice seem to have a normal phenotype (Luo *et al.* 2001), one would expect that the development of such compounds is safer and more promising.

However the hampering reasons here are structural, development of small molecules able to pass the BBB, which is quite complicated (Citron 2004). NSAIDs seem to play a role here as well, showing the ability to inhibit BACE1 by activating the PPAR- γ [peroxisome proliferated activated receptor- γ] (Sastre *et al.* 2006). Further studies need to be done in this field in order to overcome the structural complexity and to assure effectiveness and safety.

1.1.11.3 Aß clearance

Increasing Aß clearance through Aß immunization or Aß degrading enzymes could be feasible. The Aß clearance by the Aß-degrading enzymes has been thoroughly discussed before [see section 1.1.6], left to be said is that no phase II or III studies have been published on the effects of enhancing enzymatic degradation in AD patients.

1.1.11.4 Immunotherapy

As for promoting Aß clearance by immunization, this approach appears to be quite promising. In a phase II trial active immunization with Aß plus adjuvant appeared to reduce amyloid deposits in some brain regions and improve certain cognitive measures in patients who developed antibodies.

Unfortunately, the trial was discontinued due to the development of 18 cases [6%] of meningoencephalitis, which was most likely an immune response complication mediated by T helper cells (Gilman *et al.* 2005).

In order to circumvent this problem, new vaccines which lack the amino acid parts thought to be responsible for the T-cell response, but preserving the beneficial antibodies against Aß are under development.

An alternative immunotherapeutic strategy, avoiding T-cell response, is passive immunization. Unpublished data from a phase II study of the humanized monoclonal antibody bapineuzumab demonstrated that there was no improvement in cognition and functional ability (Burns 2009).

Another approach is targeting Aß aggregation. The only Aß aggregation inhibitor reaching phase III is the synthetic glycosaminoglycan 3-amino-1-propaneosulfonic acid [3APS, tramiprosate, Alzhemed]. It is designed to prevent conformational transitions that lead to the assembly of the neurotoxic oligomers and subsequently protofibrils and fibrils. A phase II trial using 3APS demonstrated a significant decrease in mean CSF Aß 42 concentration vs. baseline, but no significant differences in the cognitive and clinical assessments between the 3APS and placebo groups was noticed after 3 months of treatment. However, the disappointing results from the US phase III trial in the year 2007 led to discontinuation of the European phase III trial (McLaurin *et al.* 2006;Gervais *et al.* 2007).

Several other drugs not targeted at Aß are being examined for their efficacy. These include mainly statins, anti-oxidants, anti-inflammatory drugs, estrogens, nerve growth factor [NGF] mimics and anti-tau drugs. An old anti-histmainic drug, dimebon, has shown promising results in a clinical trial for patients with mild-to-moderate AD (Doody *et al.* 2008). Interestingly, one of the possible discussed mechanisms of action is mitochondrial protection by

preventing opening of mitochondrial pores induced by neurotoxins (Bachurin *et al.* 2003).

Concluding, there seems to be consensus that multiple drugs will be required for the treatment of AD, and more or less individually tailored drugs should be indicated. This creates imposing challenges for both researchers and pharmaceutical industries in developing such drugs.

1.2 Ginkgo Biloba

The Ginkgo tree is a living fossil, representing the family Ginkgoaceae. It is the only surviving member of this family dating back to hundreds of millions of years. Scientists thought that the Ginkgo tree became extinct, until the German physician Engelbert Kaempfer discovered its presence in Japan. Ginkgo seeds were brought to Europe from Japan by Kaempfer in the early 1700's and to the USA later that century.

Figure 1-14

Ginkgo biloba tree

The seed of the Ginkgo tree has a silvery shine [see photo below] and the name Ginkgo is thought to originate from the Japanese word Ginkyō meaning silver apricot. As for the term biloba it is a Latin word meaning 2 lobes, describing the shape of the leaves. They are leathery with a wax layer and a deep vertical slit dividing the leaf into 2 lobes and giving it its characteristic form.

Figure 1-15

Ginkgo biloba seeds and leaves

The Ginkgo biloba seeds appearing like a small apricot with a silvery shine [left] and the typical leaves with a slit dividing it into 2 lobes [left]

1.2.1 Medicinal History

The seeds of Ginkgo biloba were first described in the Chinese medicine for over 5000 years. The use of seeds was described for digestion and for the treatment of asthma, coughs, irritability of the bladder, blenorrhoa and uterine fluxes. As for the use of the leaves, this was mentioned later as an aid for blood circulation, for the lungs, for skin treatment, head sores and freckles. The first publication concerning the internal use of the leaves of Ginkgo biloba for medicinal purposes dates back to 1505 A.D, where it was first mentioned to be used for the treatment of diarrhea.

Dr. Willmar Schwabe a German physician-pharmacist introduced extracts of Ginkgo biloba leaves into medicinal practice in 1965. The extract of Ginkgo biloba leaves was first registered as a medication by Dr. Willmar Schwabe GmbH & Co. in Germany with the trade name Tebonin. Co-developed by Dr. Willmar Schwabe Company, IPSEN and INTERSAN the extract obtained the code name "EGb 761®".

Figure 1-16

Dr. Willmar Schwabe [1840-1917]

Dr. Willmar Schwabe Pharmaceuticals was found in 1866.

1.2.2 Production of EGb 761®

1.2.2.1 Harvesting and cultivation

The leaves for EGb 761® are harvested with a modified cotton-picker and air-dried to contain about 70-75 % moisture before drying and approximately 10 % after drying. Dried leaves from the plantations in Bordeaux, South Carolina and the Far East are analyzed for the contents of the active constituents. It is notable to mention the fact that the amounts of active constituents differ dramatically according to the season of harvesting.

1.2.2.2 Extraction and standardization

Like any other plant extract, toxic substances which can cause allergies or other disturbances have to be removed. In the case of Ginkgo biloba these toxic substances are mainly alkylphenol compounds. A patented invention by Schwabe in 1994 provided an extract of Ginkgo leaves that is substantially free from the alkyl phenols and containing all the ginkgolides and the flavonol glycosides present essentially in the leaves. From 100 kg Ginkgo leaves only 2.5 kg of extract are obtained.

To ensure a standardized extraction the Ginkgo leaves are harvested in summer or autumn while they are still green followed by an 18-step extraction procedure. According to the German federal health authority the composition of the extract is as follows:

“A dry extract from the dried leaves of Ginkgo biloba Linne manufactured using acetone/water and subsequent purification steps without additionally mixing concentrations or isolated active ingredients. The extract is characterized by 22-27 % flavonol glycosides, determined as quercetin, kaempferol including isorhamnetin, 5-7 % terpene lactones, of each approximately 2.8-3.4 % consist of ginkgolide A, B and C, as well as approximately 2.6-3.2 % bilobalide and below 5 ppm ginkgolic acids.

To insure consistent quality a standardized procedure is always preformed.

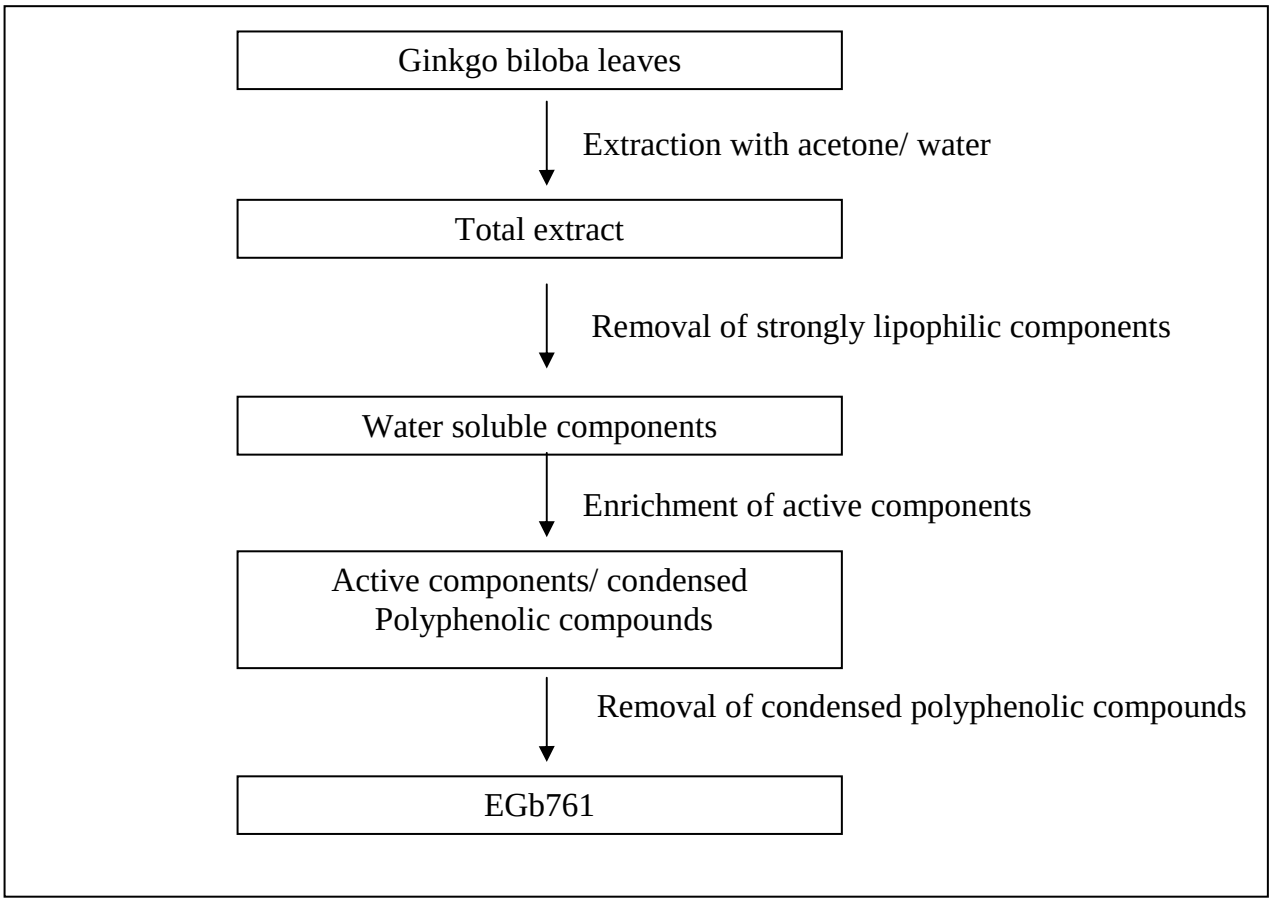


Figure 1-17

Extraction and standardization process for preparation of EGb 761®

Scheme demonstrating the extraction procedure for preparation of EGb 761® from Ginkgo biloba leaves.

1.2.3 Chemical composition

1.2.3.1 Flavonoids

The flavonoids present in EGb 761® are exclusively flavonol-o-glycosides i.e. combinations of the phenolic aglycon with sugars in different positions of the flavonol moiety. The chemical structures of the flavonoids in EGb 761® are illustrated below.

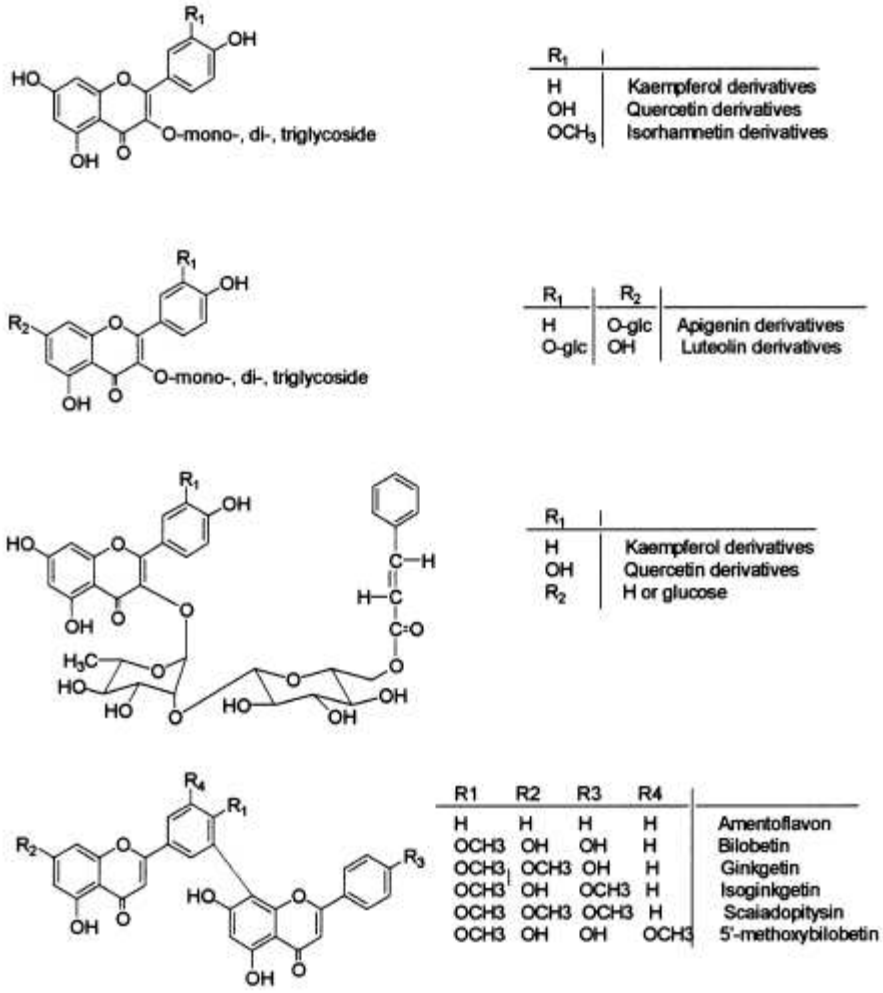


Figure 1-18

Chemical structure of flavonoid constituents

Flavonol-O-glycosides, with a glycosidic linkage normally located in position 3 or 7 of a phenolic aglycon [quercetin, kaempferol or isorhamnetin] and the carbohydrate moiety usually being D-glucose, L-rhamnose or glucorhamnose.

1.2.3.2 Terpeniods

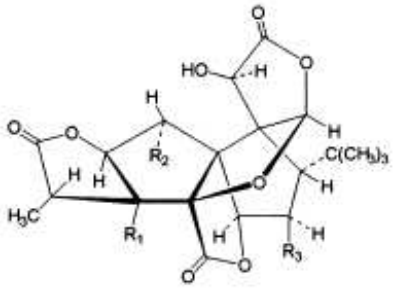
The terpeniods present in EGb 761® are Ginkgolides and Bilobalide. The Ginkgolides that are present in the EGb 761® extract are diterpenes, Biloblaide is a pentanorditerpene. These particular terpenes are found exclusively in Ginkgo biloba and they possess three lactone functions and a tertiary butyl group.

1.2.3.3 Ginkgolides

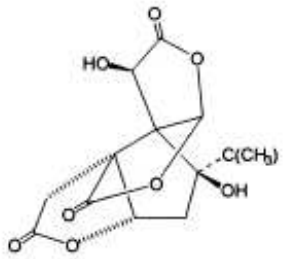
In 1967 four Ginkgolides were identified namely Ginkgolides A, B, C and M. Recently Ginkgolide J was identified as a constituent in EGb 761®. Ginkgolides A, B and C account for about 3.1 % of EGb 761®. Ginkgolides A and C are present in the highest amounts each representing ≥1 %, Ginkgolide B is ≤1% and Ginkgolide J ≤0.5 %. Ginkgolide M was only found in the roots of the Ginkgo tree, therefore it is not present in the leaf extract EGb 761® (Defeudis 1998).

1.2.3.4 Bilobalide

Bilobaide accounts for about 2.9 % of EGb 761®. Bilobalide is a pentanorditerpene and it was first isolated by Major in 1967 (Defeudis 1998).



R1	R2	R3	Ginkgolide
OH	H	H	A
OH	OH	H	B
OH	OH	OH	C
OH	H	OH	J
H	OH	OH	M



Bilabolid A

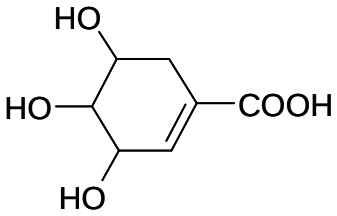
Figure 1-19

Chemical structure of terpene constituents of Ginkgo biloba

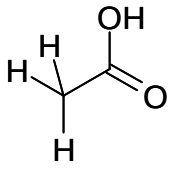
Ginkgolides A, B, C and J have been isolated from the leaves and are present in EGb 761®, Ginkgolide M is found only in the roots. Bilobalide accounts for about 3.1 % of EGb 761®.

1.2.3.5 Organic acids

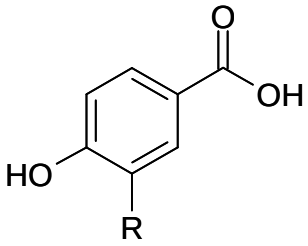
Organic acids represent about 5-10 % of EGb 761®.These include vanillic acid, protocatechuic acid, acetic acid, schikimic acid, p-hydroxybenzoic acid and in lower amounts kynurenic acid and ascorbic acid. These substances increase the solubility of the extract by increasing its acidity. Ginkgolic acid, which is toxic, is non-detectable in the EGb 761® extract.



Shikimic acid



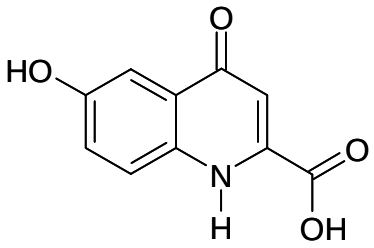
Acetic acid



R=H: 4-Hydroxybenzoic acid

R=OH: 3,4-Dihydroxybenzoic acid

R=OCH_3: 3-Methoxy-4-hydroxybenzoic acid



6-hydroxykynurenic acid

Figure 1-20

Chemical structures of acids present in EGb 761®

Acetic acid, shikimic acid, 4-hydroxybenzoic acid, protocatechouic acid [3, 4 dihydroxybenzoic acid], vanillic acid [3-methoxy-4-hydroxybenzoic acid] and 6-hydroxykynurenic acid. These acids are present in amounts >0.5 %.

1.2.4 Pharmacokinetics

1.2.4.1 EGb 761®

Determining the pharmacokinetic properties of EGb 761® is quite difficult due to the presence of several active constituents in the extract. However, the examining of the pharmacokinetic properties was already addressed in 1986. Moreau *et al* preformed their study on rats using radioactive EGb 761®. At least 60 % of the radioactivity was absorbed and was broadly distributed. In the brain radioactivity was detected in particular in the hypothalamus, striatum and hippocampus.

1.2.4.2 Flavonoids

The pharmacokinetics of the flavonoid glycosides present in the EGb 761® was thoroughly investigated. The glycosides are absorbed but also quickly metabolized. Therefore detection of glycosides and aglycons is not possible but their metabolites could be detected in plasma indicating their bioavailability. This was seen by Pietta *et al* in a 15 days treatment study on rats using EGb 761® (Pietta *et al.* 1995) and confirmed in other human studies. In human plasma, only quercetin glucuronides, but no free quercetin, could be detected after administration of quercetin plant extract to 12 healthy volunteers in a four-way crossover study (Graefe *et al.* 2001). Another study using C57BL6 mice measured the plasma concentrations of quercetin, kaempferol and isorhamnetin after administration of 36 mg/kg body weight EGb 761®. The plasma concentrations of quercetin, kaempferol and isorhamnetin were 12, 7 and 49.6 ng/ml respectively in the Ginkgo-supplemented group compared to the control group [4.8 and 3.2 ng/ml, respectively; isorhamnetin was not detectable] (Watanabe *et al.* 2001).

1.2.4.3 Ginkgolides

In an attempt to find the bioavailability of the ginkgolides and Bilobalide [BB] several pharmacokinetic studies were preformed and are described in the literature. The first pharmacokinetic study described in the literature was in 1995 by Fourtillan *et al*. In this study on human volunteers both oral and I.V EGb 761® were administered and the authors concluded that ginkgolides A [GA] and B [GB] are highly bioavailabe but C [GC] was not bioavailable.

The relative bioavailability of GA, GB and BB was demonstrated to differ in the plasma after a single oral dose of 120 mg Ginkgo biloba according to the pharmaceutical preparation applied (Kressmann *et al*. 2002).

Measuring the pharmacokinetic parameters in rats after oral administration of EGb 761® the plasma concentrations were BB> GA> GB, and GC was not detectable (Biber 2003). Other studies confirmed the bioavailability of GA, GB and BB (Mauri *et al.* 2001). Only very recently, Xie *et al* were able to report pharmacokinetic data for GC by a novel method after I.V injection of Ginkgo biloba leaf extract at a dose of 8 mg/kg (Xie *et al*. 2008).

The only study showing distribution of GB in the brain was recently published (Chen *et al*. 2007). Chen *et al* were able to demonstrate that 60 minutes after I.V administration of 12 mg/kg GB to rats, the concentration of GB in the brain was 148.9 ng/ ml, and after 6 hours GB was not detectable anymore.

1.2.5 Medicinal properties of Ginkgo biloba

Ginkgo biloba fruits and seeds have been used in Chinese traditional medicine with indications for the treatment of asthma, bronchitis and skin diseases. The use of Ginkgo biloba leaves came later into practice, where the internal use of Ginkgo leaves was first published in a text by Liu Wen-Tai. Modern Chinese pharmacopeias introduced the leaves of Ginkgo biloba for treating dysfunctions of heart and lungs (Defeudis 1998). In folk medicine Ginkgo can be used to induce labor, for the treatment of bronchitis, chronic rhinitis, arthritis and edema. However, these indications have no scientific background and are not supported by research or clinical data. Surprisingly, the indications of Ginkgo nowadays were not identified in folk medicine.

According to the WHO monograph for medicinal plants, standardized Ginkgo biloba leaves extracts have been used for symptomatic treatment of mild to moderate cerebrovascular insufficiency, different forms of dementia with the following symptoms: memory deficit, disturbance in concentration, depressive emotional condition, dizziness, tinnitus, and headache. Standardized extracts are also used in peripheral arterial occlusive disease such as intermittent claudication, Raynaud disease, acrocyanosis, and post-phlebitis syndrome, and to treat inner ear disorders such as tinnitus and vertigo of vascular and involutive origin.

Nowadays, the standardized extract of Ginkgo biloba leaves EGb 761® is becoming one of the most popular herbal medicine and dietary supplements consumed in Europe and the US. Since the major therapeutic indications for EGb 761® are associated with age-related memory impairments, more attention will be given here to the research in this field. Earlier, *in vivo* or *ex vivo* research using EGb 761® was more extensively studied, but recently the trend is moving towards cellular and molecular mechanisms of action of EGb 761® and its constituents.

1.2.6 Pharmacological effects

The pharmacological effects of EGb 761® and its constituents that most likely play a role in the treatment of memory deficits and Alzheimer's disease are discussed below.

1.2.6.1 Free radical scavenging effect

The important role of oxidative stress and ROS in aging and AD has been previously discussed in section 1.1.8. One of the highly proposed mechanisms of action of EGb 761® is its free radical scavenging effect. Several researchers have linked the neuroprotective effect of EGb 761® to its anti-oxidative effect. Previous studies have highlighted the potential of EGb 761® and its components as scavengers of free radicals such as $OH^{\cdot}$, O_2^{-} and NO in both *in vitro* acellular and cellular studies, as well as *in vivo* studies (Defeudis 1998).

For instance, Smith and Luo have demonstrated that a pre-treatment with EGb 761® significantly lowered ROS levels both in a cellular model stably expressing an AD associated double mutation and in *C.elegans* AD model (Smith and Luo 2003). EGb 761® and α-tocopherol exhibited similar inhibitory effects *in vitro* on lipid peroxidation in rat brain synaptosomes and human lymphocytes (Sram *et al.* 1993).

In post-mortem tissue from AD patients' it was shown that EGb 761® prevented lipid peroxidation induced by the pro-oxidant system H_2O_2/Fe^{2+} (Ramassamy *et al.* 1999). Moreover, intracellular and mitochondrial ROS decreased when hippocampal neurons were treated for 24 hours with EGb 761® (Bastianetto *et al.* 2000a).

Not only *in vitro* studies linked the anti-oxidative effects of EGb 761® to its neuroprotective effects, but a number of *in vivo* studies supported this fact.

Treating rats with 50 or 100 mg/kg body weight for 12 weeks resulted in decrease of lipid peroxidation in the cerebral cortex, and only the higher

concentration [100 mg/kg body weight] was able to decrease lipid peroxidation in the hippocampus (Sram *et al.* 1993).

Another *in vivo* study by Holgado *et al* in 1995 showed very interesting results after treating 4 and 33 months old rats with 100 mg/kg body weight EGb 761® daily for 3 months. In the younger rats EGb 761® was able to decrease the rates of O_2^- with no effect on superoxide dismutase [SOD], catalase and glutathione peroxidase. However in the older rats EGb 761® treatment was able to decrease the rate of O_2^- generation as well as increase the scavenging activities of the 3 mentioned enzymes. Other results were also able to confirm the ability of EGb 761® to increase the activities of anti-oxidant enzymes, for example glutathione in mouse liver (Sasaki *et al.* 2002) as well as SOD and catalase (Bridi *et al.* 2001) in rats.

A more recent study showed that both Ginkgo biloba extract and vitamin E reduced the oxidative stress resulting from senile plaques *in vivo* as monitored with intracranial imaging (Garcia-Alloza *et al.* 2006).

With all the above mentioned evidence it seems that the anti-oxidant property of EGb 761® plays a significant role in its therapeutic effects, the question that arises is clearly which of EGb 761® constituents is responsible for these effects.

Many researchers have addressed this question and the majority links the radical scavenging activity of EGb 761® to the flavonoid fraction. This is partly due to their chemical structure.

In acellular models and test-tube reactions EGb 761® scavenges hydroxyl radicals and superoxide radicals efficiently, while the terpenoid fractions fails (Pietri *et al.* 1997).

Ramassamy *et al* were able to show that the flavonoid fraction of EGb 761® has higher ability to prevent oxidative-induced damages than the terpene fraction (Ramassamy *et al.* 1993). In another study, the anti-lipoperoxidative

effect of the terpenoids and the flavonoids were determined in isolated hepatocytes and the terpenoid fraction showed no effect while the flavoniods were effective (Joyeux *et al.* 1995). Moreover, the flavonoid fraction [CP 205], but not the terpenoid constituents [CP 160 and BN 52021], was also able to decrease the Aß-induced ROS production in hippocampal cells (Bastianetto *et al.* 2000a).

Although many studies assert the anti-oxidative effect of EGb 761® to the flavonoids, it seems that the terpenoid fraction also play a role here.

In an acellular model it was shown that BB, GB, GC and GJ but not GA have radical scavenging activities (Scholtyssek *et al.* 1997) when using DMSO as a solvent instead of water.

In vitro, Zhou *et al* were also able to show that BB can protect neurons against oxidative stress (Zhou and Zhu 2000). Some studies showed that the flavonoid fraction alone was not able to exert the same effect as the whole extract. For example, the H_2O_2-induced toxicity in hippocampal cells was attenuated by a co-treatment with EGb 761®; however the flavonoid fraction CP 205 was not effective (Bastianetto *et al.* 2000a).

Therefore, the primary assumption that the anti-oxidative properties of EGb 761® are only due to the presence of its flavonoid fraction is imprecise.

Concluding, it seems that EGb 761® has indeed free radical scavenging and anti-oxidant properties, regardless of the exact constituents that are implicated. It appears that the flavonoid fraction exert a direct radical scavenging activity which could be partly explained by its chemical structure, and that the terpenoids seem to somehow decrease the generation of free radicals.

Although EGb 761® is known to act as a vasodilator, and can influence the endothelial nitric oxide synthase induced NO production (Li *et al.* 2001), it is suggested that excessive NO production stimulated by inducible nitric oxide

synthase could be controlled by EGb 761®. The deleterious effects of excessive NO have also been previously discussed [section 1.1.8] and fortunately EGb 761® shows not only anti-oxidative properties but also can protect cells from nitrosative stress. In 1994, EGb 761® was described as an NO scavenger in acellular system (Marcocci *et al.* 1994). Later on other *in vitro* studies showed the ability of EGb 761® to protect cells against NO damage in PC-12 cells and hippocampal neurons (Eckert *et al.* 2005;Bastianetto *et al.* 2000b).

As for the EGb 761® constituents, in PC 12 cells BB was able to inhibit the NO induced toxicity, on the other hand in hippocampal neurons the flavonoid fraction was most effective and the terpenoid and GB were not effective (Song *et al.* 2000;Bastianetto *et al.* 2000b).

To sum up EGb 761® and its components are capable of scavenging reactive oxygen species and reactive nitrogen species, preventing their damage on cells. Which constituent/ constituents play a part here is highly debatable. In order to reach such conclusions all components should be evaluated under the same conditions. Meaning the method used, cell model, experimental procedures and all influencing factors should be identical.

1.2.6.2 Mitochondrial protection and anti-apoptotic effects

Mitochondrial dysfunction has been lately associated with the aging process as well as with neurodegenerative diseases as described in section 1.1.9. The role of EGb 761® in protecting the mitochondria is receiving much more attention lately. Previously the effect of EGb 761® on cells was mainly attributed to its radical scavenging activity, but recently studies are pointing out to a direct effect on the mitochondria. Some of these direct effects, such as stabilization of mitochondrial membrane potential, ATP levels and protection against complexes inhibitors were shown in PC12 cells (Eckert *et al.* 2005).

Also *in vivo* protection was observed, for instance age-related decrease in respiratory functions and morphological changes of both brain and liver rat mitochondria were prevented by treatment with EGb 761® [100 mg/kg body weight] (Sastre *et al.* 1998). EGb 761® treatment also modulated synaptic and mitochondrial plasticity in rats with vitamin E deficiency (Bertoni-Freddari *et al.* 2002).

Not only direct mitochondrial effects were described for EGb 761®, but also anti-apoptotic effects. It was able to prevent serum deprivation and staurosporine-induced mitochondrial damage and attenuated cytochrome C and DNA fragmentation (Smith *et al.* 2002;Massieu *et al.* 2004). The ratio of the pro-apototic protein Bax to the anti-apoptotic protein Bcl-2 was decreased by EGb 761® in all brain regions in senescence-accelerated mice (Lu *et al.* 2006). Up regulation and down regulation of Bcl-2 and Bax respectively was previously reported (Smith *et al.* 2002;Zhou and Zhu 2000). Additionally, EGb 761® was able to decrease the activities of both caspases 3 and 9, where activation of the caspase cascade is known to lead to apoptosis and cell death (Eckert *et al.* 2005;Luo *et al.* 2002).

Here again just like the anti-oxidative effect, many tried to link the mitochondrial protective effects of EGb 761® to one or more of its components. In contrast to the free radical scavenging effect, the terpenoid fraction is highly associated to the mitochondrial protection properties of EGb 761® and BB being the most nominated candidate. *In vitro* BB inhibited hypoxia-induced decreases in ATP content in endothelial cells, protected complexes 1 and 3 against complex inhibitors and was able to increase complex 1 activity in liver mitochondria (Janssens *et al.* 1999;Janssens *et al.* 1995). BB also reduced ROS-induced elevation of Bax and activation of caspase-3 effectively in PC 12 cells.

Ex vivo, the respiratory control ratio of liver mitochondria was increased in rats treated with BB (Janssens *et al.* 1995). Another 2 *in vitro* studies carried

out on most of the terpenoids showed discrepancies, one study indicated that BB was the most potent anti-apoptotic constituent (Ahlemeyer *et al.* 1999) , where as another study revealed that GB, but not BB, was effective in opposing apoptosis (Rapin *et al.* 1998). However, the study by Ahlemeyer *et al* indicated also the anti-apoptotic effects of GJ and GB. Moreover, GB inhibited apoptosis induced by 6-hydroxydopamine [6-OHDA] and decreased the activity of caspase-3 in PC 12 cells (Meng *et al.* 2007).

From the above studies it appears indeed that the terpeniods of EGb 761® all except maybe GA (Ahlemeyer *et al.* 1999), show mitochondrial protection and anti-apoptotic properties *in vitro*. Depending on the cell model used and the methodologies it is still ambiguous which of the terpenoids is most effective, BB, or GB or is it a synergistic action of two or more terpeniods.

But it would be too simple to exclude the flavonoid fraction from the mitochondrial protective effects! So just to complicate things, there is also evidence that the total flavonoid component of EGb 761® and a mixture of flavonoids and terpenes protected cerebellar granule cells from apoptosis induced by hydroxyl radicals. Surprisingly, total terpenes of EGb 761® did not protect against apoptosis in the same study (Xin *et al.* 2000)!

1.2.6.3 Neurotransmitter systems

Neurotransmitter systems regulate CNS activities. Aging is associated with changes in neurotransmitter systems. In rodents, aging is highly associated with the decrease in the number of neurotransmitter receptors. While in humans data are more complicated, nevertheless neurotransmitter receptor changes have also been noticed in post-mortem tissue from AD patients (Mash *et al.* 1985).

Interestingly, EGb 761® is able to increase the maximum number of receptor binding sites [B max] and the receptor density of a number of neurotransmitters (Defeudis 1998) in aging models. This effect of EGb 761®

has been shown in *ex vivo* studies demonstrating that it has the ability of increasing the B max of muscarinic, serotoninergic and alpha adrenergic receptors (Taylor 1986;Huguet and Tarrade 1992;Huguet *et al.* 1994).

A dramatic degeneration of the cholinergic system especially in the hippocampus and cerebral cortex is a well known characteristic for AD and cognitive decline. For example, it was previously reported that the number of muscarinic receptors of rats subjected to cholinergic denervation is decreased (Mash *et al.* 1985). Also the muscarinic receptor binding was found to be lower in the brains of aging rodents (Defeudis 1998). These findings were confirmed in post-mortem tissue form AD patients (Mash *et al.* 1985).

Relevant to these findings, the effect of EGb 761® on the cholinergic system has been extensively studied. EGb 761® was shown to increase the B-max of high affinity choline uptake *in vitro* in hippocampal synaptosomes as well as *ex vivo* after treatment of old rats with EGb 761® for 30 days (Kristofikova and Klaschka 1997). This implies that treatment with EGb 761® might cause activation of cholinergic nerve terminals. Taking into account that high affinity choline uptake is decreased during aging (Defeudis 1998), the former results suggest that EGb 761® can cause deceleration of brain neurodegenerative processes.

Some of the constituents of EGb 761® have also shown effects on the neurotransmitter systems. An *in vitro* study on hippocampal slices, showed that BB prevented hypoxia and NMDA-induced release of choline and activation of phospholipase A2 (Klein *et al.* 1997;Weichel *et al.* 1999). Enhanced 5-HT uptake in synaptosomes from mouse cortex was related to the flavonoid fraction and not the terpeniods (Ramassamy *et al.* 1992).

These effects of EGb 761® on neurotransmitter systems especially the cholinergic system could be involved in the positive effects of the extract on aging, cognition and dementia.

1.2.6.4 Receptors: Platelet activating factor receptor

Platelet activating factor [PAF] receptor is a member of G-protein coupled receptors. Specific PAF receptors have been identified in the CNS, localized in both synaptic endings and intracellular membranes (Marcheselli *et al.* 1990). PAF is an alkyl phosphoglyceride produced by a variety of cells. PAF is involved in numerous disorders including acute allergy, inflammation, asthma, and ischemic injury. It also appears to augment neurotransmission involving excitatory amino acids. It was previously reported that PAF enhances excitatory synaptic transmission in cultured rat hippocampal neurons (Clark *et al.* 1992). Some of the actions related to PAF are its ability to induce intracellular Ca^{2+} mobilization, long term potentiation and modulation of apoptosis (Maclennan *et al.* 2002). Most of these actions were shown to be antagonized by the most potent PAF antagonist GB.

One of the earliest mechanisms described for GB was its ability to act as a PAF receptor antagonist (Braquet *et al.* 1985). Several other studies have come to show that all terpeniods act as PAF antagonist *in vitro* but with different affinities (Koch 2005). Stromgaard *et al* reported that the most potent terpenoid was GB with a Ki value of 0.56 µM, while GA was slightly less potent. GC and GJ are significantly less potent and BB had Ki value larger than 50 µM (Stromgaard *et al.* 2002). Although the exact mechanisms of PAF in the CNS are still not clear, there is no doubt that this mechanism of action is specific only for the terpenoids and no other constituents in EGb 761® (Steinke *et al.* 1993).

1.2.6.5 Receptors: Glycine receptor

Glycine receptors belong to the pentameric nicotinic acetylcholine receptor super family. Glycine receptors are known to mediate synaptic inhibition in spinal cord, brainstem and also in higher brain regions such as the hippocampus and developing cortex (Betz and Laube 2006). The neuropharmacology and functional importance of glycine receptors in higher brain regions is unfortunately not well characterized (Chattipakorn and McMahon 2002). Recently GB has been reported to inhibit glycine receptor mediated currents in acutely isolated hippocampal pyramidal neurons (Kondratskaya *et al.* 2002) and cortical slices (Ivic *et al.* 2003) in a noncompetitive fashion. It was also reported that GB and GC are significantly more potent than GA and GJ (Ivic *et al.* 2003;Chatterjee *et al.* 2003).

1.2.6.6 Amyloid precursor protein and Amyloid beta

We have already highlighted the toxicity of amyloid beta and the role it plays in AD [1.1.6]. In this section we will concentrate on the effects of EGb 761® on Aß-induced toxicity.

Quite a few *in vitro* studies showed the toxicity of Aß in *in vitro* models and the ability of EGb 761® to hinder its effect. Aß–induced increase in ROS, apoptosis, glucose uptake, decrease mitochondrial membrane potential and decrease in neurogenesis (Yao *et al.* 2001;Bastianetto *et al.* 2000a;Tchantchou *et al.* 2007) were all attenuated by EGb 761® extract.

What is more interesting is that EGb 761® is not only able to protect against Aß induced toxicities but also shows a direct effect on Aß production and aggregation. Interestingly, patients who received Ginkgo for more than 2 years had significantly lower levels of Aß 42 in plasma (Blasko *et al.* 2005). Furthermore, *in vivo* studies reported that EGb 761® was able to direct the APP metabolism towards the non-amyloidogenic alpha secretase pathway without affecting APP levels and another study showed decrease in both the amount of Aß and APP (Colciaghi *et al.* 2004;Yao *et al.* 2004). On the contrary, treating transgenic mice with EGb 761® showed unaltered Aß levels although spatial learning and memory were enhanced (Stackman *et al.* 2003).

The toxicity of Aß varies tremendously depending on its form. Aß self-assembles into oligomeric structures, protofibrils and fibrils which are the main constituents of amyloid plaques. Aß monomers have little or no toxic effects; it is the aggregates which are highly toxic. Therefore mechanisms which try to prevent aggregation of Aß could be of therapeutic relevance. Interestingly, *in vitro* studies were able to demonstrate that EGb 761® decreased Aß fibrillogenesis (Luo *et al.* 2002;Longpre *et al.* 2006). Moreover, Luo *et al* together with others were able to show that EGb 761® inhibits the

formation of the toxic oligomers or the so called ADDLs (Luo *et al.* 2002;Yao *et al.* 2001) in cell models.

These findings were confirmed *in vivo*, after treating 12 months old tg mice for 1 month with EGb 761®, levels of the 21 kDa oligomer was found to be reduced (Tchantchou *et al.* 2007).

From the above-mentioned evidence one may state that EGb 761® is protective against Aß-induced cell toxicity and alters either production and/or aggregation of Aß. Trying to relate these effects to a certain component showed inconsistency.

Aß induced cell toxicity and fibril formation were decreased by the flavonoid fraction and not the terpenoids (Bastianetto and Quirion 2002;Longpre *et al.* 2006). Moreover, a very recent study illustrated several mechanisms of myricetin [a flavonoid constituent of EGb 761®] against Aß-induced neurotoxicity. They were able to show that myricetin prevented Aß induced apoptosis, caspase 3 activation and also activated and up-regulated α-secretase and inhibited ß-secretase (Shimmyo *et al.* 2008). Quite the opposite is seen in other studies were myricetin and quercetin show no effect on Aß-induced toxicity and on the other hand GA and GB are protective (Bate *et al.* 2004).

Therefore, the role of the terpenoids here can not be left out. GJ, GA, and GB are capable of blocking the Aß 42 induced damage to synaptic plasticity in cell culture, with GJ showing the highest effects (Vitolo *et al.* 2009). On the contrary GC and BB exhibited no effects here. In transgenic *C. elegans* GA was the most effective against Aß-toxicity and to some extent GJ, but both were able to decrease the formation of Aß oligomers (Wu *et al.* 2006).

It appears quite complicated in this matter to try to assert these effects to one of the components of EGb 761®, the discrepancies seen are due to the methodologies used or the research models. Another factor which has to be

taken into consideration is the fact that all the tests carried out with the constituents are *in vitro* and their *in vivo* significance is still questionable!

1.2.6.7 Gene Expression

The gene-regulatory actions of EGb 761® has been addressed and proposed as one of its protective mechanisms that may actually oppose some gene expression abnormalities that cause neurodegeneration or cognitive impairments.

One of the major mechanisms previously discussed is the protective effect of EGb 761® against excess NO. As described before in section 1.2.6.1 EGb 761® was able to counteract some of the deleterious effects of NO. Excessive NO production can be a result of activation or over expression of enzymes which catalyze its formation.

There are 3 different forms of these enzymes which are known as NO synthases. These are,

Constitutive endothelial cells NOS [eNOS]

Inducible macrophage type NOS [iNOS]

Brain or neuronal NOS [nNOS] [inducible]

EGb 761® down regulates iNOS *in vitro* and *ex vivo*, the *ex vivo* effect was also seen with nNOS (Wadsworth *et al.* 2001;Sharma *et al.* 2000). Noteworthy here is to mention that traumatic brain injury and acute restraint stress have been shown to cause increase in nNOS RNA (de Oliveira *et al.* 2000;Rao *et al.* 1999), pointing out the neuroprotective effect of EGb 761®.

As for the constituents of EGb 761®, GA, GB and BB were able to down-regulate iNOS *in vitro*, and GB down-regulated iNOS and nNOS in rat brains *ex vivo* (Sharma *et al.* 2000).

The anti-oxidant effect of EGb 761® and its flavonoid fraction has been previously mentioned [section 1.2.6.1]. What is left to say in this section is

that EGb 761® is able to up-regulate some anti-oxidant enzymes such as mitochondrial Mn superoxide dismutase and regulatory subunit-1 of gamma-glutamyl-cysteinyl synthetase [GCS] which is the rate controlling enzyme for glutathione synthesis (Gohil *et al.* 2000). Down-regulation of this enzyme was noticed with age in cerebral cortex, hippocampus and cerebellum of rats, and this change is correlated with decreases in the gamma GCS activity and the GSH content (Liu 2002), encouraging the use of EGb 761® during aging.

Moreover Chen *et al* demonstrated that treating cells *in vitro* with Ginkgo extract induced glutathione peroxidase [GPx] gene expression (Chen *et al.* 2001) whereas GB and BB showed no effect. With regards to the fact that over-expression of GPx increases the resistance of PC12 cells and rat embryonic cultured cortical neurons to Aß-induced damage, this indicates a protective role of EGb 761® against Aß-toxicity and its benefit in AD (Barkats *et al.* 2000).

EGb 761® and its constituents influence mitochondrial and anti-apoptotic genes. EGb 761® was able to up-regulate the anti-apoptotic bcl-2 protein. Furthermore, a two-fold increase in subunit 1 of NADH dehydrogenese [complex 1] was observed by EGb 761® and BB *in vitro* (Tendi *et al.* 2002). BB affects another complex of the respiratory chain; it causes a significant increase in the cytochrome c oxidase subunit 3 COX III [complex 4] mRNA, this effect was specific to BB and not shared by GB.

Interestingly, brains from AD patients showed a 50-65 % decrease in mRNA levels of COX I and III in the middle temporal association neocortex as compared to control brains. These outcomes add to the mitochondrial protective effects of EGb 761® and its indication as a therapy for AD.

Transthyretin which is known to sequester Aß and decrease amyloid plaque formation (Tsuzuki *et al.* 2000;Schwarzman *et al.* 1994;Stein *et al.* 2004), was up regulated in mice hippocampus after feeding them for 4 weeks with EGb

761® (Watanabe *et al.* 2001). In the same study Watanabe *et al* were also able to demonstrate that mRNAs of tyrosine/threonine phosphatase 1 and microtubule–associated tau were both increased by 7 and 4 folds respectively. This is quite interesting because microtubule associated tau over expression can lead to formation of the neurofibrillary tangles found in the AD brain. On the other hand, up regulation of tyrosine/threonine phosphatase 1 could be beneficial since hyper-phosphorylated tau isolated from AD patients has been shown to be efficiently dephosphorylated by phosphatases (Gong *et al.* 2000). In relation to Aß only one study lately tested the effect of EGb 761® and its constituents on Aß production by measuring the ß-secretase which is responsible for the amyloidogenic cleavage of APP leading to formation of Aß. They concluded that neither EGb 761® nor its constituents affect ß-secretase mRNA and enzyme activity levels in cultured neurons and in mice (Augustin *et al.* 2008).

From the foregoing section it is obvious that EGb 761® and some of its constituents exert modifications in mammalian gene expression. The activation or inhibition of genes that maybe involved in diseases such as AD should be examined. Although the importance of Aß and tau in the pathogenesis of AD is well known, a few researches have been directed towards the genes responsible for production and/or clearance of these proteins.

1.2.7 Clinical evidence

1.2.7.1 EGb 761® in healthy subjects

Concerning the clinical efficacy of EGb 761® many studies were carried out on older participants with cognitive impairment, and fewer on healthy subjects. Regarding the trials on healthy subjects, a recent review updated to January 2007 from 15 randomized clinical trials declares that these studies provide no convincing evidence that G. biloba extracts taken either as a single dose or over a longer period has a positive effect on any aspect of cognitive performance in healthy people under the age of 60 years.

However, some of the earlier single-dose studies have indeed reported positive effects of Ginkgo, but due to weak methodologies such as low sample size their relevance is underestimated (Hindmarch 1986;Subhan and Hindmarch 1984;Canter and Ernst 2007). Recent studies are very contradicting, they reported subjective positive effects (Kennedy *et al.* 2002) unreliable positive results (Elsabagh *et al.* 2005) and no significant effects of EGb 761® in healthy adults (Jezova *et al.* 2002).

As for long-term studies on healthy subjects the evidence is more negative in respect to EGb 761® (Burns *et al.* 2006;Mattes and Pawlik 2004;Elsabagh *et al.* 2005;Kunkel 1993).The only positive study was by Stough *et al.* where 5 of 11 measured outcomes were in favor of Ginkgo (Stough *et al.* 2001).

The available trials imply that both short-term and long-term administration of Ginkgo in healthy young adults doesn't appear to enhance memory and/ or cognitive functions.

Nevertheless, it appears that healthy adults over 60 years could benefit from EGb 761®. This observation was made by Mix *et al* in a 6-week trial with a daily dose of 180 mg EGb 761® (Mix and Crews, Jr. 2002) where the authors concluded that EGb 761® is efficient in enhancing certain neuropsychological/memory processes in healthy adults over 60. In

accordance to this study are the findings of Burns *et al* that long-term memory assessed by associational learning tasks showed improvement with Ginkgo over 12 weeks using only 120 mg/day in subjects aged 55 to 79 years (Burns *et al.* 2006). However in the same study there was no difference between the EGb 761® and placebo group in other measured parameters. Another 6 week study applying 120 mg Ginkgo daily also found no effects on cognitive performance (Solomon *et al.* 2002).

The fact that in these 3 trials, using moderate doses [180 mg/kg] for 6 weeks showed optimum results (Mix and Crews, Jr. 2002) a lower dose 120 mg/day for longer time [12 weeks] showed effect only on long term memory (Burns *et al.* 2006) and low dose [120 mg/day] for short period [6 weeks] showed no effect (Solomon *et al.* 2002). These results can lead us to the conclusion that Ginkgo should be used for longer periods of time [at least 12 weeks] or in doses more than 120 mg/day in order to demonstrate effects in healthy adults over 60.

1.2.7.2 EGb 761® in demented patients

Many clinical trials were preformed to assess the efficacy and safety of Ginkgo biloba for dementia or cognitive decline. Due to the vast number of studies on this aspect with different criteria, diverse patient groups and variable time spans it is quite difficult to reach a veraciuos conclusion.

The most recent meta-analysis by Birks, Grimley Evans and Lee published online on 18 April 2007 states that Ginkgo biloba appears to be safe in use with no excess side effects compared with placebo, and the evidence that Ginkgo has predictable and clinically significant benefit for people with dementia or cognitive impairment is inconsistent and unconvincing. 35 studies were included in this review, from those only 29 contribute data to the meta-analyses, and of these 15 studies contribute very few data. Noteworthy, is the fact that, in the feedback the review was criticized and that the

reviewers are in the process of reconsidering the inclusion criteria. The updated review should be recently online.

Taking into consideration other meta-analysis, for example one which was carried out in 2002 by the same authors who carried out the above mentioned review (Birks *et al.* 2002), most are in favor of Ginkgo (Oken *et al.* 1998;Kleijnen and Knipschild 1992a;Kleijnen and Knipschild 1992b). Another example is the meta-analysis carried out in 1992 by Kleinjneen and Knipschild which had very stringent criteria. Here only eight of the 40 trials, generally conducted for at least 4-8 weeks with 120 mg/day of either an EGb 761® product were well performed and of acceptable quality. Interestingly seven of these "acceptable" trials showed effects of EGb 761® that were superior to placebo and significant enough to establish a clinically relevant effect of the extracts for controlling symptoms associated with cerebral insufficiency.

The most recent clinical trials which were not included in any of the above mentioned meta-analysis are discussed below.

Mazza el al preformed a 24-week randomized, placebo-controlled, double-blind study. Patients aged 50–80 years, suffering from mild to moderate dementia, were allocated into one of the three treatments: Ginkgo biloba [160 mg daily dose], Donepezil [5 mg daily dose], or placebo group. Their results suggest that there is no difference in the efficacy of EGb 761® and Donepezil in the treatment of mild to moderate Alzheimer's dementia (Mazza *et al.* 2006).

Another randomized, placebo controlled double-blind study in 513 out-patients with uncomplicated dementia of Alzheimer type was carried out for 26 weeks using 120 mg/day or 240 mg/day EGb 761®. The authors conclude there is no significant difference between placebo and Ginkgo. But in a subgroup with neuropsychiatric disorders there was a greater decline in

placebo group and better cognitive performance for Ginkgo group (Schneider *et al*. 2005).

Most likely, based on this result Napryeyenko *et al* carried out another randomized, double-blind, 22-week trial using 400 patients with dementia associated with neuropsychiatric features. Patients were treated with EGb 761® [240 mg/day] or placebo. EGb 761® was significantly superior to placebo with respect to primary and all secondary outcome variables.

These 3 aforementioned most recent clinical trials were included in the last Cochrane meta-analysis [in the process of updating] but with restrictions. For example in the Schneider trial the unplanned subgrouping of neuropsychiatric syndrome was considered inappropriate and therefore not taken into consideration! The Napryeyenko study which was restricted to participants with neuropsychiatric features of dementia, was not merged in the meta-analysis with the reasoning that "the results were strongly in favor of Ginkgo and so different statistically from the findings of the non-selective studies"!

On the other hand, both Napryeyenko and Schneider trials were included in a very recent meta-analysis completed by the Institute for Quality and Efficiency in Health Care [IQWIG] in Germany. The aim of this research was to assess the benefits of long-term treatment with Ginkgo compounds in AD compared with a) placebo, or b) a different drug or non-drug treatment option. The authors concluded that a dose of 240 mg daily shows advantage in "activities of daily living". Moreover, indications of a benefit for "cognitive function", "general psychopathological symptoms", and quality of life of caregivers" was demonstrated in the high-dose Ginkgo group. However there is possibility that the advantage of Ginkgo is only present in patients with accompanying psychopathological symptoms. Furthermore, due to diversity between the analyzed studies no conclusion could be drawn on the benefits of low-dose Ginkgo [120 mg daily].

Summing up, the fact that a vast number of clinical studies and reviews about Ginkgo are present with major differences in subjects, time spans and outcome measures makes it much more difficult to try to reach a satisfactory conclusion. However, a reasonable one would be that Ginkgo seems to be safe in use with no excess adverse effects compared with placebo. Moreover, there is promising evidence of improvement in cognition and function in patients with dementia and/or cognitive decline especially those with neuropsychiatric features.

2 Aims of the thesis

The fact that Ginkgo biloba has been used thousands of years ago in traditional medicine makes it difficult to accept that its mechanisms of action have not been fully elucidated. This is partly due to the complexity of the extract with several components contributing to its actions.

Nowadays, the standardized extract of Ginkgo biloba leaves EGb 761® is becoming one of the most popular herbal medicine and dietary supplements consumed in Europe and the US. The major therapeutic indications for EGb 761® are associated with CNS disturbances including dementia and AD. Earlier studies attempting to explicate the cellular and molecular mechanisms behind the actions of EGb 761® and its constituents pointed out its radical scavenging effect. Since ROS is associated with the mitochondria and EGb 761® has proven to act as an anti-oxidant, the protection of mitochondrial function was subsequently addressed. Previously, our group observed mitochondrial protective actions on PC12 cells *in vitro*.

Since the goal of the present study was to uncover the mechanisms of action of EGb 761® that are related to cognitive impairment and AD, therefore, the *in vitro* findings on cell culture were extended on animal models *in vitro* and *ex vivo*, as well as in transgenic animal models for AD. In this study mitochondrial functions were assessed in dissociated brain cells by measuring viability, ATP levels and mitochondrial membrane potential.

Influence of EGb 761® on the mitochondria was evaluated after additional external stress; both H_2O_2 and NO, which play a role during aging and AD, were applied.

The role of age was also addressed in this study by comparing the outcome after EGb 761® treatment in both young and older mice models as well as in senescence accelerated mouse model. However in the senescence accelerated mouse model treatment was implemented for 5 months in order to evaluate the long-term influence of EGb 761® on aging.

Acknowledging that EGb 761® is composed of several constituents, the effect of each constituent on mitochondrial functions was studied. Noticing from previously reported studies that methodologies employed and incubation schemes contribute to the efficacy of the single constituents of EGb 761®, all the constituents were tested under the same conditions, and measuring the same parameters.

Moreover, two different treatment methods were implemented, either post-treatment or pre-treatment. The motive behind this was not only to determine the relative activities of the EGb 761® components but also to find out the mechanism of action. Here again the same mitochondrial parameters for EGb 761® were determined for the constituents, namely viability, ATP levels and mitochondrial membrane potential.

The next step was then directed towards one of the major hallmarks of AD; the amyloid beta peptide, and the influence of EGb 761® on its production, aggregation and clearance. This was based on 2 reasons, first of all that EGb 761® is mainly indicated for dementia and AD, and secondly because recently researchers linked Aß and its precursor protein APP to the mitochondrion. Moreover, mitochondrial accumulation of APP directly correlates with mitochondrial dysfunction in various brain regions in AD.

Consequently transgenic mice over producing human Aß were treated for 21 days with EGb 761®. Aß levels, relative mRNA levels of proteins and

enzymes involved in Aß production and degradation were examined and compared to a placebo-treated group.

In conclusion, this study emphasized on exposing the means by which EGb 761® and its constituents act, on both the cellular and molecular level. For these investigations aging mice and a transgenic AD mouse model were utilized.

3 Materials and Methods

3.1 Materials

3.1.1 Apparatus	
Branson Sonifier, Cell Disruptor B15	Branson Ultrasonics Corp., Danbury
Centrifuge model Allegra 6R	Beckmann GmbH, Munich
CO_2 B-incubators Heraeus, Type BB 6220	Heraeus Instruments GmbH, Hanau
Combitips	Eppendorf-Netheler-Hinz-GmbH, Hamburg
Cryo-vial, model Cryo-S,	Greiner, Frickenhausen
Disposable Pasteur pipettes	Elkay, Ireland, UK
Fluorescence spectrometer, SLM Aminco Bowman Series 2	SLM instruments, Urbana. USA
Fluorescence spectrometer, Victor Multi-label counter	Perkin Elmer, Juegesheim
Fridge [4-8°C], type Glassline KGT 3546	Liebherr-Hausgeraete GmbH, Ochsenhausen
Freezer [-20 °C]	Liebherr-Hausgeraete GmbH, Ochsenhausen
Freezer [-80 °C]	Liebherr-Hausgeraete GmbH, Ochsenhausen
Horizontal slab gel electrophoresis apparatus 10x15 cm	PeqLab Biotechnologies GmbH, Erlangen
Heating Block Unitek HB-130	CLF, Emersacker Germany
Homogenizer, Potter S	B.Braun-Melsungen, Düsseldorf
Inverse microscopes, models TMS I04 and TS100	Nikon, Japan
Ice machine Ziegra	ZIEGRA Frankfurt
Laminar flow hood	Heraeus Instruments GmbH, Hanau
Latex examination gloves	Ansell GmbH, Munich
Magnetic agitator, type Poly 15	Merck Eurolab GmbH, Frankfurt
Microtiter plates IWAKI, 96 well with flat bottom	Dunn Labortechnik GmbH, Asbach
Microtiter plate, U-shaped bottom, sterile	Greiner Labortechnik, Frickenhausen
Microfuge R centrifuge	Beckmann GmbH, Munich
Mini Galaxy A CO_2 incubator	RS Biotech, UK
Minishaker MS2	IKA® Werke GmbH & Co. KG, Staufen
Photometer for microplate model Digiscan	Asys Hightech, Eugendorf
Pipetus-P®, Hirschmann	Merck Eurolab GmbH, Frankfurt
Pipettes model Pipetman	Abimed, Langenfeld
Pipettes sterile packed	Greiner Labortechnik, Frickenhausen
PP-tubes 15 ml and 50 ml, Cellstar®	Greiner, Frickenhausen

PCR tubes	Greiner Labortechnik, Frickenhausen
Rotor-Gene 3000 thermo-cycler	Corbett Research, Sydney, Australia
Thermocycler, GeneAMP PCR-9700	Perkin Elmer Bio Systems, USA
Transferpette®	Brand, Merck Eurolab GmbH, Frankfurt
UV Transilllumniator, UVT-20 M/W	Herolab GmbH, Weisloch
Water bath, type Thermomix 1441	B.Braun-Melsungen, Düsseldorf
Water bath, type 1003, GFL	Merck Eurolab GmbH, Frankfurt
Weighing scale model Ab204 and AT261	Mettler, Gießen

3.1.2 Chemicals

Acetic acid	Merck, Darmstadt
Agarose	Merck, Darmstadt
Bovine Serum Albumin [BSA]	Sigma-Aldrich, Munich
Calcium chloride dihydrate	Merck, Darmstadt
Dimethylsulfoxide [DMSO]	Merck, Darmstadt
Diphenylhexatriene [DPH]	Sigma-Aldrich, Munich
Di-sodium hydrogen phosphate	Merck, Darmstadt
Dulbecco's Modified Eagle Medium	Invitrogen, Karlsruhe
Ethidium bromide	Sigma-Aldrich, Munich
Ethanol	Merck, Darmstadt
Ethylene-diamine-tetraacetic acid	Merck, Darmstadt
Fetal calf serum [FCS]	Sigma-Aldrich, Munich
Forene [isofluran]	Abbott GmbH & co., Wiesbaden
Geneticine sulfate [G418]	Life Technologies, Karlsruhe
Ginkgo-biloba- Extract [EGb 761®]	Schwabe, Karlsruhe
Glucose monohydrate	Merck, Darmstadt
Guanidine	Merck, Darmstadt
Hank's Balanced Salt	Sigma-Aldrich, Munich
Hydrogen peroxide 30%	Sigma-Aldrich, Munich
Hydrochloric acid	Merck, Darmstadt
4-[2-hydroxyethyl]-1-piperazine-ethane-sulfonic acid HEPES	Merck, Darmstadt
Horse Serum [HS]	Invitrogen, Karlsruhe
Magnesium Chloride, hexahydrate	Merck, Darmstadt
Penicillin /Streptomycin- solution (Pen/Strep)	Life technologies, Karlsruhe
Phosphate buffered saline [PBS]	PAA laboratories GmbH, Cölbe
Potassium Chloride	Merck, Darmstadt
Potassium dihydrogen-phosphate	Merck, Darmstadt
Primers [oligonucleotides] for PCR	MWG, biotech AG

Rhodamine 123	Calbiochem, Darmstadt
Sodium hydrogen carbonate	Merck, Darmstadt
Sodium dodecyl sulfate [SDS]	Merck, Darmstadt
Sodium nitroprusside [SNP]	Sigma-Aldrich, Munich
Sterilium® disinfectant	Merck, Darmstadt
Sucrose	Merck, Darmstadt
Trimethylammonium Diphenylhexatriene [TMA-DPH]	Sigma-Aldrich, Munich
Tris[hydroxylmethyl]-aminomethane	Merck, Darmstadt
Tween 20	Sigma-Aldrich, Munich
Trypan blue solution	Biochrom AG, Berlin

3.1.3 Buffers and Media

All buffers and media were prepared in de-ionized water and pH was adjusted with pH meter before use. The pH meter was calibrated daily prior to utilization.

TAE Buffer [pH 7.4] for DNA gel electrophoresis

4.84 g Tris

1.142 ml acetic acid

1 mM Na EDTA in 1 l de-ionized water

Medium I [pH 7.35] for dissociated brain cells

138 mM NaCl

5.4 mM KCl

0.17 mM Na_2HPO_4

0.22 mM KH_2PO_4

5.5 mM Glucose · H_2O

58.4 mM Sucrose

Medium Π [pH 7.4] for dissociated brain cells

110 mM NaCl

5.3 mM KCl

1.8 mM $CaCl_2 \cdot 2\ H_2O$

1 mM $MgCl_2 \cdot 6\ H_2O$

25 mM Glucose $\cdot\ H_2O$

70 mM Sucrose

20 mM HEPES

Hank's Balanced Salt Solution [HBSS, pH 7.4]

1 vial of HBSS powder was dissolved in 1 l de-ionized water.

Supplemented with:

10 mM HEPES

1 mM $CaCl_2$

0.5 mM $MgSO_4$

Dulbecco's Modified Eagle Medium [DMEM] for dissociated brain cells

Supplemented with:

10 % [V/V] fetal calf serum [FCS]

5 % [V/V] horse serum [HS]

Dulbecco's Modified Eagle Medium [DMEM] for Hek sw cells

Supplemented with:

10 % [V/V] FCS

50 units/ml penicillin

50 μg/ml streptomycin

400 μg/ml G418

MTT solubilization reagent

90 ml N, N-Dimethyl-formamide

90 ml Solubilization Solution [Kit]

27g SDS

5 M guanidine HCl/ 50 mM Tris HCl [pH 8]

477.65 g guanidine dissolved in 50 mM Tris HCl

BSAT-DPBS [pH 7.4]

Dulbecco's phosphate buffered saline [10 xs] was diluted with de-ionized water- Supplemented with:

5% BSA

0.03% Tween-20

Tris-buffered saline [TBS, pH 7.4]

150 mM NaCl

25 mM Tris HCl

3.1.4 Kits

Bio-Rad DC Protein Assay [Lowry]	Bio Rad, Munich
Cell Proliferations Kit I [MTT-Assay]	Roche, Mannheim
DNeasy® Tissue Kit	Qiagen, Hilde
Master Taq Kit [PCR]	Eppendorf, Hamburg
Beta-Amyloid [Aβ] [1-40] colorimetric ELISA	Invitrogen, Karlsruhe
Beta-Amyloid [Aβ] [1-42] colorimetric ELISA	Invitrogen, Karlsruhe
ViaLight ® kit [ATP]	Cambrex, Belgium
Micro BCA Protein Assay kit	Pierce, Ireland
Quanti-Tec ® SYBR®Green RT-PCR kit	Qiagen, Hilde

3.2 Cell culture

3.2.1 Hek cells

Hek cells are human embryonic kidney cells. The Hek cells which were used in this thesis were transfected with DNA constructs harboring human mutant APP [APP-sw, K670M/N671L] gene inserted downstream of a CMV promoter, using FUGENE technique [Roche Diagnostics]. The Hek cells were transfected by Dr. Barbara Steiner, and characterized by Dr. Astrid Bonert. The cells were cultured in Dulbecco's modified Eagle's medium [DMEM] supplemented with 10 % heat inactivated fetal calf serum, 50 units/ml penicillin, 50 µg/ml streptomycin, and 400 µg/ml G418 at 37°C in a humidified incubator containing 5 % CO_2.

3.2.2 Cryopreservation

Cells were collected in 15 ml Falcon tubes and centrifuged at 1000 rpm for 5 minutes. Medium was aspirated; cells were re-suspended in 1 ml freezing medium and transferred to cryotubes. After storage overnight in a freezing box at -80°C the cells were transferred to liquid nitrogen until needed.

3.2.3 Thawing cells

The frozen cells were gently thawed in a water bath at 37°C. They were then transferred to a falcon tube containing 10 ml warm medium. Cell suspension was then centrifuged at 1000 rpm for 5 minutes, medium was aspirated and cells were re-suspended in 1 ml fresh medium. The cell suspension was then transferred to a Petri-dish containing 10 ml medium and incubated in the incubator.

3.3 Methods

3.3.1 Animals and housing

Mice were housed in cages [Makrolon, type II, Altromin GmbH, Germany]. Each cage was supplied with a plastic tube and cellulose sheets. Mice were kept at room temperature and constant humidity under a 12 hour dark/light cycle. Food [Standard 1320 Maintenance diet rat/mouse, Altromin GmbH, Germany] and water *ad libitum* were supplied to all cages. Cages were changed once weekly, cleaned and disinfected with perform. All experiments described in this thesis are in accordance to animal protection laws and requirements. Healthy mice only were used for the experiments, mice showing any visible skin lesions or tumors were excluded.

3.3.1.1 NMRI mice [Naval medical research Institute mice]

The original colony of Swiss mice was brought from Lausanne, Switzerland, in 1926 by Clara Lynch. In 1937 introduced to Poiley from Lynch. They were inbred by Poiley and known as NIH/PI. At F51 brought to US Naval Medical Research Institute and known as NMRI mice. Then introduced to the “Bundes-Forschungsanstalt fuer Viruskrankheiten” in 1955. The mice were then presented to the Central Institute for Laboratory Breeding in 1958, and finally to Winkelmann in 1981 [now Harlan Winkelmann]. The NMRI mice were purchased from Harlan Winkelmann.

3.3.1.2 Senescence accelerated mouse [SAMR1, SAMP8]

The senescence-accelerated mouse [SAM] is a model of accelerated senescence that was established through phenotypic selection from a common genetic pool of AKR/J strain of mice (Takeda *et al.* 1981). In 1975, early senescence was noticed in littermates of AKR/J mice with a shorter life

span. Five of these litters which become senile at an early age were selected as the progenitors of the senescence-accelerated-prone mice [SAMP]. Three litters with normal aging process were also selected as the progenitors of the control senescence-accelerated-resistant mice [SAMR] (Takeda *et al.* 1981;Miyamoto 1997). The establishment of the SAM mouse model was complete in 1981, including nine major SAMP sub-strains and three major SAMR sub-strains, each of which exhibits characteristic disorders. The SAMP8 model employed in this study is characterized by acquiring age-related learning and memory deficits and age-dependent deposition of Aß. Moreover the life span of SAMP8 mice ranges from 10-17 months which is much shorter that of the normally aging SAMR1, which ranges from 19-21 months (Flood and Morley 1998).

The author would like to acknowledge Dr. Jan Frank and his coworkers for the housing and feeding of the SAM mice.

3.3.1.3 C57BL/6 mice

The C57BL/6 mouse was developed by C.C. Little in 1921 after mating the female N°57 with the male N°52 from a commercial breeding centre in the USA [Miss Abby Lathrop]. They were introduced into the Jackson laboratory in 1948. Then to NIH in 1951 and finally to Charles River in 1974. C57BL/6J mice were purchased from Charles River laboratories.

3.3.1.4 Thy1-APP transgenic mice

Our transgenic mice were C57Bl/6 mice with both the Swedish double mutation [KM670/671NL] and the London mutation [V717I]. The 751 amino acid form of human APP with both mutations was generated under

the control of a murine Thy-1 promoter as described by Blanchard and co-workers (Blanchard *et al.* 2003). The Thy-1 promoter leads to high and selective expression in neurons. Furthermore, a Kozack element was introduced into the 5'-UTR region of the APP gene for optimization of expression. The 751 amino acid form of APP contains the 56 amino acid Kunitz protease inhibitor [KPI] region and has been shown to result in altered APP processing with a higher yield of Aβ levels in cell culture (Ho *et al.* 1996). Plaque formation is accelerated with first plaques appearing already at an age of 6 months.

3.3.2 Genotyping of transgenic mice

3.3.2.1 DNA isolation from rodent tails

DNA was isolated from rodent tails using the DNAeasy tissue kit according to the supplier's manual. This is an advanced silica-gel membrane technology for rapid and efficient purification of total cellular DNA. All steps were conducted in aseptic conditions and great care was taken to minimize possible cross-contamination between samples. Briefly, mice were weakly anaesthetized by isoflurane inhalation. Tissue samples were obtained by tail biopsy and mice were marked either with numbered ear clips or with ear piercing for identification. Tail samples were lysed by addition of lysis buffer and proteinase K during an overnight incubation in a 55°C water bath. Lysate was loaded onto the DNAeasy mini spin column. DNA is selectively bound to the DNAeasy membrane as contaminants pass through. DNA was then eluted and was ready for use.

3.3.2.2 PCR

The DNA elute obtained was used in PCR reactions for detection of human APP. PCR was performed utilizing MasterTaq Kit from Eppendorf. The PCR components were mixed according to the manufacturer's instruction to contain in an end volume of 25 µl [per probe] the following:

1 U of Taq-DNA-polymerase

2 µM of sense-primer

2 µM of anti-sense-primer

0.2 mM dNTPs

1 µl of sample DNA elute

Buffer

Samples were heated to 94°C for 5 minutes and PCR was done in 35 cycles of 1 minute at 94°C, 1 minute at 55°C and 1.5 minutes at 72°C. After the reaction, samples were kept at 4°C until gel electrophoresis was done for the detection of DNA bands.

The following primers were used:

APP genotyping:
Sense: 5'-GTA GCA GAG GAG GAA GAA GTG-3'

Anti-sense: 5'-CAT GAC CTG GGA CAT TCT C-3'

Positive controls with APP 751 DNA template and negative controls with sterile distilled water were used for controlling the PCR reaction and for exclusion of possible contamination of reagents.

3.3.2.3 DNA Gel electrophoresis

Gels were prepared by boiling 1 g agarose in 100 ml TAE buffer for a short interval followed by addition of 8 µl ethidium bromide solution after the gel solution has cooled down to approximately 70°C. Gels were cast into a 10 x 25 cm BioRad sub cell model 96 gel tray with two 51-well combs and left to cool down for 1 hour. The gels were then placed in the sub cell chamber filled with an appropriate amount of TAE buffer to ensure complete submersion of gels. PCR samples were mixed with 1 µl Blue juice 10 x gel loading buffer and loaded onto the gel. The electrophoretic separation was accomplished using Bio Rad Power Pac 300 set at 90 Volts for approximately 45 minutes. Gels were then placed on a UV trans-illuminator [Herolab] and bands were photographed with a video camera [Kodak] and printed for analysis. APP 751 positive controls always gave bands at 492 bp. No other bands were detected indicating specificity of primers. Negative controls with de-ionized water instead of DNA templates always gave negative results.

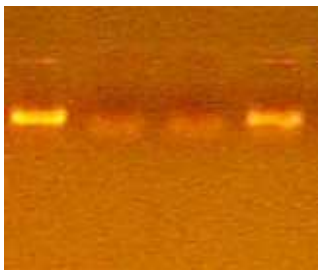


Figure 3-1

DNA gel electrophoresis for Thy-1 APP mice

PCR products of DNA samples obtained from the rodent tail were separated electrophoretically on agarose gel stained with ethidium bromide. Lane 1: positive control obtained from a transgenic Thy-1 APP mouse, lane 2: negative control [water], lane 3: no band indicating a sample from a non-transgenic mouse and lane 4: band at 492 bp showing a sample from a transgenic Thy-1 APP mouse.

3.3.3 qRT-PCR

3.3.3.1 RNA isolation

Lipid Tissue Protocol® total RNA was extracted according to the RNeasy [Qiagen]. DNA digestion was done with RNase-Free DNase Set [Qiagen]. The concentration of isolated RNA was determined by measuring the absorbance at 260 nm and the purity was determined by the ratio of 260/280 nm in a spectrophotometer [Beckmann Instruments; Munich, Germany]. RNA aliquots were stored at –80 °C until PCR analysis.

Primer sequences for real-time RT-PCR experiments were designed with primer 3 software. Primer pairs in table below were obtained from MWG Ebersberg, Germany].

Gene	Name	mRNA sequence	Source	Sequence FW	Sequence RV	Product size
ADAM10	alpha secretase	NM_007399.	primer3	CCATGCTCATGGAAGACAGTT	CCTTCTTCACCATAAATATGTCCA	144bp
BACE1	beta-secretase	NM_011792.	primer3	GGAGCATGATCATTGGTGGT	ACTCCTTGCAGTCCATCTTGA	144bp
muAPP	Murine amyloid-precursor protein	NM_007471.	primer3	CCGTTGCCTAGTTGGTGAGT	GCTCTTCTCGCTGCATGTCT	142bp
NEP	Neprilysin	NM_008604	primer3	CATTTTGACCAGCCTCGACT	GGCAAACTTTGTTCCTGACG	137bp
IDE	Insulin degrading enzyme	NM_031156	primer3	GGTTTTCCAAGAGTGCAAGG	TCAGCTGTGAGCACTCCATT	132bp
ECE-1	Endothelin converting enzyme	NM_199307	primer3	GCCATTTTATACCCGCTCTTC	TCCCATCCTTGTCGTACTCC	119bp
APPbp1	APP binding protein 1	NM_144931	primer3	GCTGCCAGGTATTGGATCAT	GCTCGGTTCTTGCCAATACT	108bp
b-actin	ACTB, actin, beta	NM_001101	primer3	GGATGCAGAAGGAGATCACTG	CGATCCACACGGAGTACTTG	90bp
Hu APP	Human Amyloid precursor protein	NM_201414 NM_201413 NM_000484	primer3	ACCGCTGCTTAGTTGGTGAG	GGTGTGCCAGTGAAGATGAG	113bp

3.3.3.2 Real-time qRT-PCR

One-step quantitative reverse transcriptase PCR was carried out with the Quanti-Tec®SYBR®Green RT-PCR kit [Qiagen]. Each PCR reaction [final volume 20.0 µl] contained 0.45 µl of the respective forward and reverse primer, 22.5 µl of Quanti-Tec® SYBR® Green RT-PCR Master Mix, 0.45 µl Quanti-Tec RT-Mix, 18.0 µl of RNA dilution and 3.15 µl of water.

Real-time PCR amplification was performed in a Rotor-Gene 3000 thermo-cycler [Corbett Research, Sydney, Australia]. Relative mRNA levels of genes were quantified as the ratio between the amount of target gene and the amount of a housekeeping gene [beta-actin].

The author would like to thank Dr. Patricia Huebbe for her assistance in conducting the real-time RT-PCR.

3.3.4 Preparation of dissociated brain cells

Mice were sacrificed by decapitation and brains were immediately dissected on ice [method modified after Stoll *et al* (Stoll *et al.* 1992)]. After removing the cerebellum, brains were minced using a scalpel and suspended in ice-cold medium I. Tissue was dissociated by triturating through a nylon mesh [210 µm pore diameter] with a Pasteur pipette. The resulting suspension was filtered by gravity through another nylon mesh [102 µm pore diameter] and the dissociated cell aggregates were washed twice with 20 ml of ice-cold medium II by centrifugation [2000 rpm for 5 mins at 4°C]. 50 µl of the suspension were used for protein determination. The prepared dissociated brain cells were re-suspended in 6 ml DMEM for the whole cerebrum or in 3 ml for the hemisphere. The cell suspension was distributed in 48-well plates, 250 µl per well for measurement of mitochondrial membrane potential, or 50 µl in 96-well plates for measurement of ATP levels and MTT assay. Plates were then incubated for 4 hours at 37°C and 5 % CO_2. Vitality of the cells

was tested previously using the Trypan blue exclusion method and was shown to be > 90% (Hartmann *et al.* 1996).

3.3.5 Determination of protein content

Protein levels were determined using either modified Lowry (LOWRY *et al.* 1951) or BCA (Smith *et al.* 1985) protein assay according to sample compatibility. Bovine serum albumin [BSA] was used as protein standard solution and dilutions were prepared using the same buffer or medium used for the tested samples.

3.3.5.1 Lowry Assay

In the first step of this assay proteins react with copper tartrate in alkaline solution. In the second step, Folin reagent is added and reduced by the protein-copper-complexes resulting in a characteristic blue color due to several reduced species. The color shows an absorbance minimum at 405 nm and a maximum at 750 nm (Peterson 1979). We used the commercially available BioRad DC Protein Assay Kit. Briefly, 5 µl of sample replicate were mixed with 25µl of reagent A [copper tartrate solution] and 200µl of reagent B [Folin reagent] in a micro-titer plate. If detergents were present in the samples, reagent A was supplemented with 3 % reagent S. Standards and samples were assayed in triplicates. Plates were mixed thoroughly on a plate shaker for 15 minutes at room temperature. Absorption was read using the ASYS Hi-tech Digiscan microtiter plate reader with a 620 nm filter. Protein concentration was calculated from the standard curve.

3.3.5.2 BCA Assay

The first step in the BCA assay is the chelation of copper with protein in an alkaline environment to form a blue colored complex that absorbs light at 540 nm. In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate. In the second

step of the color development reaction, bicinchoninic acid [BCA], a highly sensitive and selective colorimetric detection reagent reacts with the cuprous cation [Cu^{1+}] that was formed in step 1. The purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The commercially available Pierce® BCA Protein Assay Kit was used. The working solution was prepared by mixing BCA Reagents A and B in the ratio of 50:1 respectively. 25 µl of each standard or unknown sample replicate was placed into a micro-titer plate well and 200 µl of the working solution was added to each well. Standards and samples were assayed in triplicates. Plates were mixed thoroughly on a plate shaker for 30 seconds, and then incubated for 30 minutes at 37°C. Absorption was read by means of the ASYS Hi-tech Digiscan micro-titer plate reader using a 570 nm filter. The standard curve was used to calculate the protein concentrations.

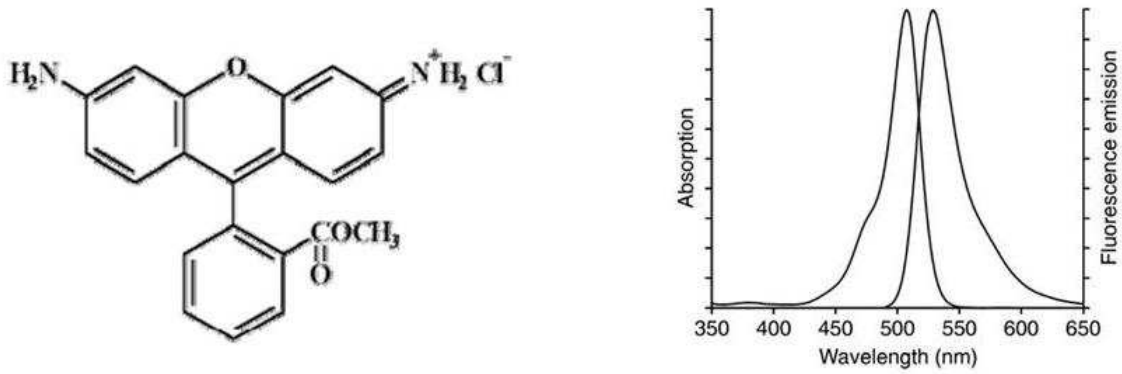
3.3.6 *In vitro* treatment schemes

For pre-treatment studies, DBCs were incubated with EGb 761® or its constituents and 30 minutes later H_2O_2 or SNP was added. Plates were incubated for 4 hours at 37°C and 5 % CO_2, followed by measurement of mitochondrial membrane potential, ATP levels or viability [MTT assay] as described below.

For post treatment studies, DBCs were first stressed with H_2O_2 or SNP for 30 minutes, followed by addition of EGb 761® or its components. Plates were incubated for 4 hours at 37°C and 5% CO_2. Consequently mitochondrial membrane potential, ATP levels or viability [MTT] were measured as described below.

3.3.7 Mitochondrial membrane potential

The mitochondrial membrane potential was measured using the fluorescence dye Rhodamine 123 [Rh-123] (Baracca *et al.* 2003). Rh-123 is a cationic lipophilic dye that accumulates in the mitochondrial matrix driven by the electric gradient following the Nerst equation. The higher the mitochondrial membrane potential, the more Rh-123 is taken up into the matrix.



Molecular structure and spectra of Rhodamine 123

For the measurement of mitochondrial membrane potential the dissociated brain cells that were incubated in 48-well plates were removed from the incubator and centrifuged at 1500 rpm for 5 minutes. The medium was aspirated carefully and 250 µl HBSS were added to each well. 5µl of Rh-123 [end concentration of 0.4 µM] was placed in each well and the plates were incubated for 15 minutes with the dye on a plate shaker. After the incubation time, plates were centrifuged at 1500 rpm for 5 minutes, HBSS was removed and again 250 µl HBSS were added. The previous wash step with HBSS was repeated and finally the plate was measured using Victor® Multi-label Counter at an excitation wavelength of 490 nm and an emission wavelength of 535 nm.

3.3.8 Measuring ATP levels

ATP levels were measured using the Via-Light HT® kit which is based upon the bioluminescent measurement of ATP (Crouch *et al.* 1993).This method utilizes an enzyme, luciferase, which catalyses the formation of light from ATP and luciferin according to the following reaction:

The emitted light is linearly related to the ATP concentration and can be measured using an illuminometer. The dissociated brain cells were incubated in 96-well white walled plates for measurement of ATP levels. The plates were removed from the incubator and left to cool down to room temperature. Cell lysis reagent was added as instructed to each well and after 10 minutes ATP monitoring reagent was added. The luminescence was measured directly using Victor® Multi-label Counter.

3.3.9 MTT assay

The cytotoxicity was measured using the MTT assay kit. This assay which measures metabolic activity is based on the cleavage of the yellow tetrazolium salt MTT into purple formazan by metabolically active cells. This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH. A decrease in the number of living cells results in a decrease of total metabolic activity which leads to a weaker color formation. For the respective experiments dissociated brain cells were incubated in 96-well plates. MTT reagent was added at a final concentration of 1.0 mg/ml 2 hours before the end of the incubation period. After incubation the formazan crystals were solubilized by adding 100 µl of a 20%SDS/50% N, N-dimethly-formamide solution (Mosmann 1983). The absorption of the solubilized formazan was measured at 570 nm using ASYS Hitech Digiscan microtiter plate reader.

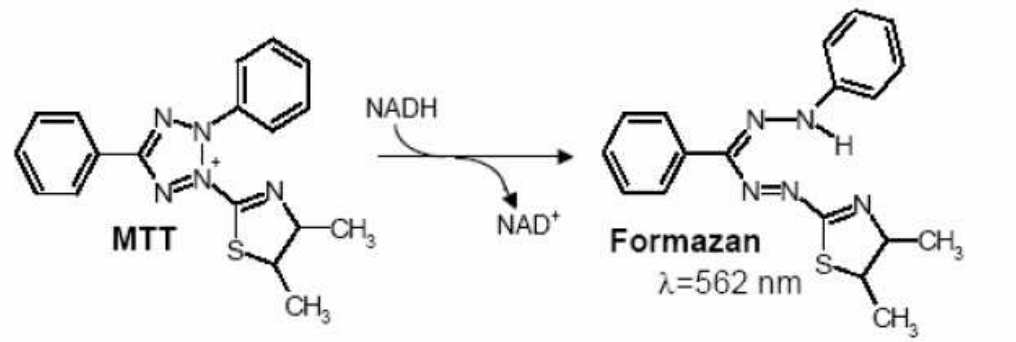


Figure 3-2

Reduction of MTT reagent to Formazan

3.3.10 Determination of membrane fluidity

3.3.10.1 Tissue preparation

For membrane fluidity measurements, brain hemispheres were homogenized in 15 ml buffer [5 mmol/liter Tris-HCl] and centrifuged at 48,000 g for 20 min. The formed pellet was re-suspended in 20 ml buffer and centrifuged. Protein content was determined according to Lowry method.

3.3.10.2 Fluorescent probes

1,6-Diphenyl-1,3,5-hexatriene [DPH] and its cationic derivative which contains trimethylammonium [TMA-DPH] were provided for the measurement of anisotropy. DPH and its derivatives are cylindrically shaped molecules with absorption and fluorescence emission transition dipoles aligned approximately parallel to their long molecular axis. Consequently, their fluorescence polarization is high in the absence of rotational motion and is very sensitive to re-orientation of the long axis resulting from interactions with surrounding lipids. Intercalation of DPH and its derivatives into membranes is accompanied by strong enhancement of their fluorescence; their fluorescence is practically negligible in water. DPH is highly lipophilic and can reside in the center of the lipid bilayer parallel to the surface. It is generally assumed to be oriented parallel to the lipid acyl chain axis.

As for TMA-DPH, due to the cationic substitute it is more water soluble and acts as a surface anchor. Staining of cell membranes by TMA-DPH is much more rapid than staining by DPH. However, the duration of plasma membrane surface staining by TMA-DPH before internalization into the cytoplasm is quite prolonged.

3.3.10.3 Anisotropy measurement

Membrane pellets were re-suspended accordingly to obtain 300 µg/ml protein. 100 µl of membrane suspension were incubated for 45 mins at 37 °C with 1000 µl buffer, 900µl of 1:150 DPH [stock solution 5mmol/l in

tetrahydrofuran] or 1:300 TMA-DPH [stock solution 5 mmol/l in dimethyl formamide]. The steady-state anisotropy was measured using excitation and emission wavelengths of 360 nm and 450 nm [slits 4 nm] respectively.

The steady-state fluorescence polarization (*P*s) was expressed as the anisotropy (*r*s) of the probe, using the following equation:

$$rs = 2Ps/3 - Ps$$

The author would like to thank Claudia Jordan for her assistance in performing the membrane fluidity experiments.

3.3.11 Quantification of Beta Amyloid

The Biosource Human beta Amyloid [Hu Aß] kits, 1-40 and 1-42 were used for the determination of beta amyloid amounts in the tissues.

Both kits are solid phase sandwich Enzyme Linked Immuno-Sorbent Assays [ELISA]. A monoclonal antibody specific for the NH_2-terminus of Hu Aß has been coated onto the wells of the microtiter strips. Standards, control specimens and unknowns were pipetted into these wells and co-incubated with a rabbit antibody specific for the COOH-terminus of either the 1-40 Aß sequence or the 1-42 Aß sequence. The bound rabbit antibody is detected by the use of a horseradish peroxidase-labeled anti-rabbit antibody. After removal of excess anti-rabbit antibody, a substrate solution was added, which is acted upon by the bound enzyme to produce color. The intensity of the colored product is directly proportional to the concentration of Hu Aß present in the original specimen. The optical density was measured at 450 nM.

3.3.11.1 Soluble Amyloid beta

Hek-293 cells

The Hek-sw cells were cultured in Petri-dishes in the incubator until the plates were approximately 70 % confluent. The medium was aspirated and new medium was added to the cells in the Petri-dishes with EGb 761® in different concentrations. 24 hours later the cell suspension was collected in falcon tubes and centrifuged at 1000 rpm for 5 minutes. 1 ml from the supernatant was transferred to cryotubes and 10 µM PMSF was immediately added. The tube was frozen at -80 °C until the ELISA was carried out.

The cell pellet was re-suspended in PBS buffer and centrifuged at 1000 rpm for 5 minutes. PBS was aspirated and the protein content of the cell pellet was determined by Lowry's method.

Mouse brain tissue

For the determination of the levels of soluble Aß, the wet mass of the mice brain samples were determined. 10x volume of Tris-Buffered Saline supplemented with Complete® protease inhibitor cocktail was added and homogenized using Potter S homogenizer [10 strokes, 1200 rpm]. The homogenate was centrifuged at 15,000 x g for 30 minutes at 4°C. The supernatant was collected and stored at -80°C until performing the $Aß_{1-40}$ ELISA.

3.3.11.2 Total amyloid beta

Total Aß was extracted from the tissues using 5 M guanidine HCl/ 50 mM Tris HCl solution. The mice brain samples were homogenized with Potter S homogenizer [10 strokes, 1200 rpm]. The homogenate was mixed at room temperature for 4 hours using a plate shaker and stored at -20°C. Directly before use, the samples were diluted with the reaction buffer BSAT-DPBS, so that guanidine concentration does not exceed 0.1 M. Diluted samples were centrifuged at 16,000 x g for 30 minutes at 4°C. ELISA was then preformed.

3.3.12 *Ex vivo* treatment studies

Ex vivo studies were carried out in order to test the effect of EGb 761® after oral application. For these studies mice were treated *per os* daily either with EGb 761® or with the vehicle only for the control groups. The solutions were prepared directly before treatment. Mice were weighed daily before treatment and were given 100 mg/ kg body weight EGb 761® or 0.2 % agarose solution. Mice were sacrificed 24 hours after the last treatment.

Study designs

Study 1

Testing the effect of EGb 761® [100 mg/kg] treatment on mitochondrial functions in 2 different age groups

3 and 15 months old NMRI female mice were treated daily for 14 consecutive days with either EGb 761® [100 mg/kg] solution in agarose or with the vehicle alone for the control group. Mice were sacrificed 24 hours after the last treatment and dissociated brain cells were prepared. Total number of mice tested was 10 mice per group. Mice showing lesions or tumors were excluded from the experimental results.

	Control [0.2 %Agarose]	EGb 761® [100 mg/kg]
2-3 months	10	10
15-16 months	10	10

Study 2

Testing the effect of EGb 761® [100 mg/kg body weight] treatment on mitochondrial functions in transgenic APP mice and non-transgenic littermates

Tg-APP mice [6 months old] and their corresponding littermates were treated daily for 14 consecutive days with either EGb 761® [100 mg/kg] solution in agarose or with the vehicle for the control group. Total number of mice tested was 18 transgenic and 18 non-transgenic as shown in the table below. Mice were sacrificed and dissociated brain cells were prepared. Mice showing lesions or tumors were excluded from the experimental results.

	Control	EGb 761® [100mg/kg]
Tg-APP	9	9
Non-Tg littermates	9	9

Study 3

Testing the effect of long-term treatment with EGb 761® on SAMP8 and SAMR1 mice

Mice were treated for 5 months with western diet pressed in pellets. Western diet contains 21% butterfat, 0.15% cholesterol, 35 mg/kg vitamin E and 3000 IE/Kg vitamin A. However it was devoid of anti-oxidants, flavonoids and vitamin C. The amount of EGb 761® present in the pellets was 1g/ g western diet. Given that on average basis one mouse consumes 3.5 g/day; the calculated daily intake of EGb 761® would be 150 mg/ kg body weight. Both SAMR1 and the placebo-treated mice received western diet devoid of EGb 761®.Total number of mice was 23.

After 5 months treatment mice were sacrificed and the mitochondrial functions were directly assessed in a hemisphere. Half of the other hemisphere was rapidly frozen for membrane fluidity studies.

	Control	EGb 761® [100mg/kg]
SAMR1	10	-
SAMP8	7	6

Study 4

Testing the effect of EGb 761® [100 mg/kg body weight] on amyloid beta levels and gene expression in transgenic mice

6 months old Tg-APP and their corresponding littermates were treated daily *per os* for 21 consecutive days with either EGb 761® [100mg/kg body weight] solution in agarose or with the vehicle only for the placebo group. Total number of mice was 36. Mice were sacrificed. One hemisphere was stored directly at -80 °C until further use for the ELISA. The second hemisphere was stored in RNAlater solution for 24 hours at room temperature, and then stored at -20 °C for RT-PCR.

	Control	EGb 761® [100mg/kg]
Tg-APP	9	10
Non-tg littermates	9	8

3.3.13 Software and statistics

Microsoft excel was used to calculate mean values from measured duplicates or triplicates. Calculated means were then transferred to Graphpad prism [version 4.03]. Data are represented as means ± standard error of mean [S.E.M]. Statistical analyses were preformed also using the Graphpad prism program, p values less than 0.05 were considered significant. For analysis of significant correlations between variables, linear regression was calculated. It was also assumed that data are sampled from Gaussian populations.

4 Results

4.1 Dissociated brain cells: Experimental conditions optimization

In this study mitochondrial functions were tested using dissociated brain cells [DBCs] prepared from mice. This model was previously used and described by others. Vitality of the cells was tested earlier using the Trypan blue exclusion method and was shown to be > 90% (Hartmann *et al.* 1996). Using MTT-Assay it was demonstrated that after 4 hours there is no significant decrease in the viability of DBCs. This is first noticed after 8 hours, where there exists a 25 % decrease in metabolic activity of the DBCs which indicates an approximate decrease of 25 % in the number of living cells.

In the current study, the effect of EGb 761® on mitochondrial functions and its ability to regenerate the mitochondria were investigated. Mitochondrial dysfunction was achieved by implementing either oxidative stress or nitrosative stress. Hydrogen peroxide [H_2O_2] was used to induce oxidative stress and sodium nitroprusside [SNP], a nitric oxide [NO] donor to induce nitrosative stress. As markers for the function of the mitochondria, mitochondrial membrane potential, ATP levels and metabolic activity of the cells [MTT Assay] were determined.

4.1.1 Optimization of experimental conditions for SNP

4.1.1.1 Mitochondrial membrane potential

Sodium nitroprusside was used to induce nitrosative stress on DBCs. Mitochondrial membrane potential was one of the parameters to be investigated; therefore optimization of experimental conditions for this marker was essential.

For establishment of experimental conditions 2-3 months old NMRI mice were utilized.

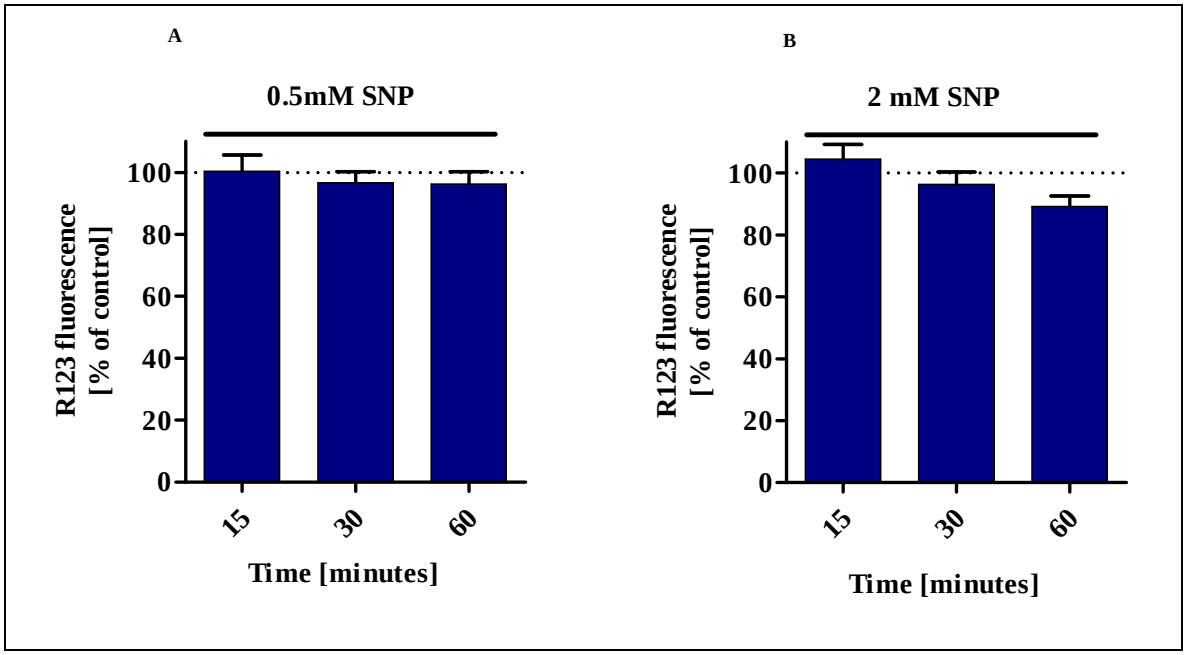


Figure 4-1

Time dependent decrease in mitochondrial membrane potential of dissociated brain cells with SNP

Dissociated brain cells were incubated with either 0.5 mM SNP [A] or 2 mM SNP [B] for 15, 30 and 60 minutes. Mitochondrial membrane potential was then determined. Data are represented as percent of control. Data are expressed as means ± S.E.M [n=15-17].

In order to choose a time frame for our experiments, DBCs were incubated with 2 different concentrations of SNP for 15, 30 and 60 minutes. Neither 0.5 mM nor 2 mM were able to influence the mitochondrial membrane potential in this short period [figure 4-1].

Consequently, the effect of these 2 concentrations was tested for a longer period. The DBCs were incubated for 1, 2, 4 and 6 hours and mitochondrial membrane potential was determined.

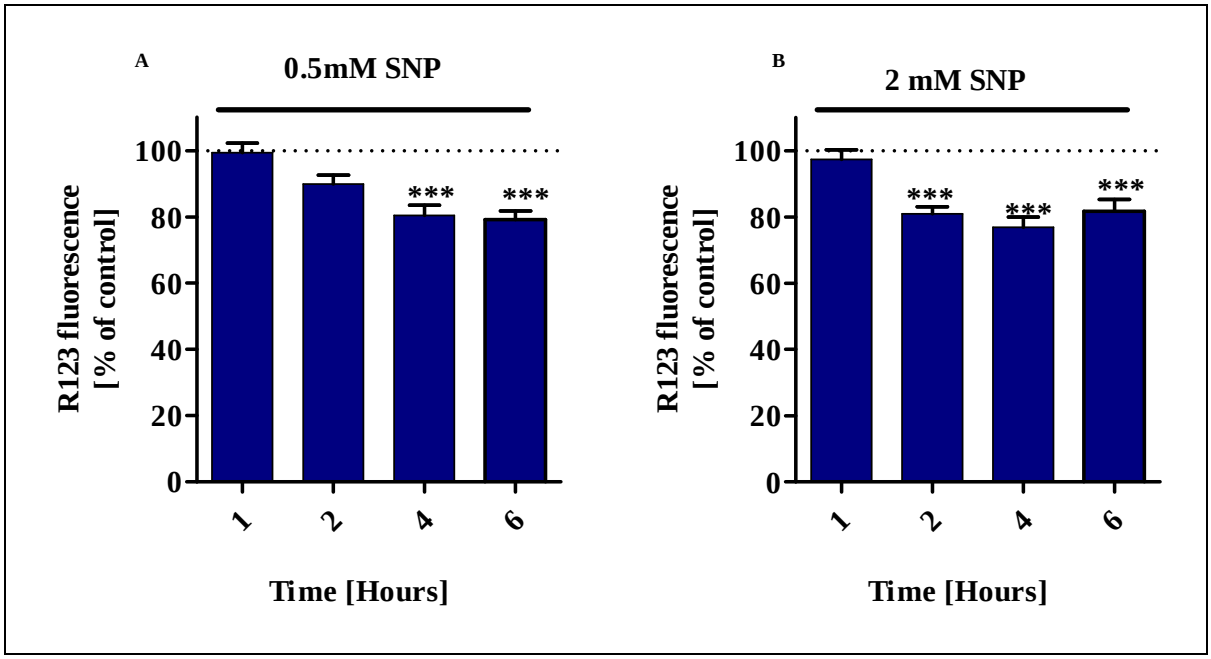


Figure 4-2

Time dependent decrease in mitochondrial membrane potential of dissociated brain cells by SNP

Dissociated brain cells were incubated with either 0.5 mM SNP [A] or 2 mM SNP [B] for 1, 2, 4 and 6 hours. Mitochondrial membrane potential was then determined. Data are represented as percent of control. Data are expressed as means ± S.E.M [n=12] [ANOVA, ***$p<0.001$ vs. untreated controls].

Based on our knowledge that a 25 % decrease in cell viability of DBCs occurs within 8 hours, our incubation periods were limited to 6 hours in order to have maximum amount of viable cells and mitochondria. As depicted above, 0.5 mM showed a significant effect at 4 hours and 2 mM SNP after a minimum of 2 hours. A more pronounced decrease in mitochondrial membrane potential was detected after 4 hours with 2 mM SNP causing approximately 24 % reduction in mitochondrial membrane potential [figure 4-2]. In light of these findings, incubation period was standardized to 4 hours.

After choosing the time frame different concentrations of SNP were examined, starting from 0.5 mM to 3 mM. DBCs were incubated for 4 hours with 4 different concentrations of SNP as shown below [figure 4-3].

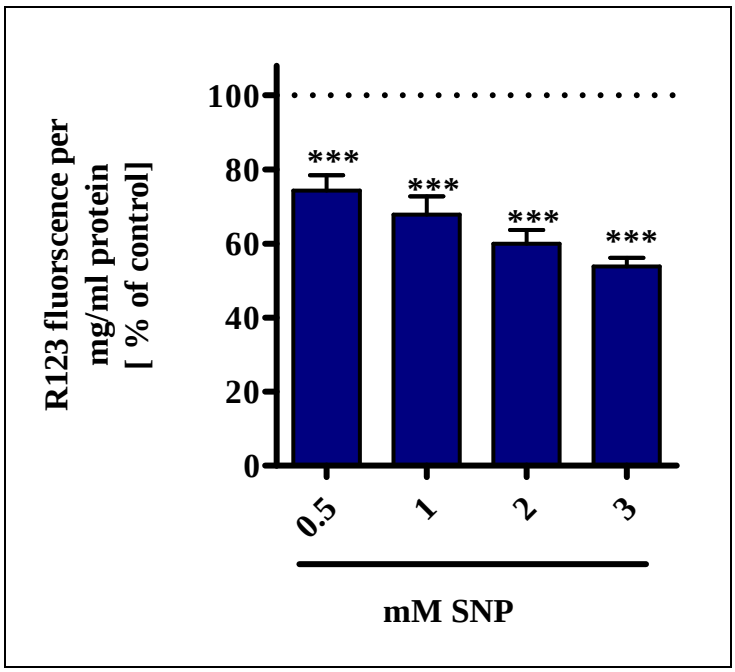


Figure 4-3

Concentration dependent decrease in mitochondrial membrane potential of dissociated brain cells by SNP

Mitochondrial membrane potential was measured after incubating dissociated brain cells with different concentrations of SNP for 4 hours. Data are represented as percent of control. Data are expressed as means ± S.E.M [n=4-7], [ANOVA, ***p<0.001 vs. untreated controls].

A concentration dependent depolarization of mitochondrial membrane potential was observed. 2 mM SNP was the concentration chosen for our up-coming experiments.

4.1.1.2 ATP levels

The second marker for mitochondrial function used in this study was ATP levels. In this experiment, the influence of different concentrations of SNP on the amount of ATP levels was tested. As shown in figure 4-4, SNP led to a dramatic decrease in ATP levels. Using 2 mM SNP lowered ATP levels by approximately 88 %, which is the same concentration causing 24 % decrease in mitochondrial membrane potential. This could be due to methodological differences, indicating that quantifying ATP levels using bioluminescence assay is a more sensitive method compared to the fluorometric measurement

of mitochondrial membrane potential using Rh 123. Consequently, a concentration of 0.2 mM SNP was chosen for our further investigations on mitochondrial functions, which causes a significant decrease in ATP levels but also allows us to see treatment effects of EGb 761®, if present.

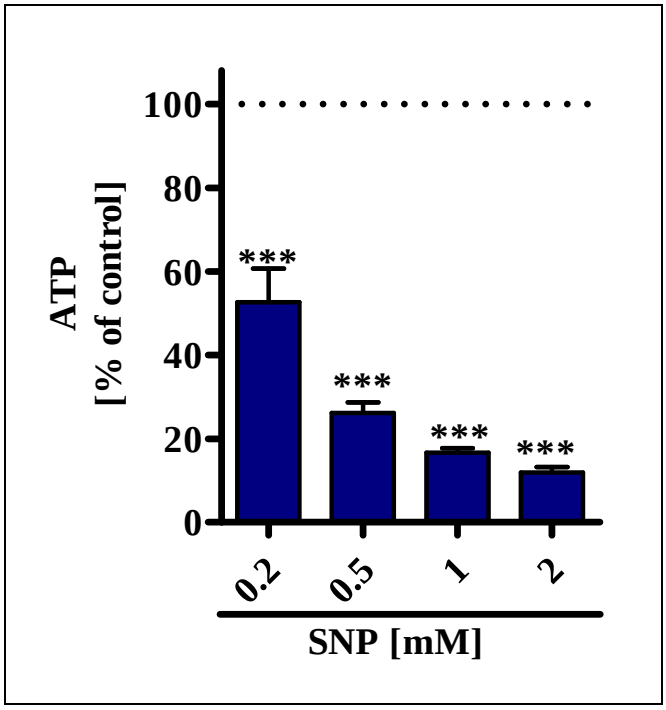


Figure 4-4

Concentration dependent decrease in ATP levels of dissociated brain cells by SNP

Dissociated brain cells were incubated with different concentrations of SNP for 4 hours and ATP levels were measured. Data are represented as percent of control. Data are expressed as means ± S.E.M [n=6] [ANOVA, ***p<0.001 vs. untreated controls].

4.1.1.3 MTT assay

The metabolic activity of the cells was measured using the MTT assay. Different concentrations of SNP were examined to determine the most appropriate concentration to use for this assay [figure 4-5]. 0.5 mM of SNP was selected to be used for all MTT assays in this study.

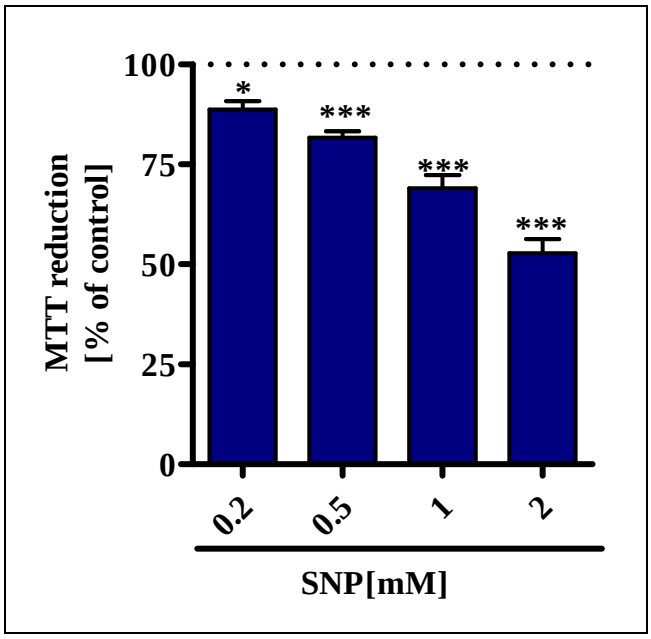


Figure 4-5

Concentration dependent decrease in cell viability of dissociated brain cells by SNP

Dissociated brain cells were incubated for 4 hours with different concentrations of SNP and cell viability was measured using the MTT assay. Data are represented as percent of control. Data are expressed as means ± S.E.M [n=4] [ANOVA, *$p<0.05$ and ***$p<0.001$ vs. untreated controls].

Concluding, in all the experiments preformed with SNP throughout this thesis, the incubation period was kept constant [4 hours] but the concentration of SNP was adjusted according to the sensitivity of the assay preformed. A minimal concentration of 0.2 mM was applied for ATP assay due to its high sensitivity and a much higher concentration of 2 mM was used for the fluorometric determination of mitochondrial membrane potential. The relative sensitivities of the different assays preformed with SNP are summarized in the table below.

	Percentage damage caused by different concentrations of SNP [% ± S.D]			
Assay	**0.2 mM SNP**	**0.5 mM SNP**	**1 mM SNP**	**2 mM SNP**
MMP	-	74.31 ± 10.8	67.83 ± 13.13	60 ± 9.71
ATP	52.65 ± 8.00	26.16 ± 2.54	16.63 ± 1.07	11.92 ± 1.32
MTT	88.69 ± 4.13	81.73 ± 3.06	69.04 ± 6.56	52.81 ± 7.01

Table 4.1

Relative sensitivities of different tests to SNP-induced damage

Dissociated brain cells were incubated for 4 hours with different concentrations of SNP, and mitochondrial membrane potential [MMP], ATP levels [ATP] and metabolic activity [MTT] were determined. The sensitivity of ATP > MTT > MMP. Data are represented as percent of control ± Standard deviation.

4.1.2 Optimization of experimental conditions for H_2O_2

In order to test the protective effects of EGb 761® on the mitochondria, not only nitrosative stress was induced but also oxidative stress. In all the experiments H_2O_2 was used as a source of oxidative stress. Hence, the experimental conditions for H_2O_2 were optimized.

4.1.2.1 Mitochondrial membrane potential

Different concentrations of H_2O_2 were applied to the DBCs and mitochondrial membrane potential was determined after 4 hours. H_2O_2 was able to influence mitochondrial membrane potential at a concentration of 1 mM but a more apparent depolarization was seen at a concentration of 2 mM [Figure 4-6].

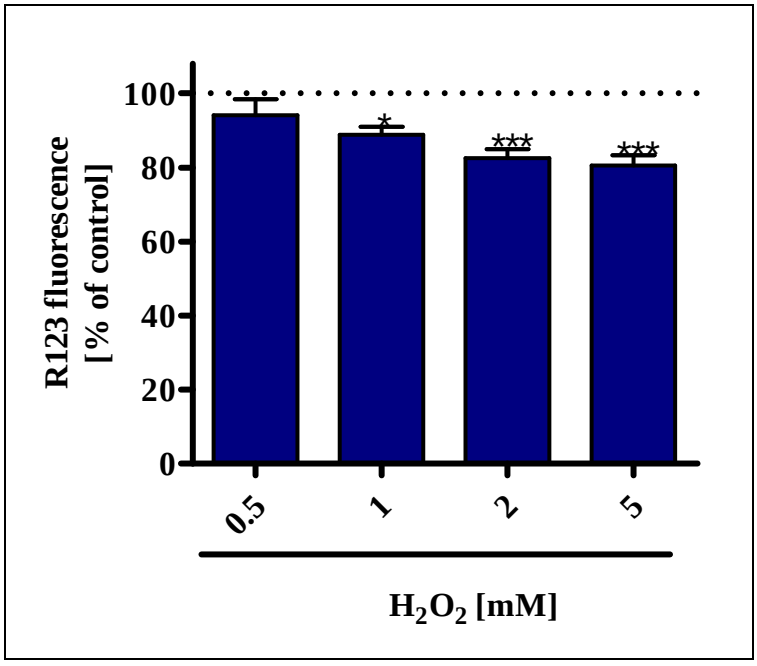


Figure 4-6

Concentration dependent decrease in mitochondrial membrane potential of dissociated brain cells by H_2O_2

Mitochondrial membrane potential was measured after incubating dissociated brain cells with different concentrations of H_2O_2 for 4 hours. Data are represented as percent of control. Data are expressed as means ± S.E.M [n=6] [ANOVA, *p<0.05, ***p<0.001 vs. untreated controls].

The 2 mM concentration was tested for shorter periods of time, 1 hour and 2 hours [figure 4-7]. Incubating the DBCs for 1 hour with H_2O_2 had no effect on mitochondrial membrane potential, but starting 2 hours the effect was

noticeable. For consistency and direct comparison with SNP, the 4 hour incubation period was selected.

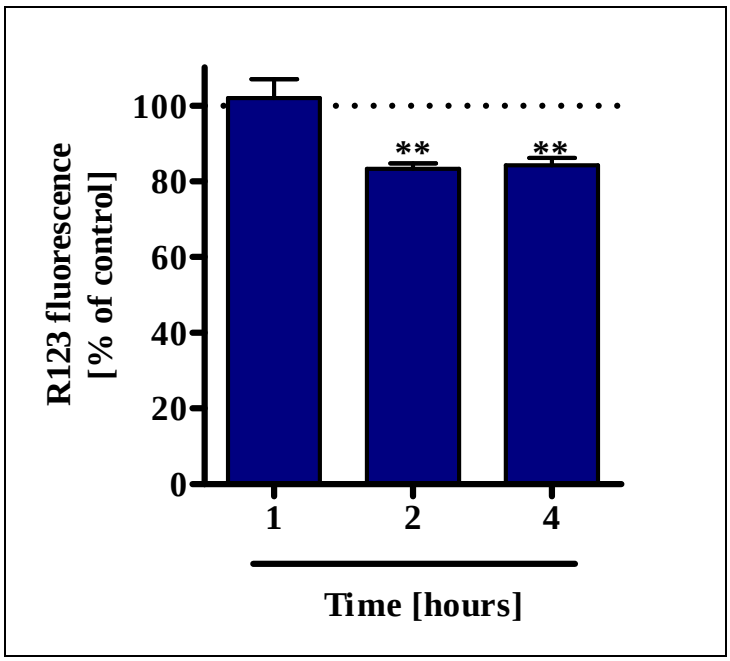


Figure 4-7

Time dependent decrease in mitochondrial membrane potential of dissociated brain cells with 2mM H_2O_2

Dissociated brain cells were incubated with 2 mM H_2O_2 for 1, 2, and 4 hours. Mitochondrial membrane potential was then measured. Data are represented as percent of control. Data are expressed as means ± S.E.M [n=6] [ANOVA, **p<0.01 vs. untreated controls].

4.1.2.2 ATP levels

DBCs were incubated for 4 hours with several concentrations of H_2O_2 [Figure 4-8]. The ATP levels were highly influenced by H_2O_2, a concentration as low as 0.4 mM was able to decrease the ATP levels significantly. This concentration was used for all future experiments.

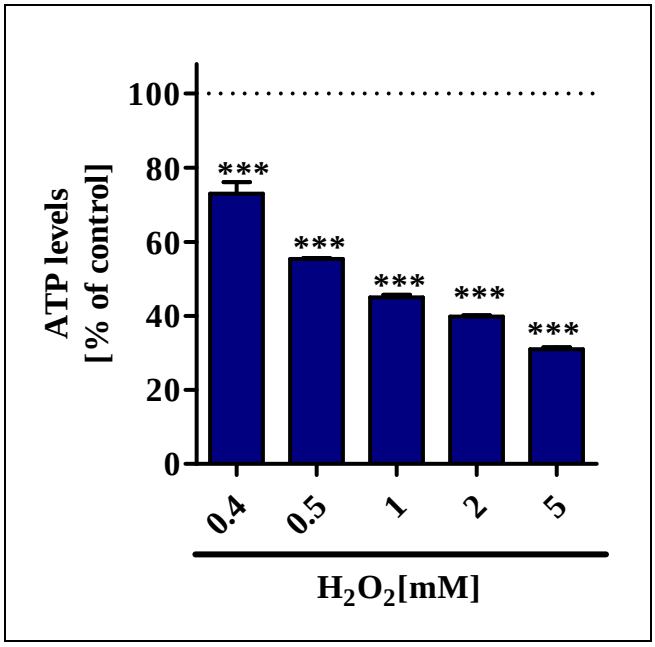


Figure 4-8

Concentration dependent decrease in ATP levels of dissociated brain cells with H_2O_2

Dissociated brain cells were incubated with different concentrations of H_2O_2 for 4 hours and ATP levels were measured. Data are represented as percent of control. Data are expressed as means ± S.E.M [n=6-8] [ANOVA, ***p<0.001 vs. untreated controls].

The discrepancy observed previously in the sensitivities of the ATP assay and the fluorometric determination of mitochondrial membrane potential, was confirmed in the above experiments. Here again extensively higher concentrations of H_2O_2 were required to cause noticeable decrease in mitochondrial membrane potential as apparent in the table below.

	Percentage damage caused by different concentrations of H_2O_2 [% ± SD]			
Test	**0.4 mM H_2O_2**	**0.5 mM H_2O_2**	**1 mM H_2O_2**	**2 mM H_2O_2**
MMP	-	94.11± 10.54	88.85 ± 5.4	82.52 ± 5.91
ATP	73.05 ± 8.60	55.37 ± 0.82	44.93 ± 1.91	39.78 ± 0.92 2

Table 4.2

Relative sensitivities of different assays to H_2O_2-induced damage

Dissociated blood cells were incubated for 4 hours with different concentrations of SNP and mitochondrial membrane potential [MMP], ATP levels [ATP]] were measured. The sensitivity of ATP > MMP. Data are represented as percent of control ± Standard deviation.

After the optimization of the experimental conditions for determining the mitochondrial functions in DBCs, the aforementioned assigned concentrations and the time intervals were kept constant throughout all the following experiments.

4.2 Effects of Ginkgo biloba extract [EGb 761®] on mitochondrial function: Protection against H_2O_2 - initiated stress

EGb 761® has been widely used in the treatment of geriatric memory disorders including vascular and neurodegenerative dementia. The major intention of this study was to examine extensively the protective properties of EGb 761® and the mechanisms lying behind these effects. Lately, it has been proposed that mitochondrial dysfunction plays a key role in aging and age-related diseases. Therefore, examination of EGb 761® effects pertaining to mitochondrial functions would be plausible.

One of the major causes of mitochondrial dysfunction is oxidative stress. Oxidative stress is known to play a key role in aging and age-related neurodegenerative diseases including Alzheimer's disease [AD] [see section 1.1.8]. The effects of EGb 761® on H_2O_2 induced mitochondrial damage was examined both *in vitro* and *ex vivo*.

4.2.1 *In vitro* findings

4.2.1.1 3 months old NMRI mice

DBCs attained from young adult female NMRI mice ranging between 2-3 months were incubated for 4 hours with either 2 mM H_2O_2 for depolarizing mitochondrial membrane potential or with 0.4 mM H_2O_2 for reducing ATP levels. After 30 minutes 0.1 mg/ml or 0.5 mg/ml EGb 761® was added. EGb 761® was able to alleviate the decrease in mitochondrial membrane potential caused by H_2O_2 but had no protective effect on the ATP levels [figure 4-9].

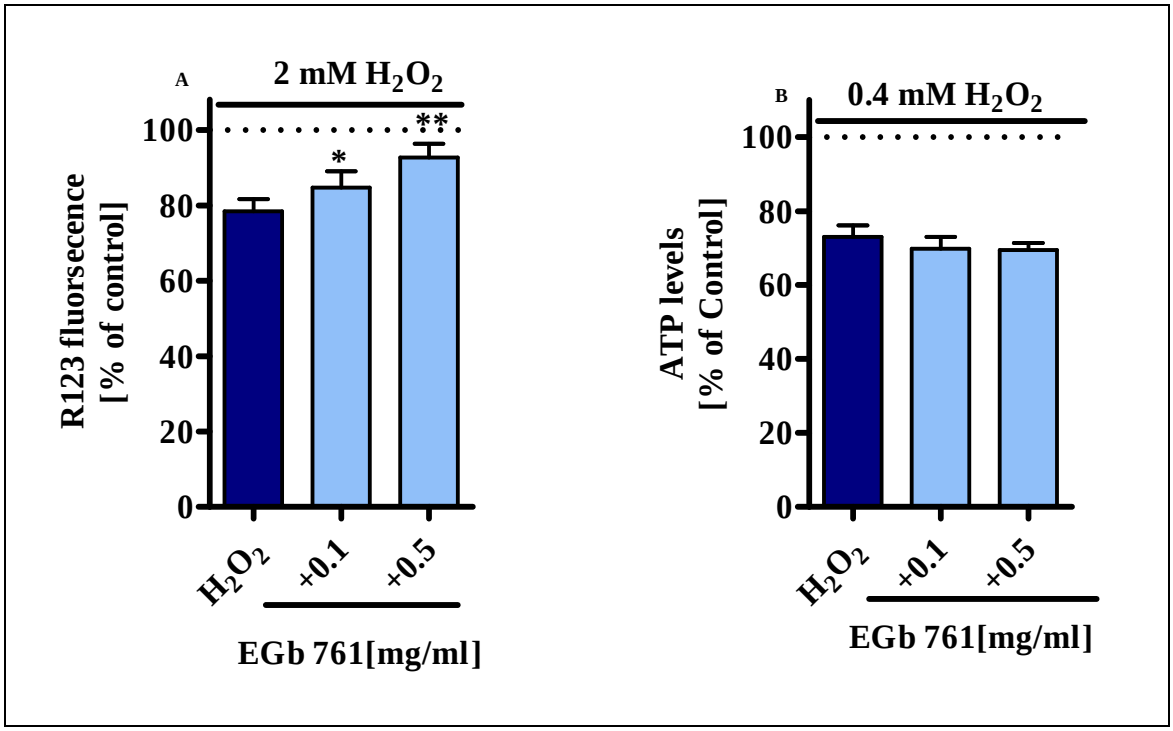


Figure 4-9

In vitro protection of mitochondrial functions against H_2O_2 induced oxidative stress in 2-3 months old mice

Dissociated brain cells were incubated for 4 hours with 2 mM H_2O_2 for determination of mitochondrial membrane potential [A] or with 0.4 mM for the measurement of ATP levels [B]. EGb 761® was added 30 minutes after incubation with H_2O_2. Data are expressed as means ± S.E.M [n=8-9] [*$p<0.05$, **$p<0.01$ vs. H_2O_2, student's t-test]

4.2.1.2 15 months old NMRI mice

The influence of EGb 761® in DBCs from older mice was then determined. The previous experiment was repeated but instead of obtaining DBCs from 2-3 months old mice, they were prepared from 15-16 months old mice. 0.1 mg/ml EGb 761® was able to slightly improve both mitochondrial membrane potential [figure 4-10A] and ATP levels [figure 4-10B].

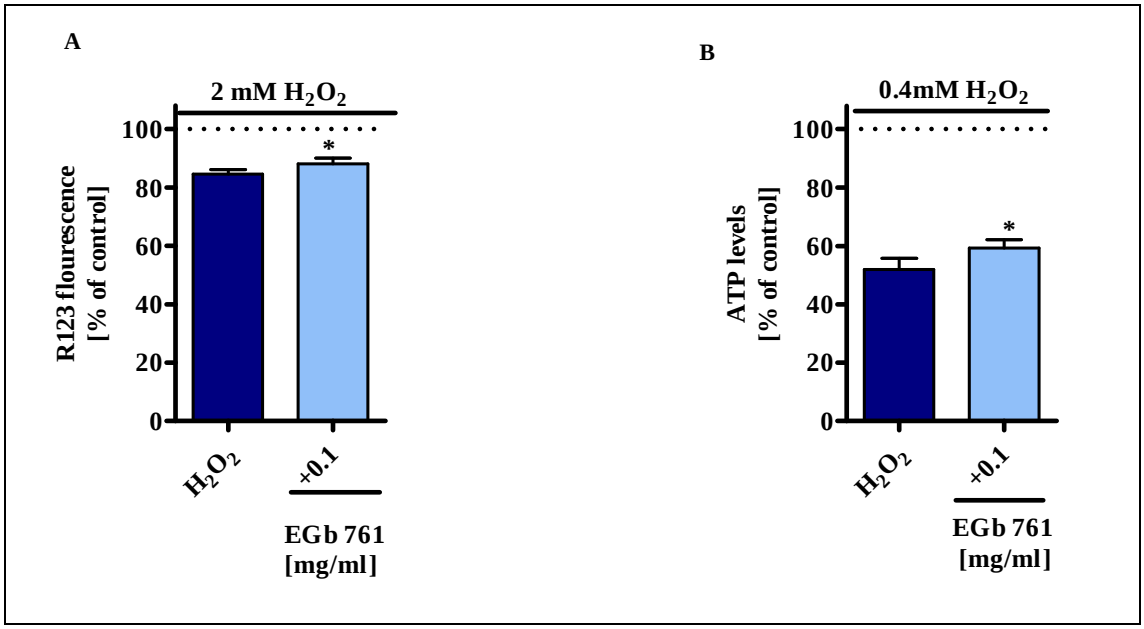


Figure 4-10

In vitro protection of mitochondrial functions against H_2O_2 induced oxidative stress in 15-16 months old mice

Dissociated brain cells prepared from 15-16 months old mice were incubated for 4 hours with 2 mM H_2O_2 for mitochondrial membrane potential [A] or with 0.4 mM for ATP levels [B]. EGb 761® was added 30 minutes after incubation with H_2O_2. Data are expressed as means ± S.E.M [n=13 and n=6 for A and B respectively]. [*$p<0.05$ vs. H_2O_2, student's t-test]

4.2.2 *Ex vivo* findings

After noticing a significant improvement on the mitochondrial functions after H_2O_2 –induced damage in both young and older animals, the *ex vivo* effects of EGb 761® were analyzed.

A preliminary test was carried out to examine the influence of oxidative stress on the 2 different age groups. Both 2-3 and 15-16 months old mice were equally sensitive to H_2O_2 generated oxidative stress as demonstrated in figure 4-11. The decrease in mitochondrial membrane potential [figure 4-11A] and ATP levels [figure 4-11B] was the same in both age groups.

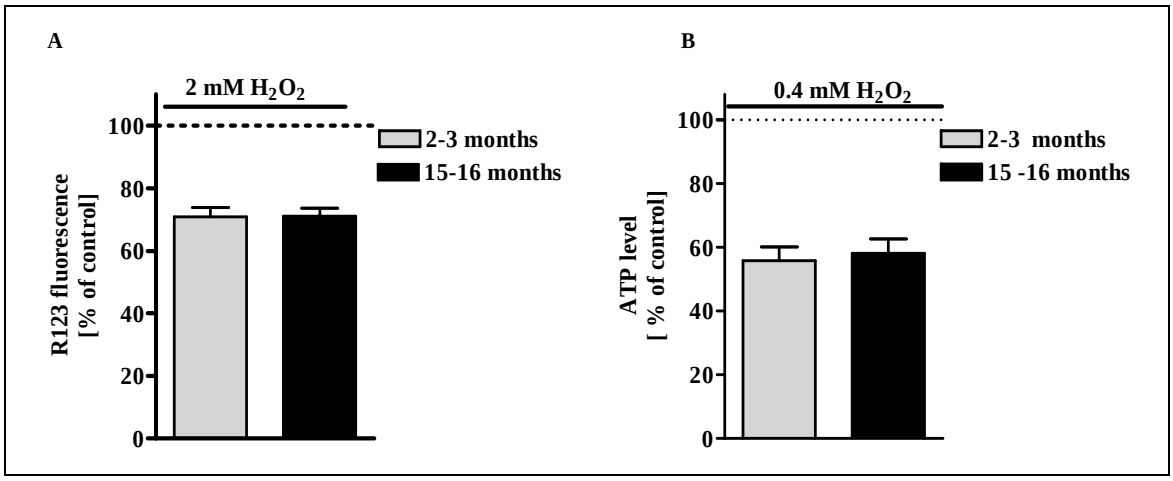


Figure 4-11

Effect of H_2O_2 on the mitochondrial functions of 2 different age groups of NMRI mice

Prepared dissociated brain cells were incubated for 4 hours with H_2O_2, followed by measuring mitochondrial membrane potential [A] and ATP levels [B]. Data are expressed as means ± S.E.M [n=8-10]

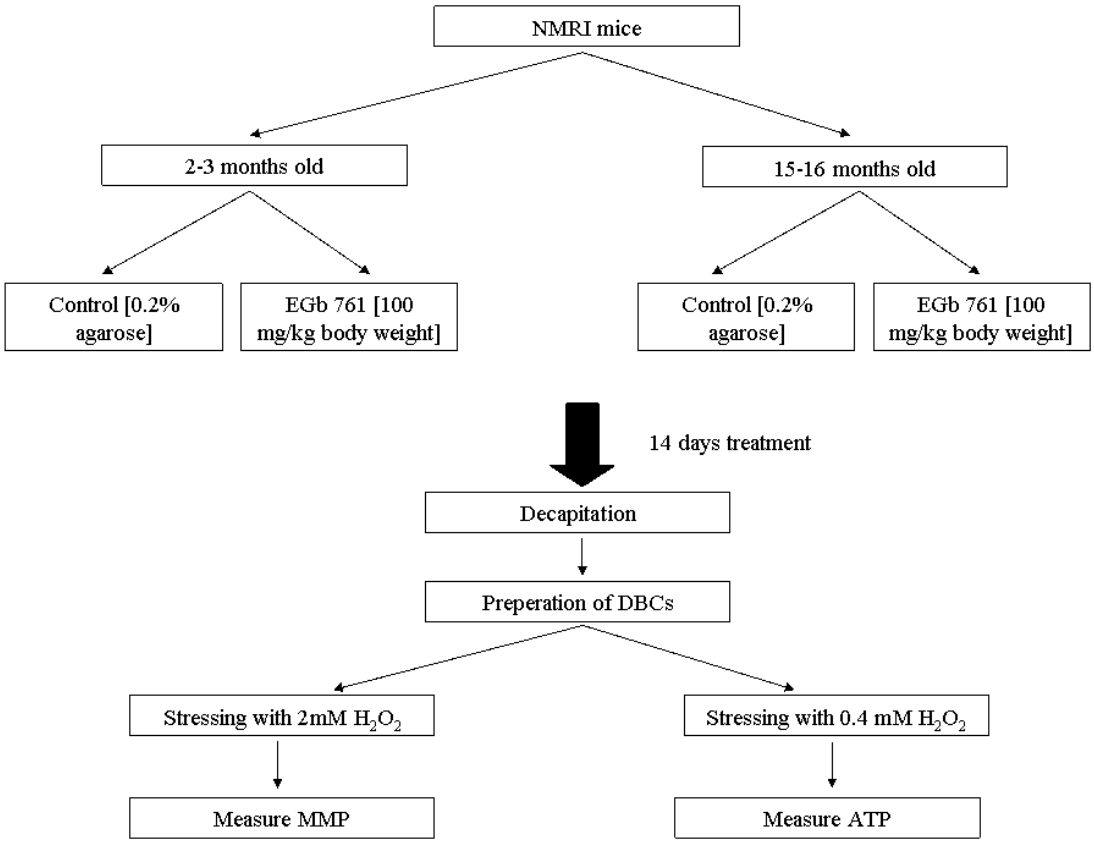


Figure 4-12

14 days treatment of NMRI mice with 100 mg/kg body weight EGb 761®

2-3 months old NMRI mice and 15-16 months old mice were treated orally with either 0.2 % agarose [control] or with 100 mg/kg body weight EGb 761® for 14 consecutive days. 24 hours after the last treatment mice were sacrificed and Dissociated brain cells were prepared. Dissociated brain cells were incubated for 4 hours with H_2O_2 followed by measurement of mitochondrial membrane potential [mmp] and ATP levels

For the treatment study, the above scheme was implemented. For each age group 18 mice were used. 9 mice were assigned to the control group, receiving a daily treatment of 0.2 % agarose solution and the other 9 mice received 100 mg/kg body weight EGb 761® solution. This is a universally applied dosage and has been previously reported to reduce ROS-induced apoptosis and improve learning deficits in aged mice (Schindowski *et al.* 2001;Stoll *et al.* 1996). Mice were treated daily for 14 consecutive days, 24 hours after the last treatment mice were sacrificed and DBCs were prepared as usual. For mitochondrial membrane potential measurement cells were

incubated with 2 mM H_2O_2 and for ATP measurement with 0.4 mM H_2O_2 for 4 hours.

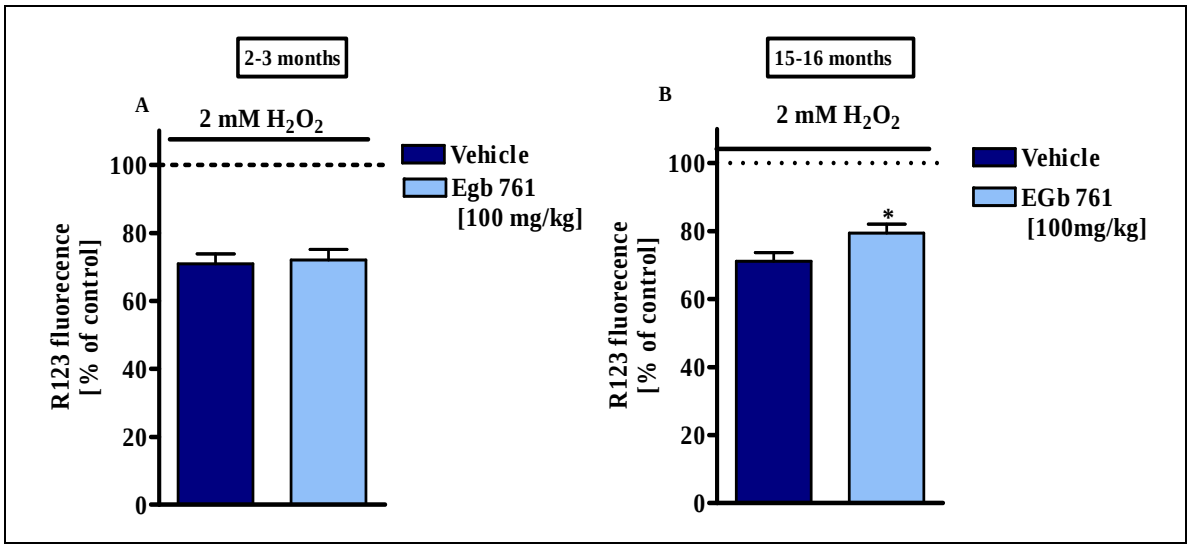


Figure 4-13

Ex vivo protection of mitochondrial membrane potential against H_2O_2 induced oxidative stress in different age groups

2-3 months old [A] and 15-16 months old [B] mice were treated for 14 consecutive days with 100 mg/kg body weight EGb 761® or with vehicle only. Dissociated brain cells prepared from the treated mice were incubated for 4 hours with 2mM H_2O_2 and then mitochondrial membrane potential was measured. Data are expressed as means ± S.E.M [n=8-9] [*$p<0.05$, vs. H_2O_2, unpaired student's t-test]

An age dependent sensitivity was observed , only the 15-16 months old group showed an improvement in the mitochondrial membrane potential after EGb 761® treatment, [figure 4-13B] but no response was observed in the young mice [figure 4-13A].

Measuring the ATP levels after the H_2O_2 triggered impairment revealed no alterations between the vehicle-treated group and the EGb 761® treated group in both age groups [figure 4-14].

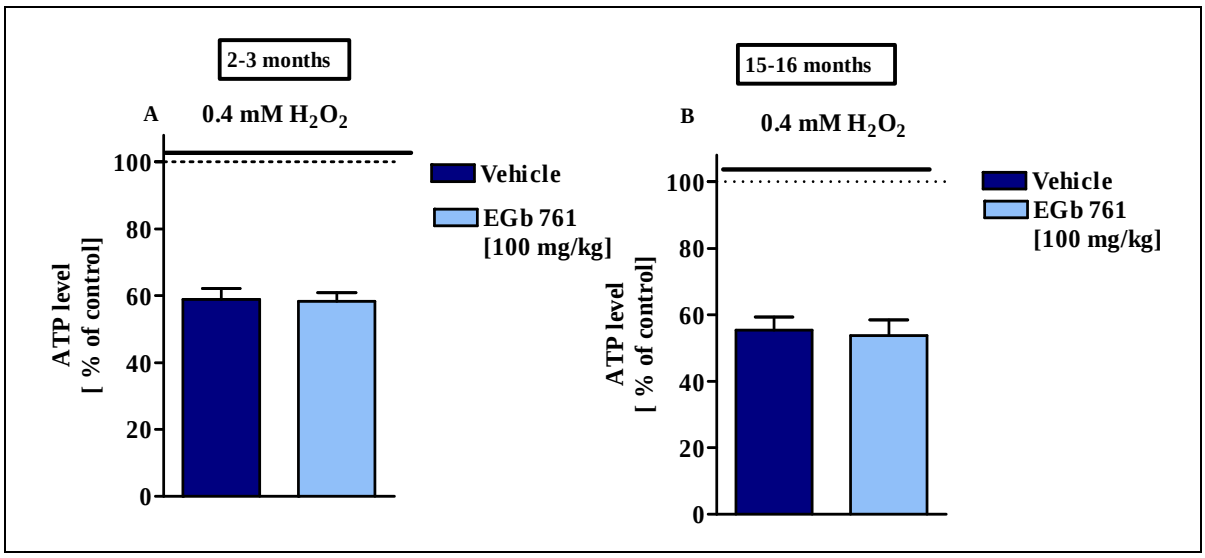


Figure 4-14

Ex vivo effect of EGb 761® on ATP levels against H_2O_2 induced oxidative stress in different age groups

2-3 months old [A] and 15-16 months old [B] mice were treated for 14 consecutive days with 100 mg/kg body weight EGb 761® or with vehicle only. Dissociated brain cells prepared from the treated mice were incubated for 4 hours with 0.4 mM H_2O_2 and then ATP levels measured. Data are expressed as means ± S.E.M [n=8-9]

After examining the effect of EGb 761® *ex vivo* in young and older mice and noticing a slight protective effect during aging we decided to examine the effect of EGb 761® in a transgenic mouse model mimicking AD. Thy-1 APP mice are transgenic; comprising both the double Swedish mutation and the London mutation. These mutations lead to higher yields of amyloid beta peptide and consequently plaque formation.

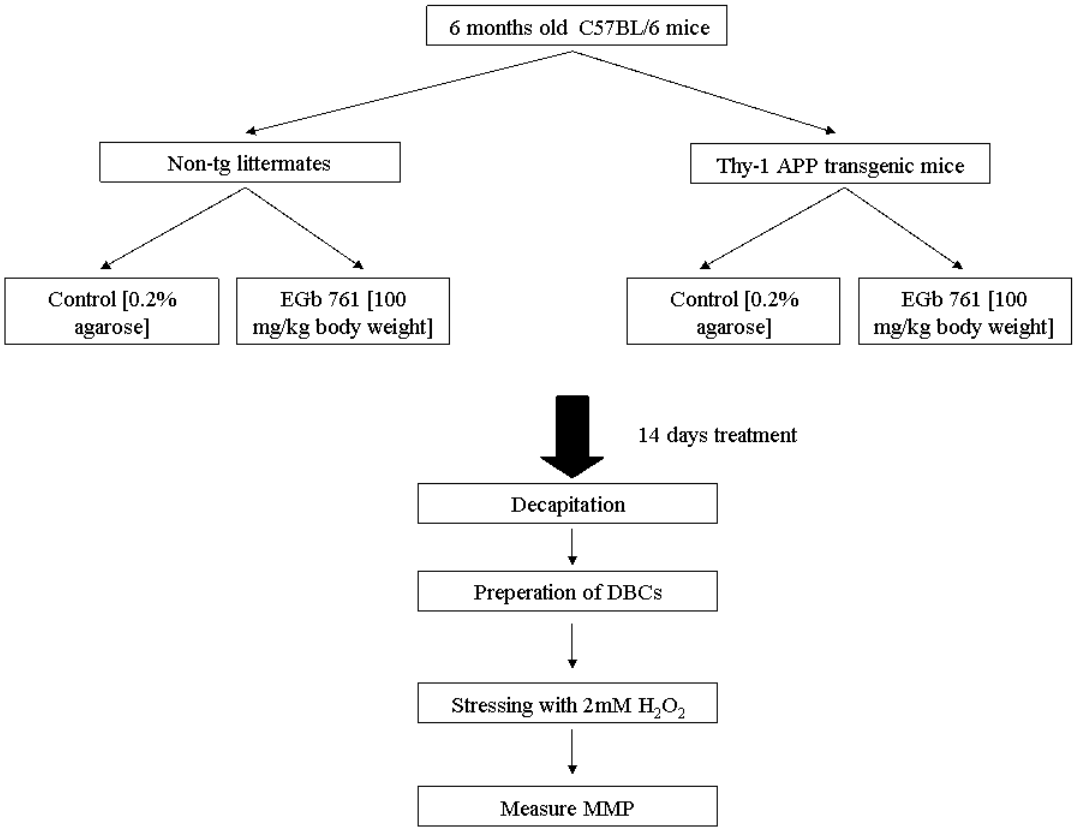


Figure 4-15

14 days treatment of Thy-1 APP mice and non-tg littermates with 100 mg/kg body weight EGb 761®

6 months old Thy-1 APP mice and non-tg littermates were treated orally with either 0.2 % agarose [control] or with 100 mg/kg body weight EGb 761® for 14 consecutive days. 24 hours after the last treatment mice were sacrificed and dissociated brain cells were prepared. Dissociated brain cells were incubated for 4 hours with H_2O_2 followed by measurement of mitochondrial membrane potential [mmp]

6 months old wild type [littermates] and Thy-1 APP female mice were both treated for 14 consecutive days with EGb 761® [100 mg/kg body weight] or with vehicle only. 24 hours after the last treatment the mice were sacrificed and DBCs were prepared.

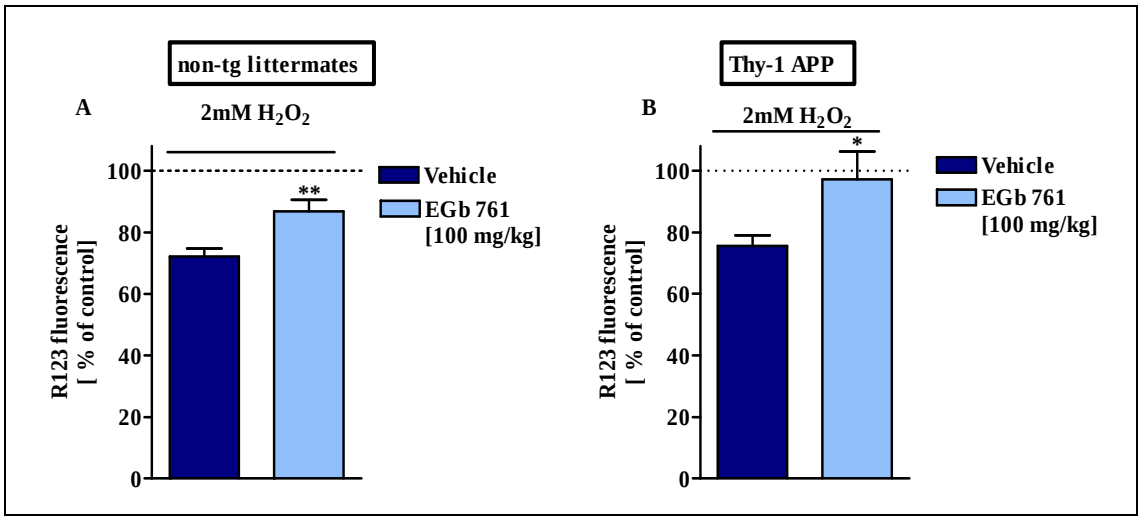


Figure 4-16

Ex vivo protection of mitochondrial membrane potential against H_2O_2 induced oxidative stress in transgenic APP mice and non-tg littermates

6 months old non.-tg littermates [A] and Thy-1 APP [B] mice were treated for 14 consecutive days with 100 mg/kg body weight EGb 761® or with vehicle only. Dissociated brain cells prepared from the treated mice were incubated for 4 hours with 2 mM H_2O_2 and then mitochondrial membrane potential was determined. Data are expressed as means ± S.E.M [n=8-9] [*p<0.05, **p<0.01 vs. H_2O_2, unpaired student's t-test]

Mitochondrial membrane potential was measured after incubating the DBCs with 2 mM H_2O_2. As depicted in figure 4-16 both wild type and transgenic mice showed higher resistance to H_2O_2 -induced mitochondrial damage after EGb 761® treatment. It is worth mentioning that the potency of EGb 761® appears to be higher in the transgenic mouse model. The effect observed in the wild type mice and was not observed in the NMRI mice could be due to the age difference [3 months vs. 6 months] or due to strain difference [NMRI vs. C57BL/6].

Summing up the previous section, EGb 761® enhanced mitochondrial membrane potential *in vitro* against H_2O_2 -induced damage in both young and old NMRI mice and improved ATP levels only in the older mice. Treating the same age groups with EGb 761® for 14 consecutive days alleviated mitochondrial membrane potential of the older group only. Unfortunately, EGb 761® was not able to protect neither the young nor the older mice against

H_2O_2 -mediated decrease in ATP levels *ex vivo*. Testing the effects of EGb 761® in an AD mouse model *ex vivo* showed alleviation in the mitochondrial membrane potential of wild type mice and the effect was more pronounced in transgenic mice.

4.3 Effects of Ginkgo biloba extract [EGb 761®] on mitochondrial function: Protection against SNP induced stress

SNP which is a well known NO donor was used to exert nitrosative stress. NO and its derivative peroxynitrite cause injury to the mitochondria, inhibiting all respiratory chain complexes. This leads to reduced ATP formation and decreases the mitochondrial membrane potential.

4.3.1 *In vitro* findings

4.3.1.1 3 months old NMRI mice

2-3 months old female NMRI mice were used in this study. DBCs were prepared from these mice and were incubated for 4 hours with either 2 mM SNP to measure mitochondrial membrane potential [figure 4-17A] or with 0.2 mM SNP for ATP levels [Figure 4-17B]. EGb 761® was added 30 minutes after incubation with SNP. As illustrated in figure 4-17, EGb 761® was able to protect the mitochondria from the SNP induced damage. Both mitochondrial membrane potential and ATP levels were improved after EGb 761® treatment.

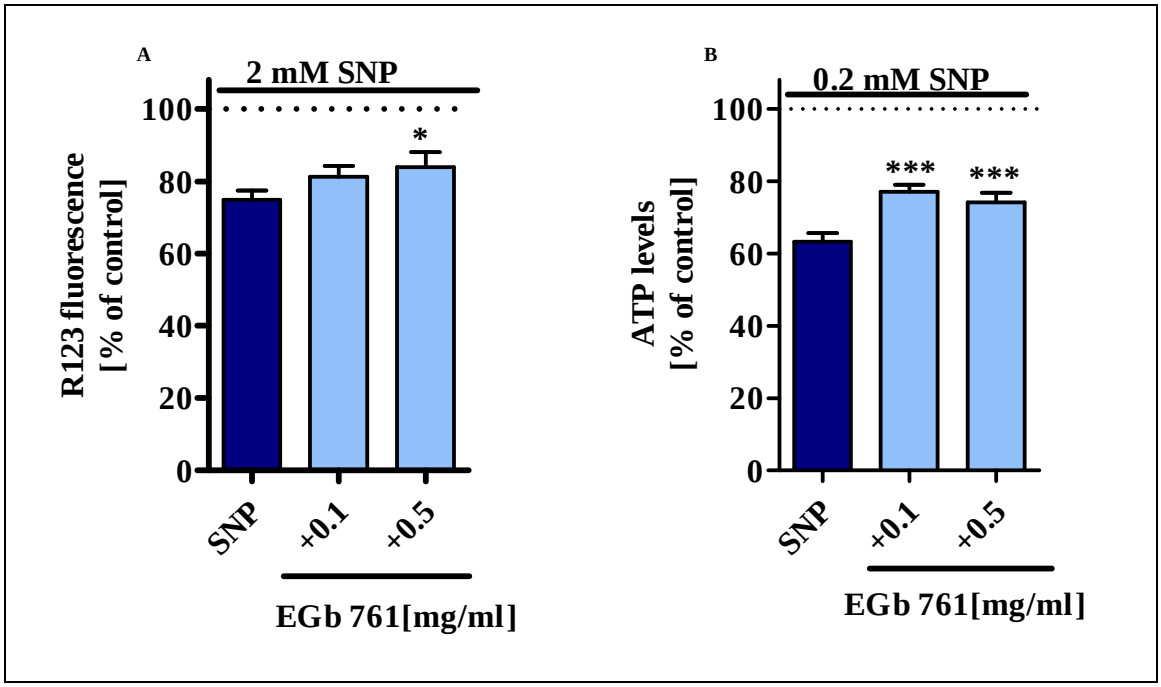


Figure 4-17

In vitro protection of mitochondrial functions against SNP induced nitrosative stress in 2-3 months old mice

Dissociated brain cells were incubated for 4 hours with 2 mM SNP for mitochondrial membrane potential [A] or with 0.2 mM for ATP levels [B]. EGb 761® was added 30 minutes after incubation with SNP. Data are expressed as means ± S.E.M [n=11-12] [*p<0.05, ***p<0.001 vs. SNP, student's t-test]

4.3.1.2 15 months old NMRI mice

To examine the effect of EGb 761® on aging, the same *in vitro* experiment was repeated but instead of using 2-3 months old mice, 15-16 months old mice were used. EGb 761® was able to improve the mitochondrial damage caused by SNP in 15-16 months old mice. A concentration of 0.1 mg/ml showed a significant effect on the ATP levels [figure 4-18B] and a concentration of 0.5 mg/ml EGb 761® was able to enhance mitochondrial membrane potential [figure 4-18A].

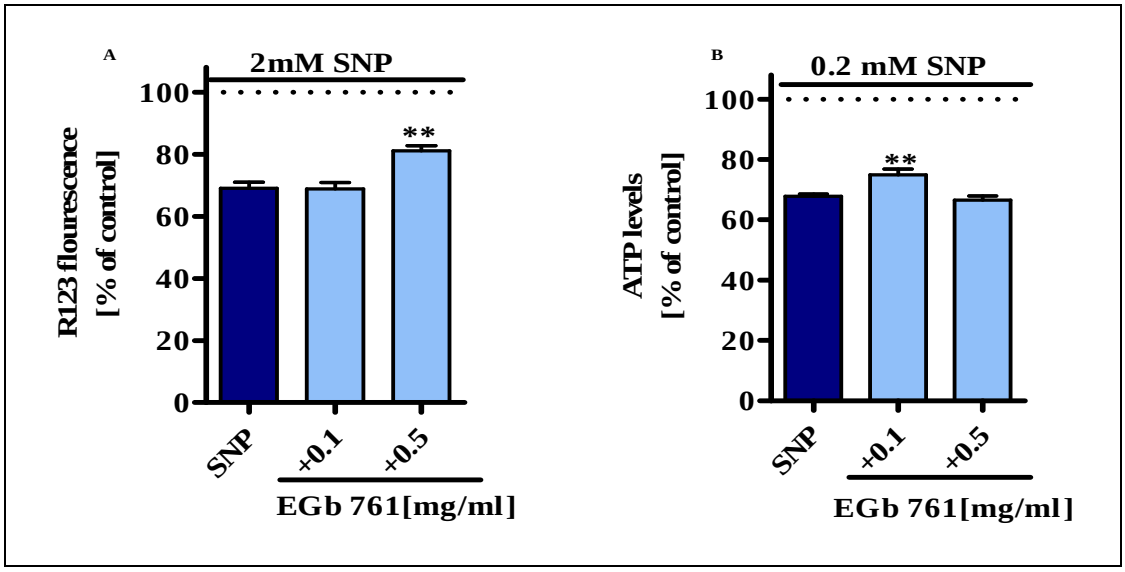


Figure 4-18

In vitro protection of mitochondrial functions against SNP induced nitrosative stress in 15-16 months old mice

Dissociated brain cells were incubated for 4 hours with 2 mM SNP for mitochondrial membrane potential [A] or with 0.2 mM for ATP levels [B]. EGb 761® was added 30 minutes after incubation with SNP. Data are expressed as means ± S.E.M [n=7-8] [**p<0.01 vs. SNP, student's t-test]

4.3.2 Ex vivo findings

A treatment study for 2 weeks was carried out to examine the influence of EGb 761® on NO generated mitochondrial dysfunction in both young [2-3 months old] and 15-16 months old mice. Each age group was divided into a control group and an EGb 761® group. After 14 days treatment the mice were sacrificed and DBCs were prepared as usual. The DBCs were then incubated for 4 hours with either 2 mM SNP for mitochondrial membrane potential or with 0.2 mM SNP for determining ATP levels.

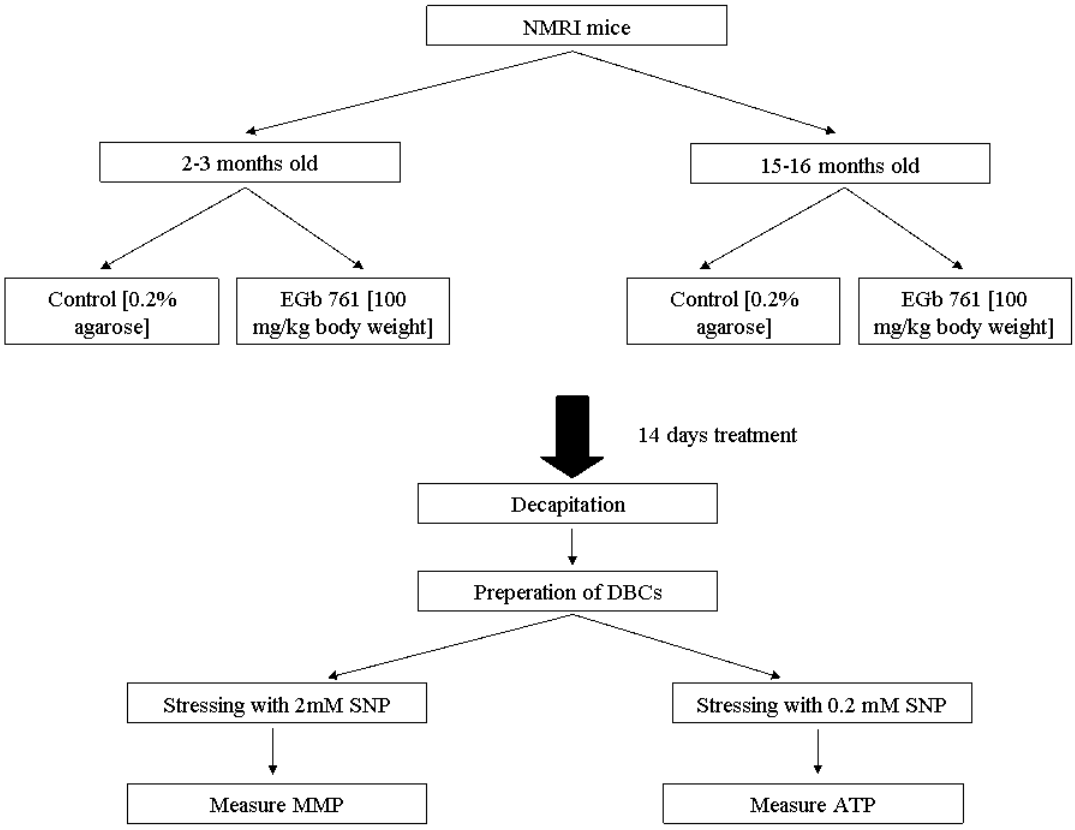


Figure 4-19

14 days treatment of NMRI mice with 100 mg/kg body weight EGb 761®

2-3 months old NMRI mice and 15-16 months old mice were treated orally with either 0.2 % agarose [control] or with 100 mg/kg body weight EGb 761® for 14 consecutive days. 24 hours after the last treatment mice were sacrificed and dissociated brain cells were prepared. Dissociated brain cells were incubated for 4 hours with SNP followed by measurement of mitochondrial membrane potential [mmp] and ATP levels.

Comparing the effect of NO on the two different age groups indicated that the younger mice were more sensitive to nitrosative stress as depicted in figure 4-20. This observation was minor on the mitochondrial membrane potential [figure 4-20A], but was highly pronounced on ATP levels [figure 4-20B].

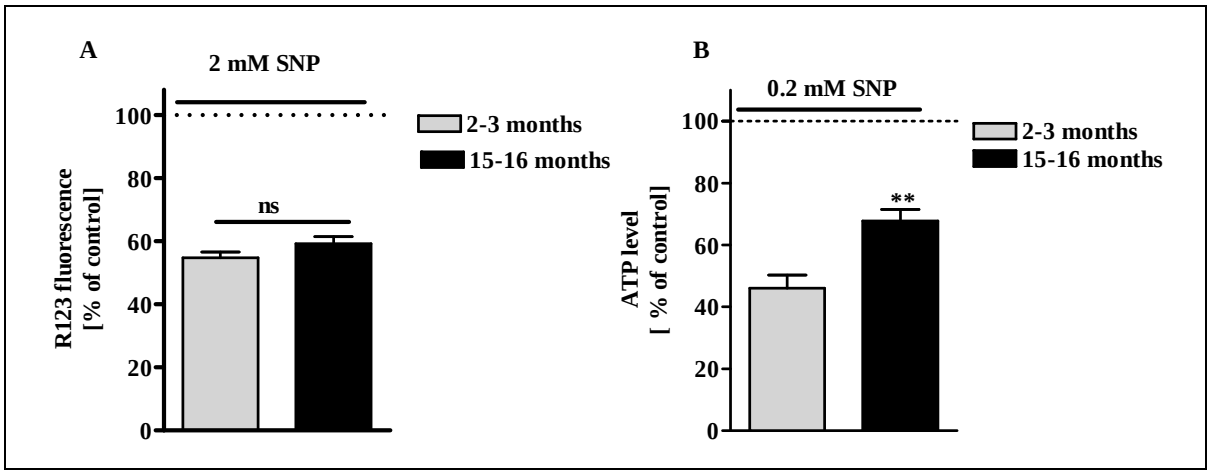


Figure 4-20

Effect of NO on the mitochondrial functions of 2 different age groups of NMRI mice

Prepared dissociated brain cells were incubated for 4 hours with SNP, followed by measuring mitochondrial membrane potential [A] and ATP levels [B]. Data are expressed as means ± S.E.M [n=8-9] [**p<0.01 vs. 2-3 months old mice, student's unpaired t-test].

After 2 weeks treatment EGb 761® was able to improve mitochondrial membrane potential only in older mice and showed no effect in the younger mice [figure 4-21].

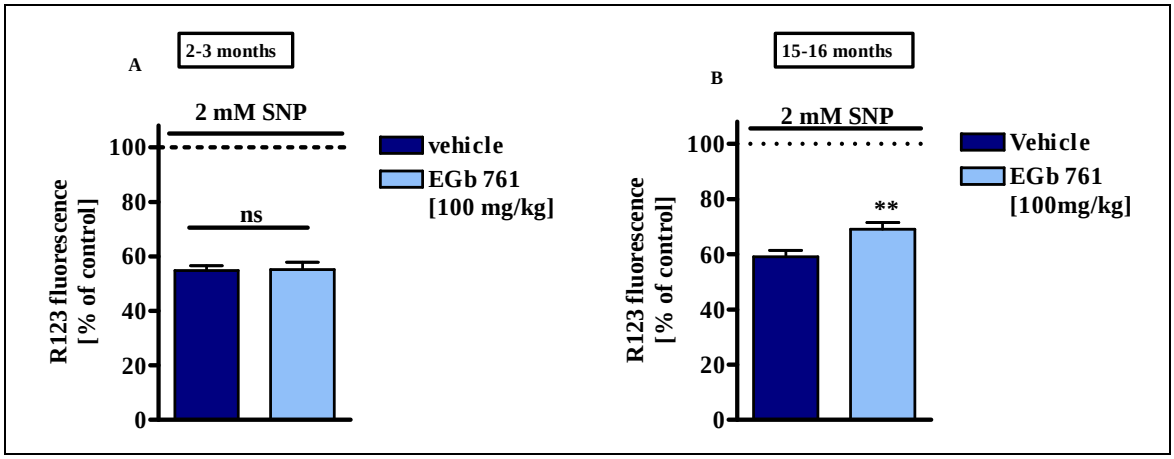


Figure 4-21

Ex vivo protection of mitochondrial membrane potential against SNP induced stress in different age groups

2-3 months old [A] and 15-16 months old [B] mice were treated for 14 consecutive days with 100 mg/kg body weight EGb 761® or with vehicle only. Dissociated brain cells prepared from the treated mice were incubated for 4 hours with 2mM SNP and then mitochondrial membrane potential was determined. Data are expressed as means ± S.E.M [n=8-10] [**p<0.01 vs. vehicle, unpaired student's t-test].

However, comparing ATP levels in the EGb 761® treated group and the placebo group after NO stress showed no difference in both age groups [figure 4-22]

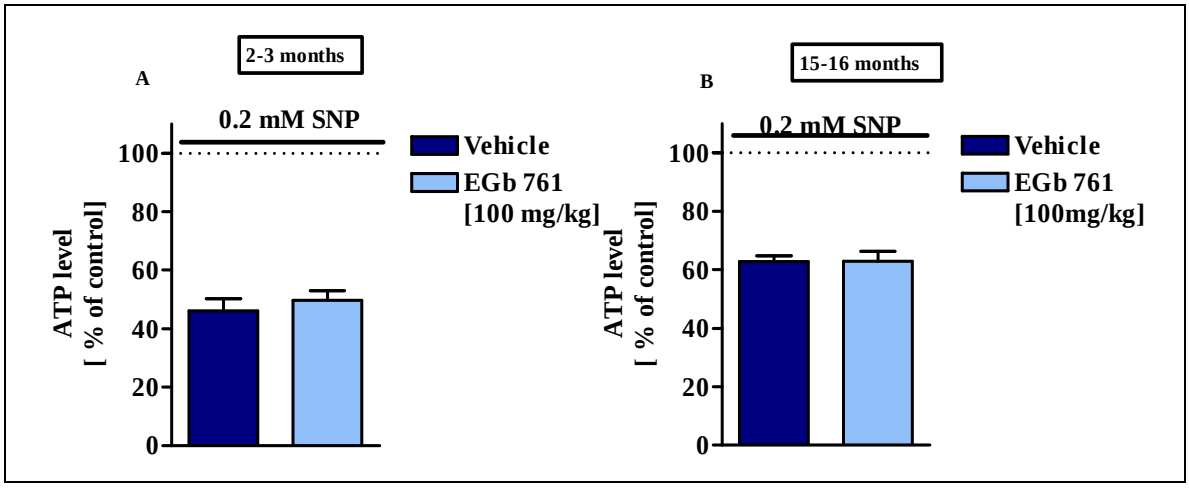


Figure 4-22

Ex vivo effect of EGb 761® on ATP levels against SNP induced nitrosative stress in different age groups

2-3 months old [A] and 15-16 months old [B] mice were treated for 14 consecutive days with 100 mg/kg body weight EGb 761® or with vehicle only. Dissociated brain cells prepared from the treated mice were incubated for 4 hours with 0.2mM SNP and then ATP levels were measured. Data are expressed as means ± S.E.M [n=8-10].

Similar to the experiments done with H_2O_2, our *ex vivo* studies with NO were extended by testing the influence of EGb 761® in AD transgenic mouse model. 6 months old female Thy-1 APP and wild type mice were treated for 14 days with EGb 761® [100 mg/kg body weight] or with vehicle. DBCs prepared from these mice were stressed for 4 hours with 2 mM SNP and mitochondrial membrane potential was then measured.

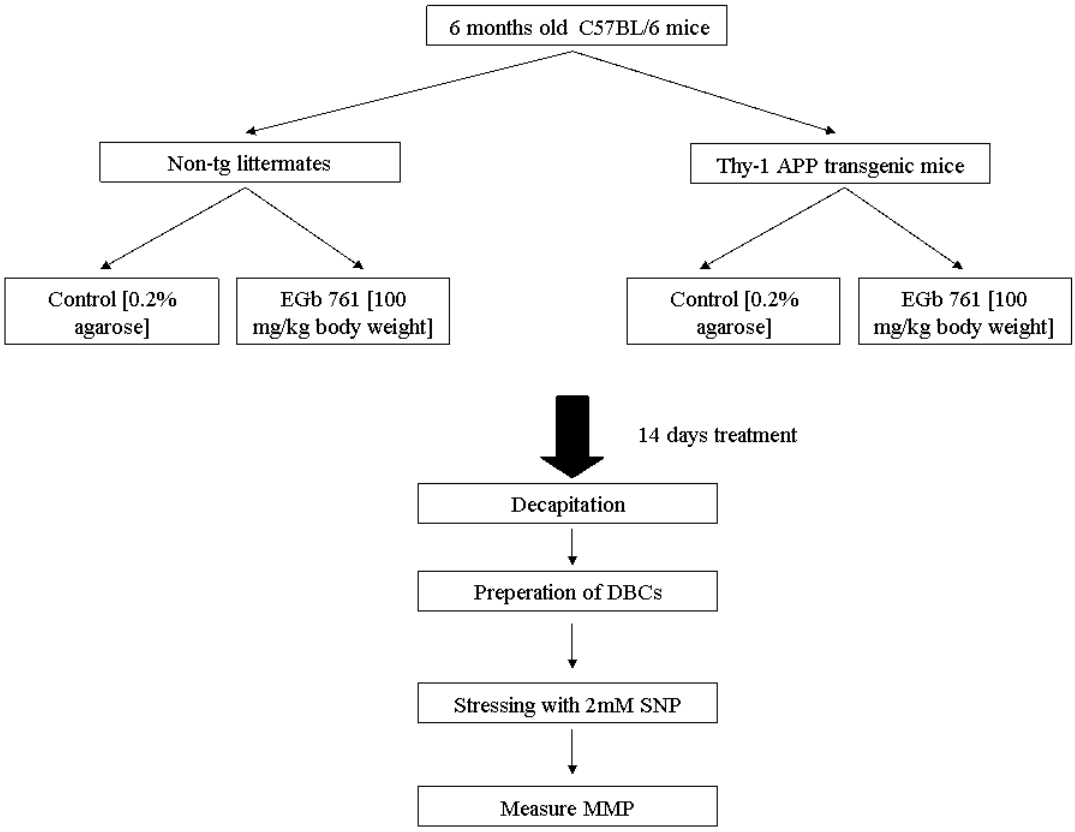


Figure 4-23

14 days treatment of Thy-1 APP mice and non-tg littermates with 100 mg/kg body weight EGb 761®

6 months old Thy-1 APP mice and non-tg littermates were treated orally with either 0.2 % agarose [control] or with 100 mg/kg body weight EGb 761® for 14 consecutive days. 24 hours after the last treatment mice were sacrificed and dissociated brain cells were prepared. Dissociated brain cells were incubated for 4 hours with SNP followed by measurement of mitochondrial membrane potential [MMP].

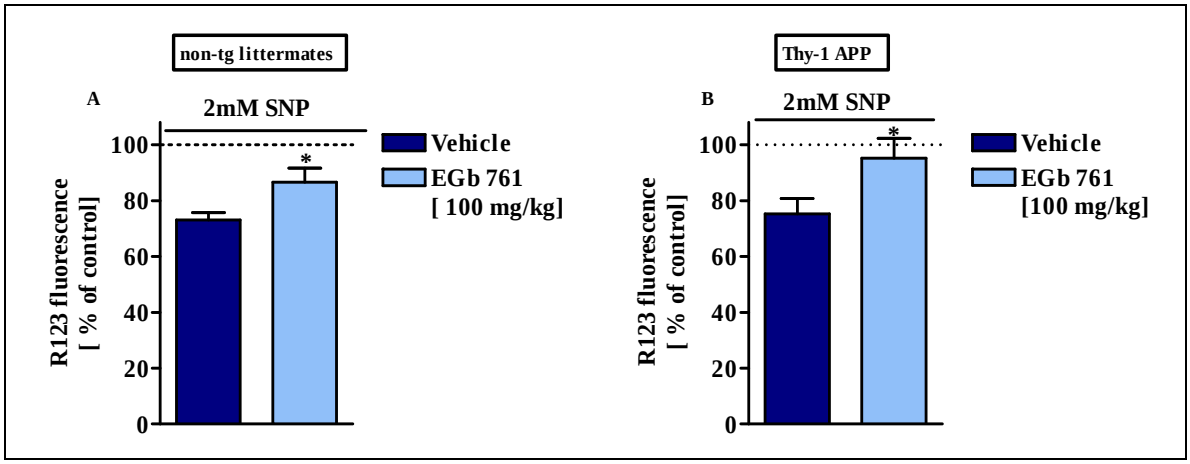


Figure 4-24

Ex vivo protection of mitochondrial membrane potential against SNP induced nitrosative stress in transgenic APP mice and non-tg littermates

Non.-tg littermates [A] and Thy-1 APP [B] mice were treated for 14 consecutive days with 100 mg/kg body weight EGb 761® or with vehicle only. Dissociated brain cells prepared from the treated mice were incubated for 4 hours with 2 mM SNP and then mitochondrial membrane potential was determined. Data are expressed as means ± S.E.M [n=8] [*p<0.05 vs. vehicle, unpaired student's t-test].

Both wild type and transgenic mice were significantly protected against the NO-induced stress after EGb 761® treatment [figure 4-24], here again with a more evident effect in the transgenic mice.

The effect of EGb 761® on NO-induced damage was similar to that seen with H_2O_2 but slightly more pronounced. Both young and old mice were protected *in vitro* against NO-induced mitochondrial damage. There was no effect noticed on ATP levels *ex vivo*, but mitochondrial membrane potential was improved in the older mice group. EGb 761® was also efficient in the AD transgenic mice model.

4.4 Effects of various components of EGb 761® on mitochondrial function: Protection against SNP induced stress

From the above findings and from previously reported results in the literature, EGb 761® has proven its ability to prevent ROS and RNS induced damage, and protect the mitochondria. However, the question which component/s is/are responsible for these effects is still not answered. Only a few scientists tried to answer this question by testing all the different components under the same conditions, some tested only the flavonoids others only bilobalide or the terpenoids. Comparing these different studies and trying to reach a conclusion makes it very difficult due to the methodological differences. In order to reach such conclusions all components should be evaluated under the same conditions.

Therefore, the impact of the single components of EGb 761® on the mitochondrial function was tested, under the same conditions and measuring the same parameters.

EGb 761® consists of two major groups of substances, the flavonoid fraction [24 %] and the terpenoid fraction [6 %]. The flavonoid fraction is primarily composed of quercetin, kaempferol and isorhamnetin glycosides and the terpenoid fraction consists of ginkgolides A, B, C, J and bilobalide. The components were tested against SNP initiated mitochondrial dysfunction, and not H_2O_2, since the influence of EGb 761® on NO-generated damage was more pronounced.

4.4.1 *In vitro* findings

In vitro experiments with DBCs were preformed using two different experimental procedures. For pre-treatment studies the ability of the EGb 761® constituents to prevent occurrence of mitochondrial damage was

examined. Here the EGb 761® constituents were first added, after 30 minutes incubation time the DBCs were stressed with NO for 4 hours.

For post-treatment studies the ability of the constituents to regenerate the mitochondrial functions after nitrosative damage was tested. The DBCs were first stressed with NO for 4 hours, 30 minutes after insult the constituents were added.

4.4.1.1 Pre-treatment studies

In this section, experiments were conducted with the help of DBCs prepared from 2-3 months old female NMRI mice. Mitochondrial function was evaluated via 3 different markers. Mitochondrial membrane potential, ATP levels and cell viability were determined for each constituent.

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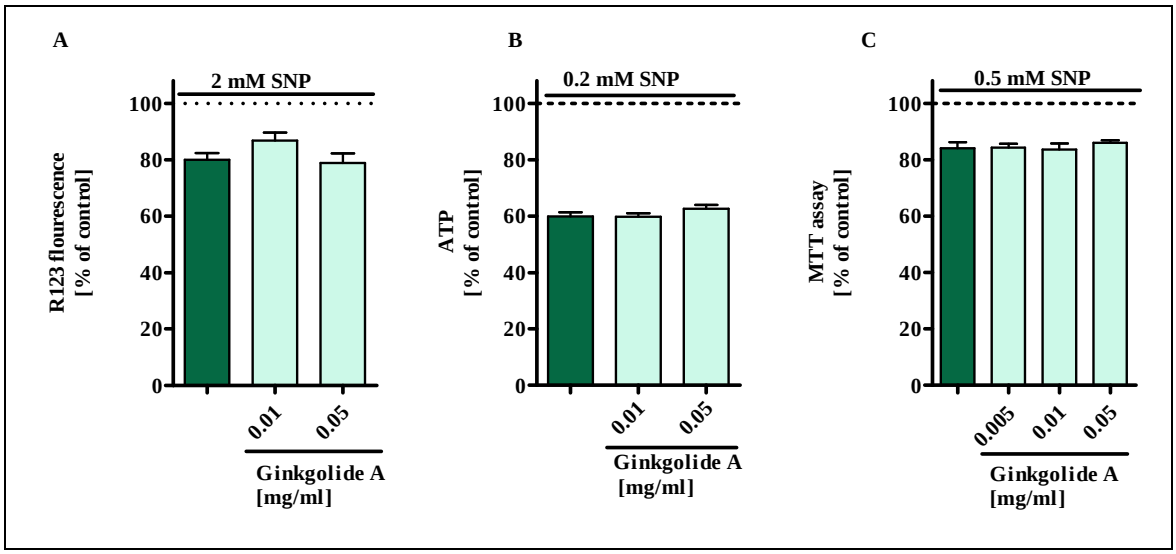

Figure 4-25

Protection of the mitochondria against NO insult by pre-treatment with Ginkgolide A

Dissociated brain cells were incubated with Ginkgolide A for 30 minutes. Then SNP was added for 4 hours. Mitochondrial membrane potential [A], ATP levels [B] and metabolic activity were determined [C]. Data are expressed as means ± S.E.M [n=5-8]

The DBCs were first incubated with GA. 30 minutes later cells were incubated with 2 mM SNP for mitochondrial membrane potential, 0.2 mM SNP for ATP levels and with 0.5 mM SNP for MTT assay. After 4 hours

mitochondrial membrane potential, ATP levels and MTT assay were measured. As shown in figure 4-25 pre-treatment with GA had no influence on mitochondrial functions.

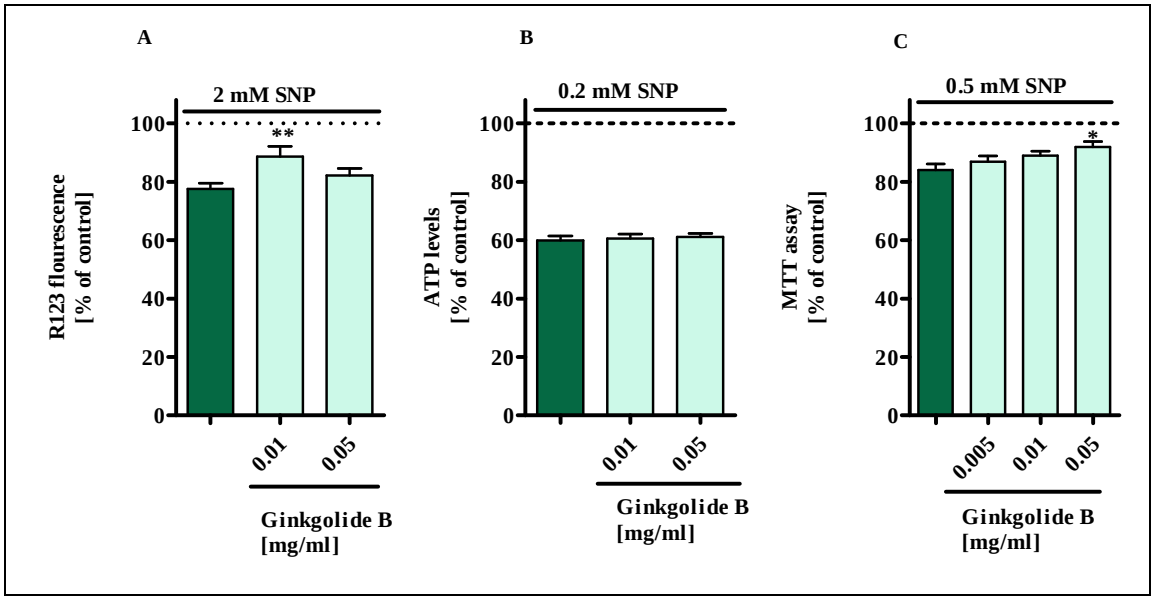

Figure 4-26

Protection of the mitochondria against NO insult by pre-treatment with Ginkgolide B

Dissociated brain cells were incubated with Ginkgolide B for 30 minutes. Then SNP was added for 4 hours. Mitochondrial membrane potential [A], ATP levels [B] and metabolic activity were determined [C]. Data are expressed as means ± S.E.M [n=6-8] [*p<0.05, **p<0.01 vs. SNP, student's paired t-test].

On the contrary, pre-treatment with GB was able to enhance mitochondrial membrane potential at a concentration of 0.01 mg/ml [figure 4-26A] and improve cell viability at a concentration of 0.05 mg/ml [figure 4-26C]. On the other hand ATP levels were not affected by the pre-incubation with GB.

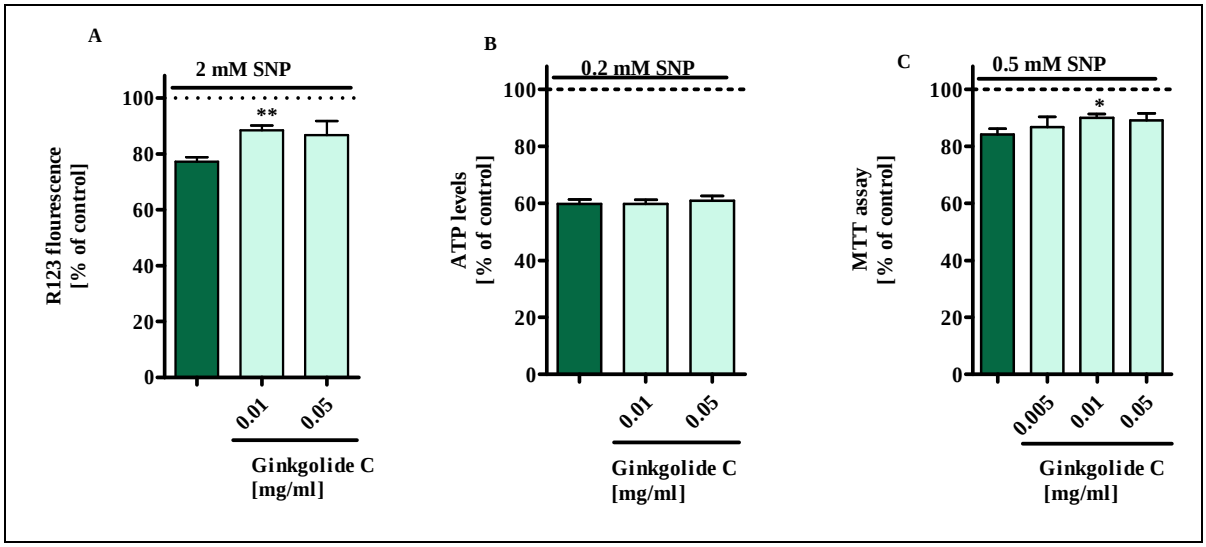

Figure 4-27

Protection of the mitochondria against NO insult by pre-treatment with Ginkgolide C

Dissociated brain cells were incubated with Ginkgolide C for 30 minutes. Then SNP was added for 4 hours. Mitochondrial membrane potential [A], ATP levels [B] and metabolic activity were determined [C]. Data are expressed as means ± S.E.M [n=6-8] [*p<0.05, **p<0.01 vs. SNP, student's paired t-test].

Both mitochondrial membrane potential and cell viability were significantly improved using 0.01 mg/ml GC [figures 4-27A&C]. Here again ATP levels were not improved by pre-treatment with GC [figure B].

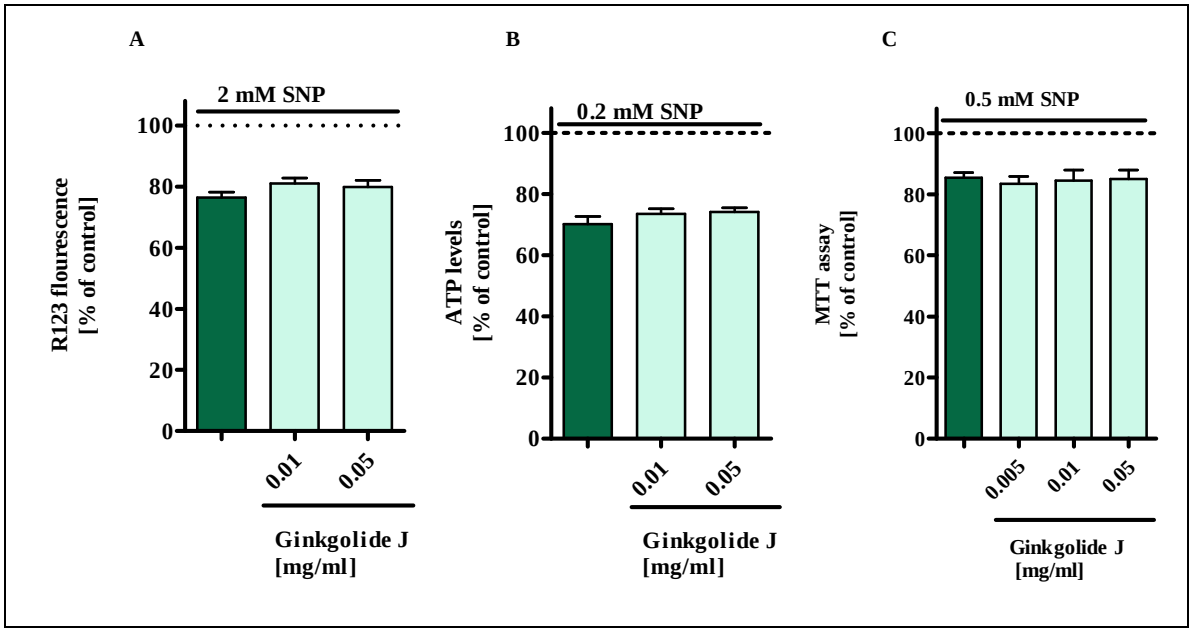

Figure 4-28

Protection of the mitochondria against NO insult by pre-treatment with Ginkgolide J

Dissociated brain cells were incubated with Ginkgolide J for 30 minutes. Then SNP was added for 4 hours. Mitochondrial membrane potential [A], ATP levels [B] and metabolic activity were determined [C]. Data are expressed as means ± S.E.M [n=5-7].

Similar to GA, pre-treatment with GJ had no influence on mitochondrial properties as depicted in figure 4-28.

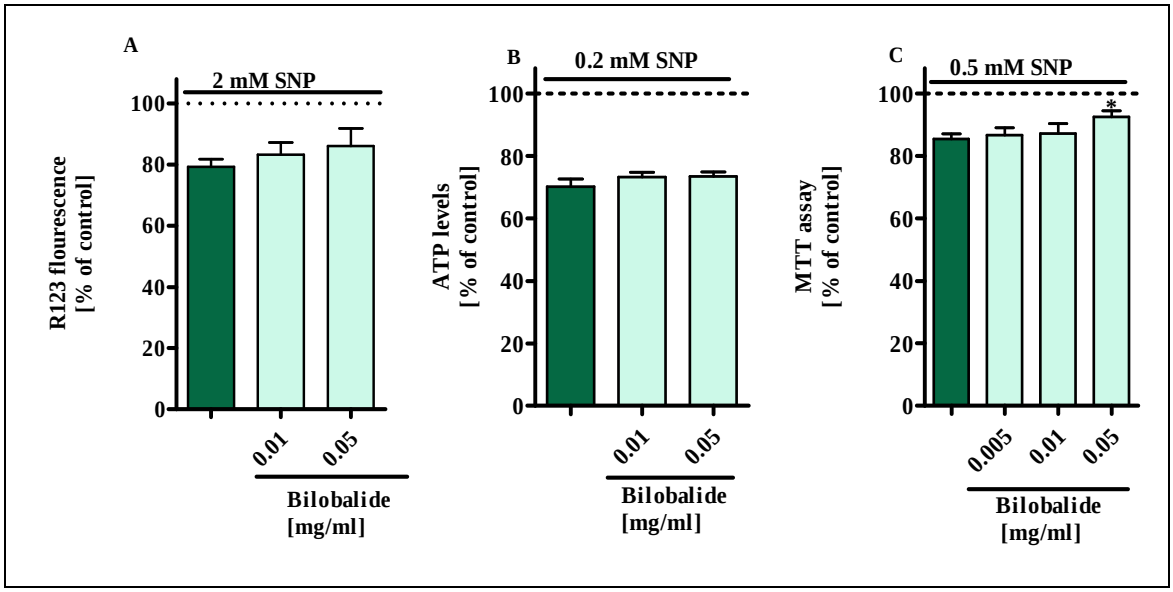

Figure 4-29

Protection of the mitochondria against NO insult by pre-treatment with Bilobalide

Dissociated brain cells were incubated with Bilobalide for 30 minutes. Then SNP was added for 4 hours. Mitochondrial membrane potential [A], ATP levels [B] and metabolic activity were determined [C]. Data are expressed as means ± S.E.M [n=5-7] [*$p<0.05$ vs. SNP, student's paired t-test].

Pre-treating the DBCs with Bilobalide demonstrated a slight improvement on the mitochondrial functions. 0.05 mg/ml showed a slight increase in mitochondrial membrane potential [figure 4-29A] which was not significant, but the same concentration was able to enhance cell viability significantly [figure 4-29C].

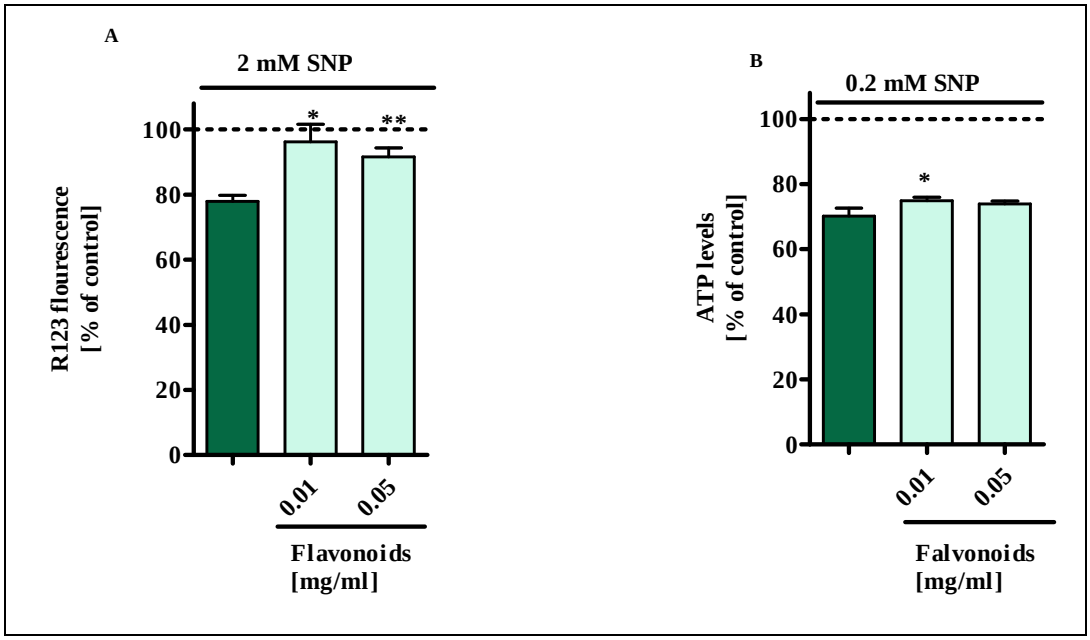

Figure 4-30

Protection of the mitochondria against NO insult by pre-treatment with Flavonoids

Dissociated brain cells were incubated with Flavonoids for 30 minutes. SNP was added for 4 hours. Mitochondrial membrane potential [A], ATP levels [B] were measured. Data are expressed as means ± S.E.M [n=6-7] [*p<0.05, **p<0.01 vs. SNP, student's paired t-test].

The flavonoid fraction had a relatively potent effect compared to the other single constituents and was able to enhance mitochondrial membrane potential to nearly control levels [100 %] as illustrated in figure 4-30A. The ATP levels were also significantly increased with a concentration of 0.01 mg/ml [figure 4-30B]. Cell viability for DBCs treated with flavonoids could not be measured, due to the interference with the MTT assay reagents.

Pre-treatment		0.005	0.01	0.05
GA	MTT	-	-	-
	MMP	-	-	-
	ATP	-	-	-
GB	MTT	-	-	*
	MMP	-	**	-
	ATP	-	-	-
GC	MTT	-	*	-
	MMP	-	**	-
	ATP	-	-	-
GJ	MTT	-	-	-
	MMP	-	-	-
	ATP	-	-	-
Bilobalide	MTT	-	-	-
	MMP	-	-	*
	ATP	-	-	-
Flavonoids	MTT	Reagents interference		
	MMP	-	*	**
	ATP	-	*	-

Table 4.3

Summary of pre-treatment with EGb 761® single constituents

Dissociated brain cells were incubated with SNP for 4 hours. The EGb 761® constituents were added 30 minutes before [Pre-treatment] incubation with SNP. Cell viability [MTT assay], mitochondrial membrane potential [MMP] and ATP levels were measured. [*$p<0.05$ and **$p<0.01$ vs. SNP, student's paired t-test].

The above table represents an overview of the outcomes of the pre-treatment study. In conclusion, it appears that the flavonoid fraction is the most potent, followed by GC. Minor protection was noticed by treatment with GB and BB. Finally GJ and GA were ineffective.

4.4.1.2 Post-treatment studies

In this section the ability of EGb 761® constituents to regenerate mitochondrial functions after nitrosative stress was tested. DBCs were incubated for 4 hours with SNP; 30 minutes after nitrosative stress EGb 761® components were added. After the 4 hours incubation period mitochondrial membrane potential, ATP levels and cell viability were measured.

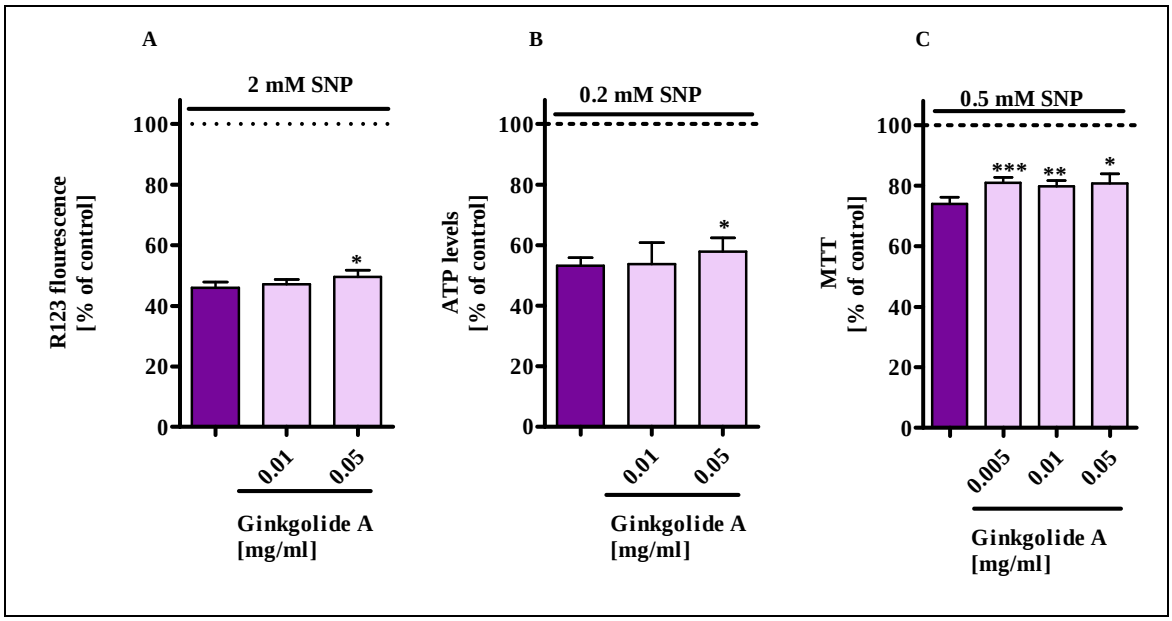

Figure 4-31

Protective effect of Ginkgolide A on mitochondrial functions after NO insult

Dissociated brain cells were stressed with SNP for 4 hours. 30 minutes after incubation with SNP Ginkgolide A was added. Mitochondrial membrane potential [A], ATP levels [B] and metabolic activity were determined [C]. Data are expressed as means ± S.E.M [n=6-11] [*p<0.05, **p<0.01 and ***p<0.001 vs. SNP, student's paired t-test].

In contrast to pre-treatment, post- treatment with GA was able to enhance the mitochondrial functions against NO damage. GA in concentrations as low as 0.005 mg/ml improved cell viability and 0.05 mg/ml improved mitochondrial membrane potential and ATP levels [figure 4-31].

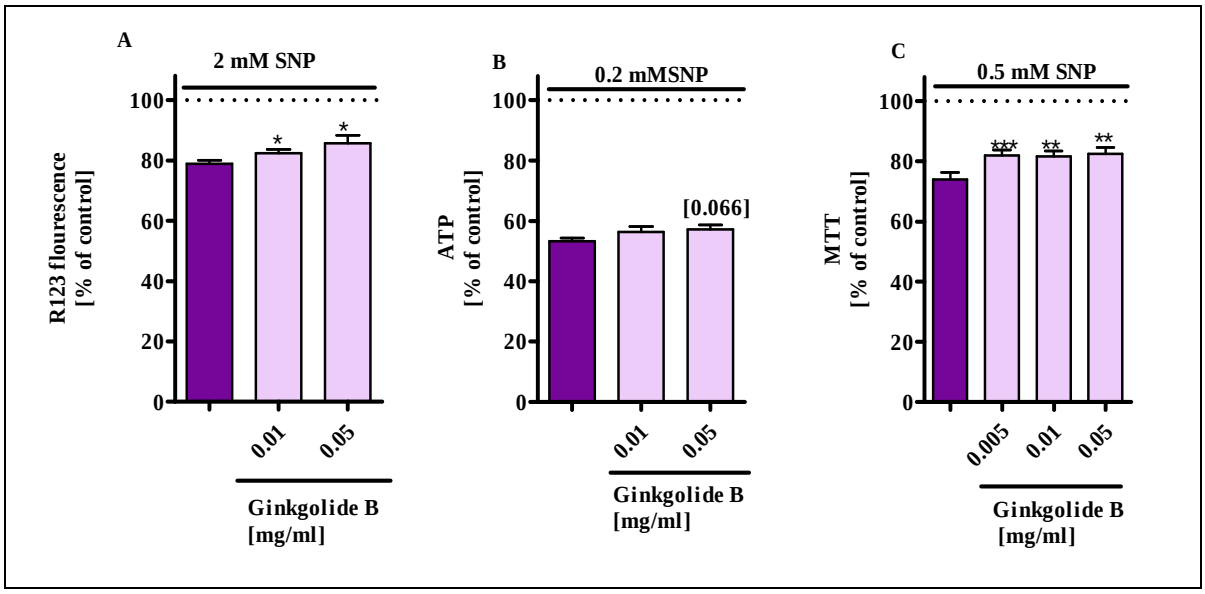

Figure 4-32

Protective effect of Ginkgolide B on mitochondrial functions after NO insult

Dissociated brain cells were stressed with SNP for 4 hours. 30 minutes after incubation with SNP Ginkgolide B was added. Mitochondrial membrane potential [A], ATP levels [B] and metabolic activity were determined [C]. Data are expressed as means ± S.E.M [n=6-8] [*p<0.05, **p<0.01 and ***p<0.001 vs. SNP, student's paired t-test].

Post-treatment with GB showed significantly higher cell viability at a concentration of 0.005 mg/ml [figure C]. 0.01 mg/ml was able to increase mitochondrial membrane potential [figure A], and only slight improvement in ATP levels was detected [figure B].

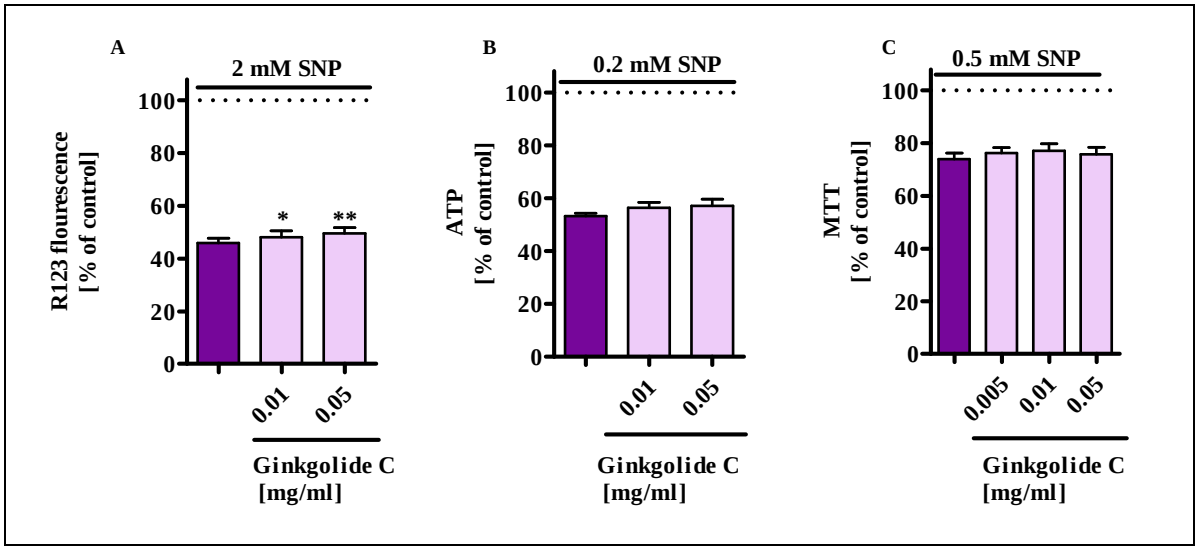

Figure 4-33

Protective effect of Ginkgolide C on mitochondrial functions after NO insult

Dissociated brain cells were stressed with SNP for 4 hours. 30 minutes after incubation with SNP Ginkgolide C was added. Mitochondrial membrane potential [A], ATP levels [B] and metabolic activity were determined [C]. Data are expressed as means ± S.E.M [n=6-12] [*p<0.05, **p<0.01 vs. SNP, student's paired t-test].

Ginkgolide C had a weak protective effect on the mitochondria. This effect was only perceived on mitochondrial membrane potential were a concentration of 0.01 mg/ml was able to alleviate the impairment caused by NO [figure 4-33].

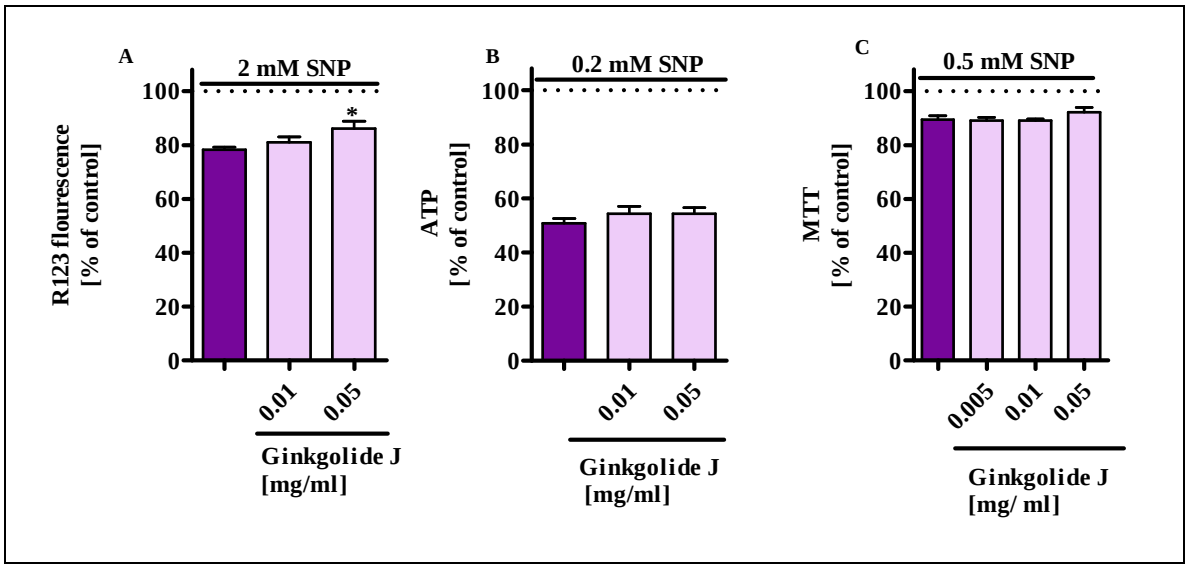

Figure 4-34

Protective effect of Ginkgolide J on mitochondrial functions after NO insult

Dissociated brain cells were stressed with SNP for 4 hours. 30 minutes after incubation with SNP Ginkgolide J was added. Mitochondrial membrane potential [A], ATP levels [B] and metabolic activity were determined [C]. Data are expressed as means ± S.E.M [n=6-8] [*$p<0.05$ vs. SNP, student's paired t-test].

Mitochondrial dysfunction caused by NO was not reduced by treatment with GJ. Slight improvement was observed using 0.05 mg/ml on mitochondrial membrane potential [figure 4-34A]. ATP and cell viability were not affected [figure 4-34B&C].

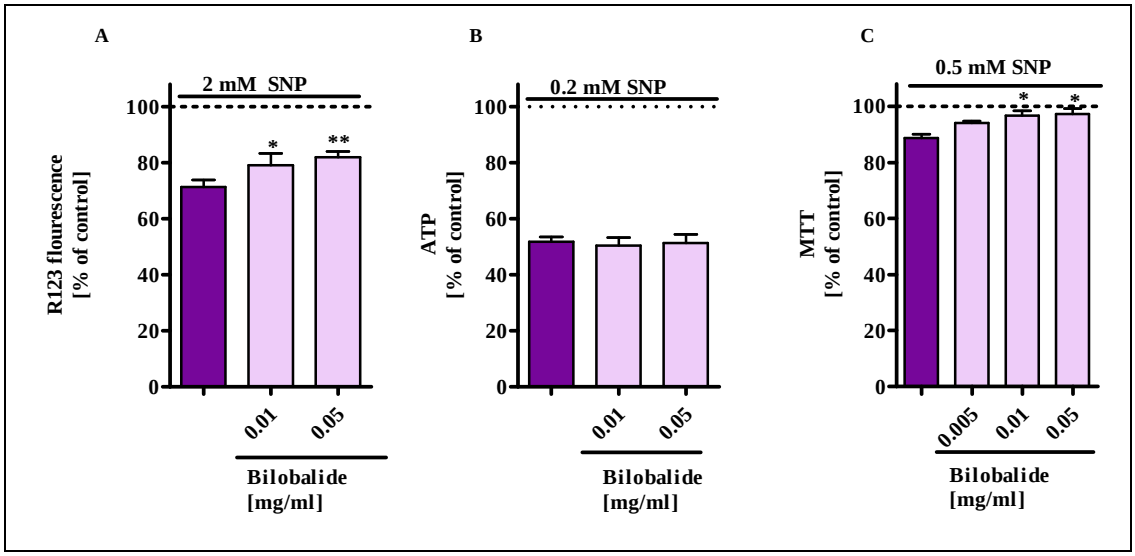

Figure 4-35

Protective effect of Bilobalide on mitochondrial functions after NO insult

Dissociated brain cells were stressed with SNP for 4 hours. 30 minutes after incubation with SNP Bilobalide was added. Mitochondrial membrane potential [A], ATP levels [B] and metabolic activity were determined [C]. Data are expressed as means ± S.E.M [n=6-7] [*$p<0.05$, **$p<0.01$ vs. SNP, student's paired t-test.

The sesquiterpenoid, Bilobalide was able to protect the mitochondria at a concentration of 0.01 mg/ml. Mitochondrial membrane potential was significantly elevated [figure 4-35A] and cell viability was increased to nearly 100 % [figure 4-35C]. Unfortunately no effect was seen on ATP levels.

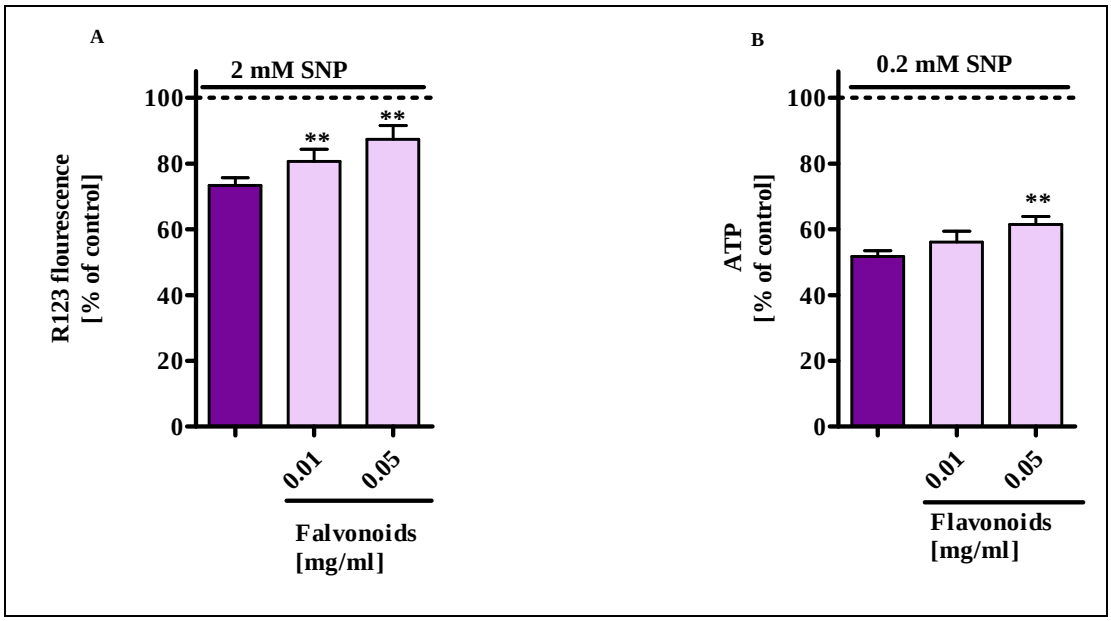

Figure 4-36

Protective effect of Flavonoids on mitochondrial functions after NO insult

Dissociated brain cells were stressed with SNP for 4 hours. 30 minutes after incubation with SNP a mixture of the Flavonoids extracted from EGb 761® was added. Mitochondrial membrane potential [A], ATP levels [B] were determined. Data are expressed as means ± S.E.M [n=6-7] [**p<0.01 vs. SNP, student's paired t-test].

The Flavonoid fraction of EGb 761® was also active in post-treatment as it was in pre-treatment. It was able to increase mitochondrial membrane potential significantly at a concentration of 0.01 mg/ml [figure 4-36A] and ATP levels were also significantly increased but at a higher concentration of 0.05 mg/ml [figure 4-36B].

Post-treatment		Concentration mg/ml		
		0.005	0.01	0.05
GA	MTT	***	**	*
	MMP	-	-	*
	ATP	-	-	*
GB	MTT	***	**	**
	MMP	-	*	*
	ATP	-	-	-
GC	MTT	-	-	-
	MMP	-	*	**
	ATP	-	-	-
GJ	MTT	-	-	-
	MMP	-	-	*
	ATP	-	-	-
Bilobalide	MTT	-	*	*
	MMP	-	*	**
	ATP	-	-	-
Flavonoids	MTT	Reagant interference		
	MMP	-	**	**
	ATP	-	-	**

Table 4.4

Summary of post-treatment with EGb 761® single constituents

Dissociated brain cells were incubated with SNP for 4 hours. The EGb 761® constituents were added 30 minutes after [post-treatment] incubation with SNP. Cell viability [MTT assay], mitochondrial membrane potential [MMP] and ATP levels were measured. [*p<0.05, **p<0.01, ***p<0.001 vs. SNP, student's paired t-test].

The above table summarizes the effects of post-treatment with EGb 761® components. All the EGb 761® constituents were are able to enhance one or more of the measured parameters. It is obvious that the ginkgolides are more effective in post-treatment than in pre-treatment.

4.5 Influence of long-term treatment with EGb 761® in a senescence accelerated mouse model

With the intention of investigating the influence of a long- term EGb 761® therapy, a 5 months feeding study was preformed. Since 5 months would only represent ¼ of the life span of mice, a special mouse model known as SAMP [senescence accelerated prone-mice] was utilized. The life span of SAMP8 mice ranges from 10 to a maximum of 17 months, and they are characterized by acquiring age-related learning and memory deficits and an increased deposition of Aß. This makes this model optimal for studying long-term effects of EGb 761® with only 5 months feeding, and representing AD characteristics. As controls SAMR1 [senescence accelerated resistant-mice] were employed, these have a normal life span ranging from 19 to 21 months, and undergo normal aging without memory impairments.

Mitochondrial functions of the SAMP8 and SAMR1 were assessed, and the influence of 5 months feeding with EGb 761® was evaluated. Given that on average basis one mouse consumes 3.5 g/day; the calculated daily intake of EGb 761® would be 150 mg/ kg body weight. Both SAMR1 and the placebo-treated mice received western diet devoid of EGb 761®.

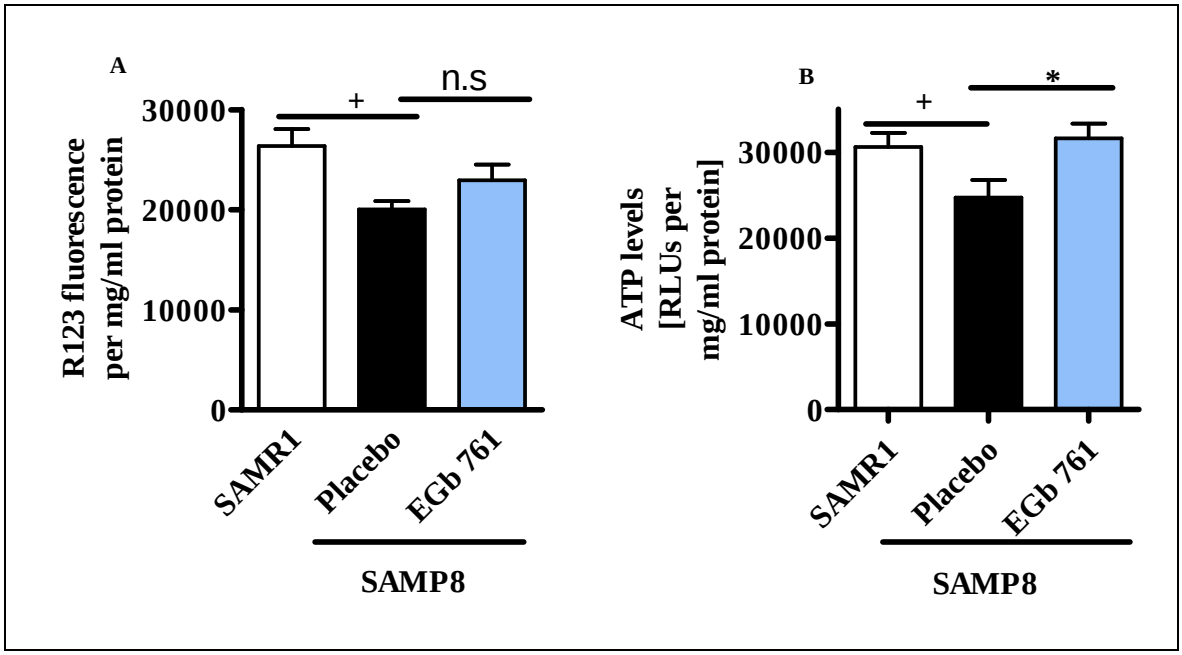

Figure 4-37

Mitochondrial functions of SAMR1 and SAMP8

The EGb 761® SAMP8 mice received 5 months food pellets supplemented with EGb 761® extract, while both the placebo SAMP8 and SAMR1 received western diet. Dissociated brain cells were prepared from SAMR1 and SAMP8 mice and mitochondrial membrane potential [A] and ATP levels [B] were determined. Data are expressed as mean ± S.E.M [n=4-6]. [+$p<0.05$ vs. SAMR1,*$p<0.05$ vs. placebo-treated, student's unpaired t-test].

The SAMP8 exhibited impaired mitochondrial functions as depicted above [figure 4-37]. They have significantly lower mitochondrial membrane potential and ATP levels compared to SAMR1 mice. EGb 761® enriched diet alleviated mitochondrial dysfunction by increasing ATP levels significantly and mitochondrial membrane potential slightly.

In view of the fact that membrane fluidity decreases during aging and since SAMP8 mice age rapidly, we thought it would be credible to compare the membrane fluidity of SAMR1 and SAMP8.

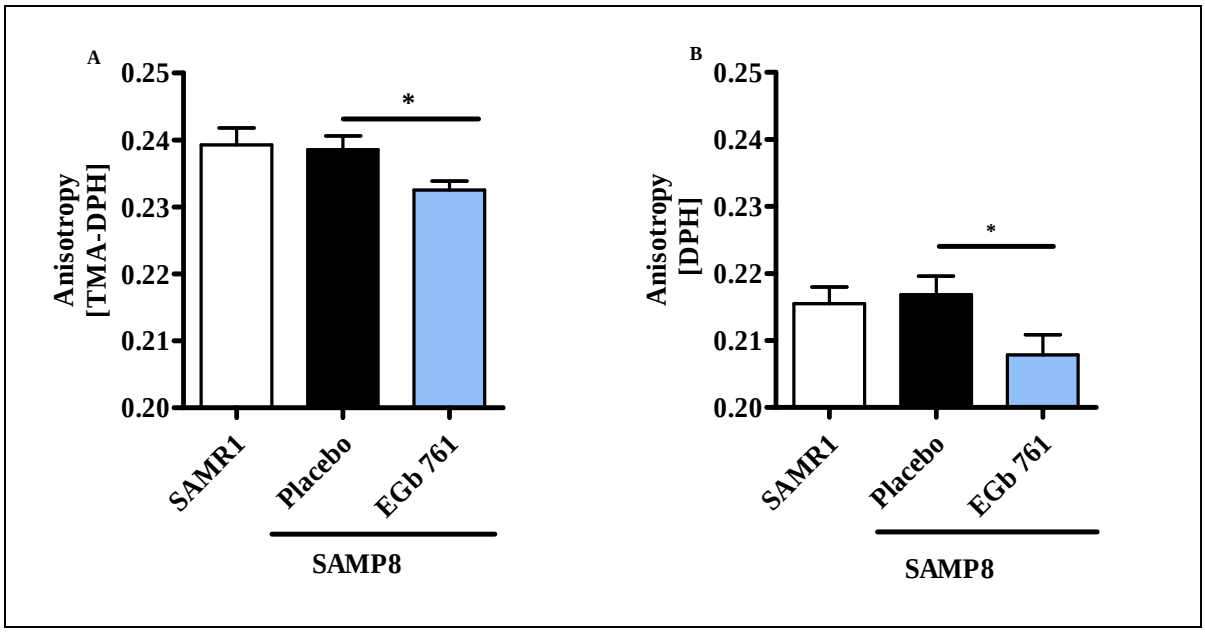

Figure 4-38

Influence of EGb 761® on the membrane fluidity in SAMP8 mice

The EGb 761® SAMP8 mice received 5 months food pellets supplemented with EGb 761® extract, while both the placebo SAMP8 and SAMR1 received western diet. Anisotropy was measured with the help of TMA-DPH [A] and DPH [B] fluorescent probes. Data are represented as mean ± S.E.M [n=6-8]. [*p<0.05 vs. placebo-treated, student's unpaired t-test].

There was no difference identified in the fluidity of the membranes obtained from SAMR1 and SAMP8 mice. However, EGb 761® was able to increase membrane fluidity in the SAMP8 mice. This observation was made by both fluorescent probes [DPH and TMA-DPH].

4.6 Effects of EGb 761® on amyloid beta production

Genetic, animal modeling and biochemical studies suggest that amyloid beta [Aβ] plays a central role in initiating Alzheimer's disease. Lately, Aβ has been reported to contribute to mitochondrial dysfunction in several models. Based on this data and the current findings that EGb 761® improves mitochondrial functions of impaired mitochondria, the influence of EGb 761® on Aβ was examined.

Aβ is derived from the amyloid precursor protein [APP] by the action of two aspartyl proteases [β- and γ-secretases]. APP is first cleaved by β-secretase shedding its large ectodomain and leaving a membrane bound C-terminal stub. This 99 amino acid long stub is subsequently cleaved by γ-secretase and Aβ is released. Depending on the cleavage by γ-secretase, two main forms of Aβ, comprising either 40 or 42 amino acid residues, are produced. The longer form [Aβ 42] is far more prone to oligomerize and form fibrils than is the more abundantly produced Aβ 40 peptide.

4.6.1 HEK cells with Swedish mutation

Preliminary experiments were carried out on a cell line model for AD. Hek-293 cells, which are human embryonic kidney cells carrying the Swedish double mutation were employed. This mutation leads to the production of large amounts of Aß, comparable to the transgenic mice model [Thy-1 APP] which is transfected with both the Swedish and the London mutations. For this purpose Hek-Swedish [Hek-sw] cells were selected as a suitable model for the preliminary experiments.

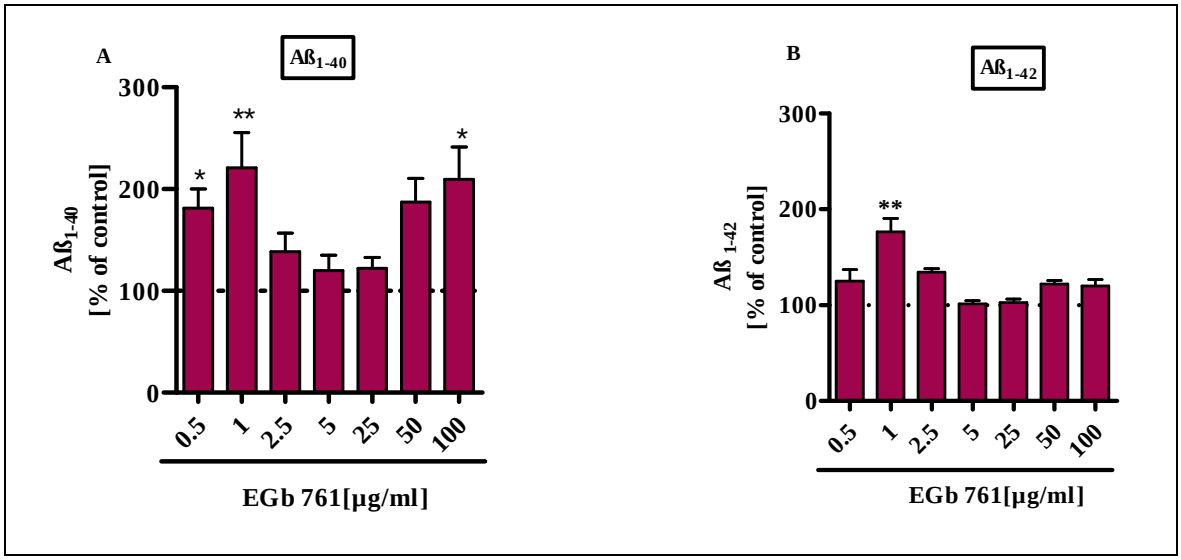

Figure 4-39

Effect of different concentrations of EGb 761® on amyloid beta Aß 1-40 production in HEK sw cells

HEK sw cells were incubated for 24 hours with concentrations ranging from 0.5µg/ml to 100 µg/ml EGb 761®. Medium was collected after the 24 hours and Aß $_{1-40}$ and Aß $_{1-42}$ levels were determined using an ELISA kit. Data are expressed as means ± S.E.M [n=3-6] [*p<0.05, **p<0.01 one way ANOVA].

For testing the influence of EGb 761® on Aß production, the Hek-sw cells were grown in petri dishes. The medium was exchanged and the new medium conferred to the cells contained different concentrations of EGb 761®. Cells were harvested and medium was collected 24 hours after incubation with EGb 761®. The amount of Aß was determined in the medium and an unexpected dramatic increase in the levels of soluble Aß 40 was observed, significant at a concentration as low as 0.5 µg/ml [figure 4-39A]. The Aß 42 values were slightly increased showing a maximum effect at 1µg/ml EGb 761®. A typical u-shape curve was noticed with both forms of Aß.

4.6.2 Thy-1 APP transgenic mice

In order to confirm the above observation with Hek-sw cells and for further investigations, a treatment study was carried out in a transgenic mice model [Thy-1 APP]. These mice are characterized by an altered APP processing leading to overproduction of Aß and plaque formation at an age of 6 months.

4.6.2.1 Soluble amyloid beta

Transgenic Thy-1 APP mice and non-transgenic littermates were treated for 21 days with EGb 761® [100 mg/kg body weight] or vehicle only. After 21 days the mice were sacrificed and brains were removed to determine Aß levels.

Comparing the Aß levels of tg-APP and non-tg mice, the soluble Aß 40 was as expected, produced only in the transgenic mice as shown in figure 4-40B.

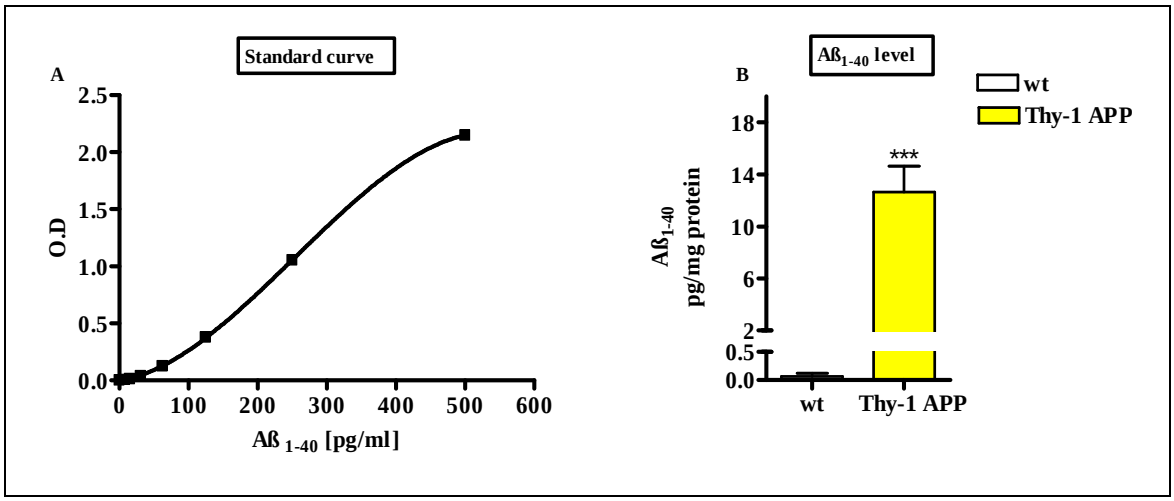

Figure 4-40

Amount of soluble amyloid beta [Aß1-40] in vehicle treated non-tg and tg-APP mice

Non-tg and tg-APP Mice were treated for 21 days with vehicle [0.2% agarose] for 21 days. ELISA was carried out on brain extracts to determine the amount of soluble Aß1-40. The levels of Aß were calculated from the standard curve [A], produced with the provided standard solution in the commercially available ELISA kit. Data are expressed as means ± S.E.M [n=8-9] [***p<0.001 vs. wild type, student's unpaired t-test].

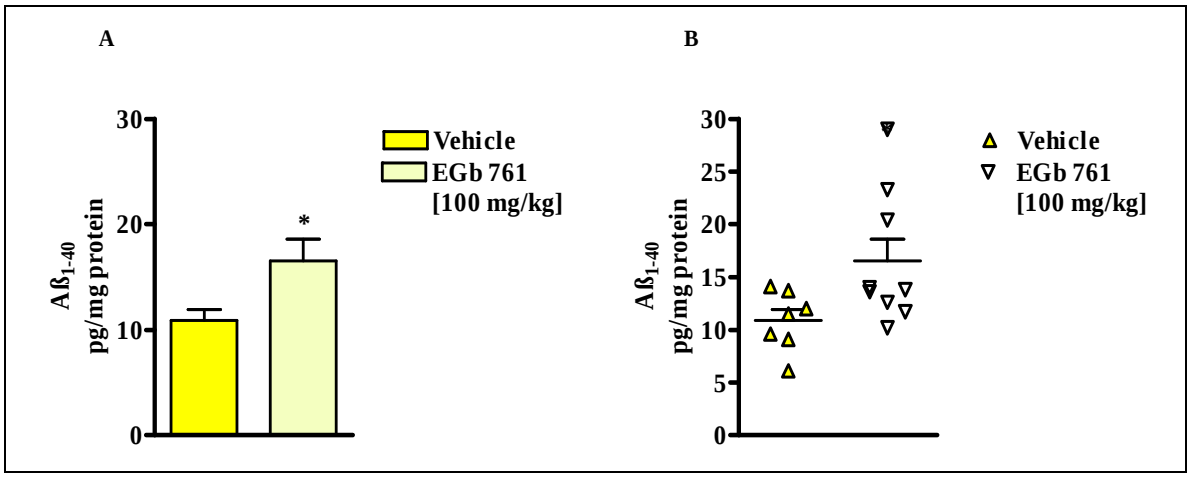

Figure 4-41

Effect of 21 days treatment with EGb 761® on the soluble Aß 1-40 levels in Tg-APP mice

After treating Tg-APP mice for 21 days with EGb 761® [100 mg/kg], soluble $Aß_{1-40}$ was determined in the brain extracts using ELISA. Data are expressed as means ± S.E.M [n=7-9] [*$p<0.05$ vs. vehicle, unpaired t-test].

Verifying our observation with Hek-sw cells, brain extracts from the EGb 761®- treated mice exhibited higher levels of soluble Aβ 40 compared to the placebo-treated group.

Note: The soluble levels of Aß 42 were below the detection level of the commercially available ELISA kit.

4.6.2.2 Total amyloid beta

Total Aß, soluble and insoluble was extracted using guanidine tris-buffer. The total amount of both Aß 40 and Aß 42 in the transgenic and wild type mice was determined and as shown in the figure below, transgenic mice showed masses of both Aß 40 and Aß 42.

Although both Aß 40 and Aß 42 levels were very high in the transgenic mice, the amount of Aß 42 was around 20 times more as Aß 40. The transgenic mice showed an approximate of 26,000 pg/mg protein Aß 40 [figure 4-42B] while the average amount of Aß 42 is 550,000 pg/mg protein [figure 4-42D].

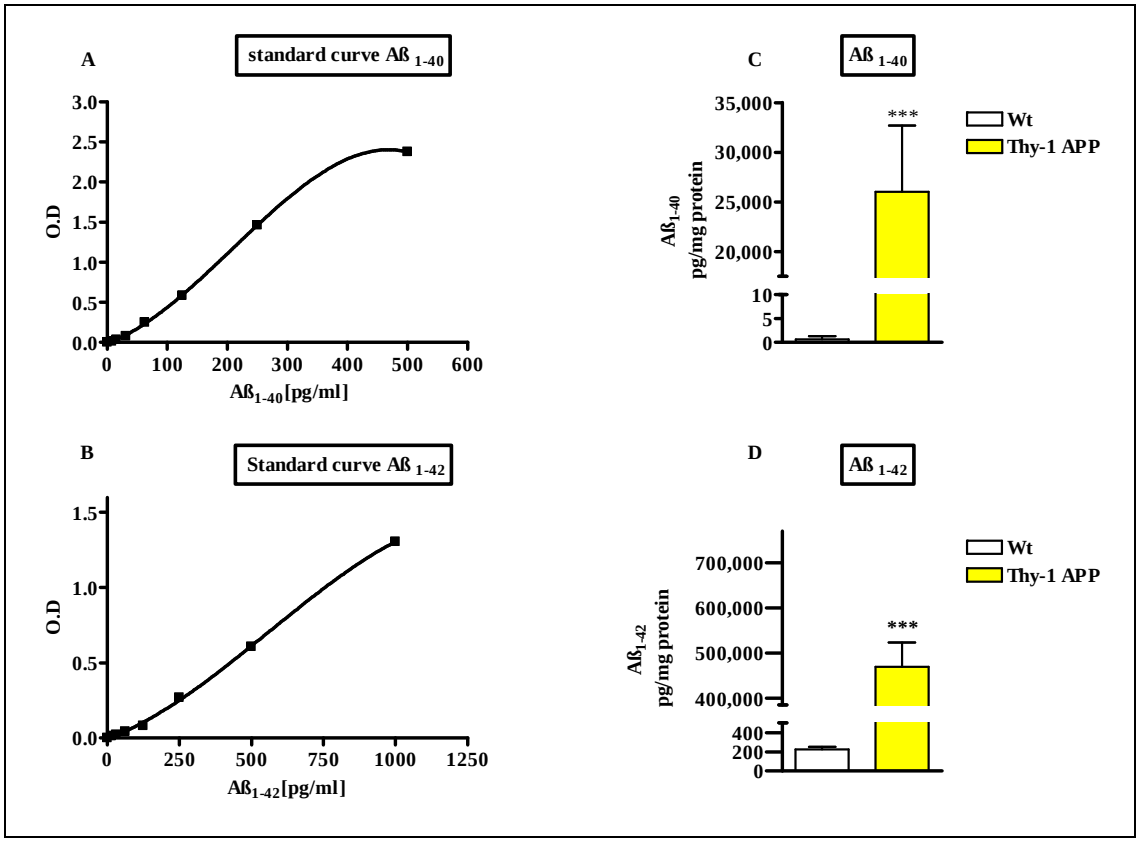

Figure 4-42

Total Amount of Aß 1-40 and Aß 1-42.in vehicle treated non-tg and tg-APP mice

Non-tg and tg-APP Mice were treated for 21 days with vehicle [0.2% agarose]. ELISA was carried out on the guanidine brain extracts and the total amount of Aß 1-40 [A & C] and Aß1-42 [B & D] were determined from their corresponding standard curves. Data are expressed as means ± S.E.M [n=8-9] [***p<0.001 vs. wild type, student's unpaired t-test].

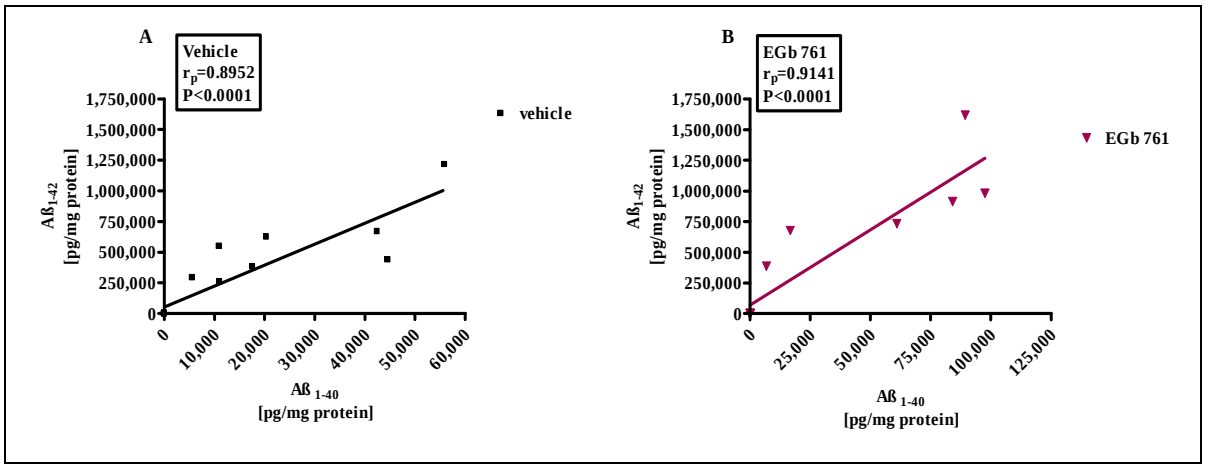

Figure 4-43

Correlation between total Aß 1-40 and Aß 1-42 in both placebo and EGb 761® treated mice

Both tg and non-tg mice were treated for 21 days either with Vehicle only or 100 mg/kg body weight EGb 761®.The amount of Aß 1-40 was plotted against the amount of Aß 1-42 for both placebo treated and EGb 761®-treated mice. Correlation r_p = 0.8952, P<0.0001 for the placebo treated group [A], and correlation r_p = 0.9141, P<0.0001 for the EGb 761® treated group [B].

Comparing the Aß 40 and Aß 42 levels, it was clear that they highly correlate linearly and positively with each other, meaning a mouse exhibiting high levels of Aß 40 has also high levels of Aß 42 [figure 4-43] and vice versa.

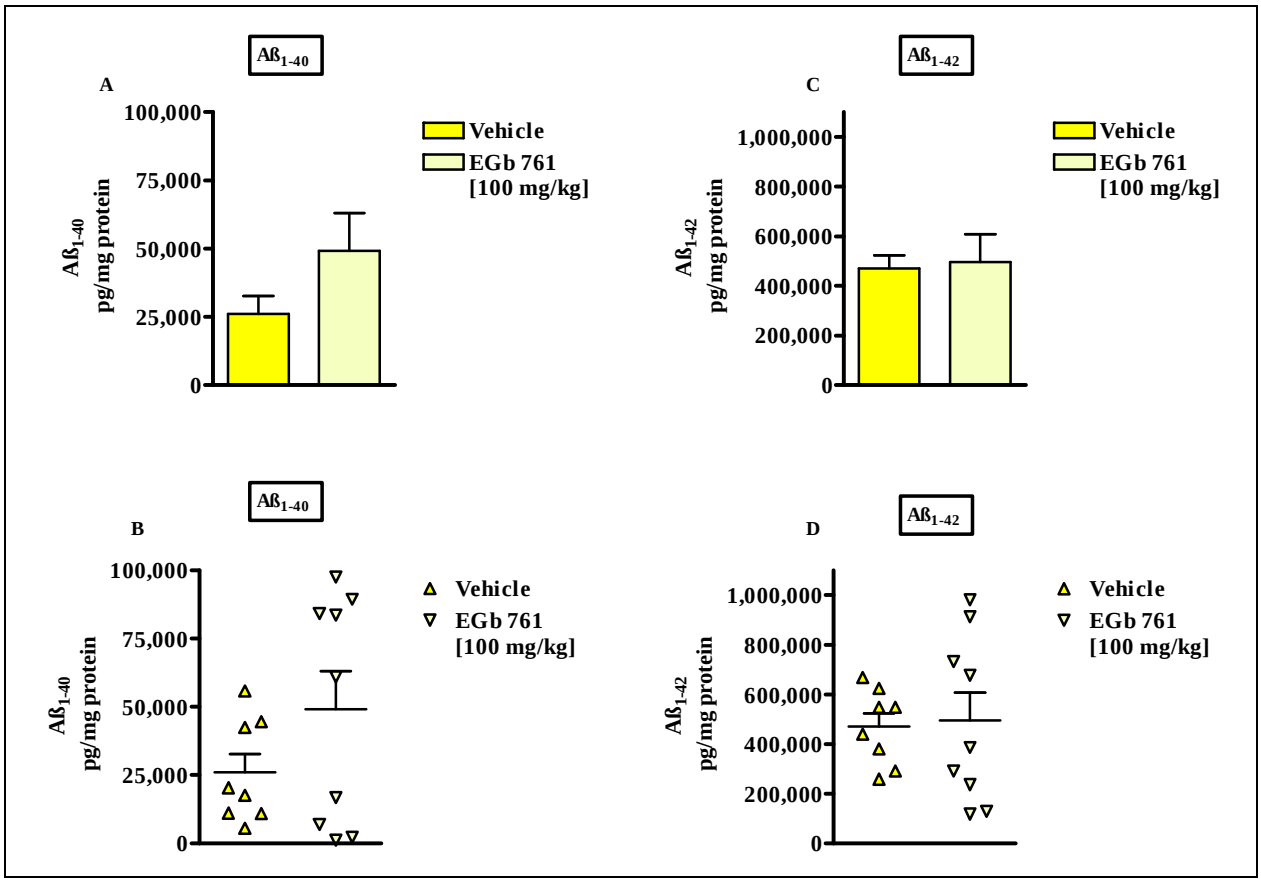

Figure 4-44

Effect of 21 days treatment with EGb 761® on the levels of Aß 1-40 and Aß 1-42 levels in Tg-APP mice

Tg-APP mice were treated for 21 days with EGb 761® [100 mg/kg]. ELISA was carried out on the guanidine brain extracts to determine the total amount of Aß 1-40 [A & B] and Aß 1-42[C & D] Data are expressed as means ± S.E.M [n=8-9].

Observing the amount of Aß 40 [figure 4-44B] it is noticeable that the levels vary greatly from one mouse to another as depicted in the scatter plots, making it difficult to draw a conclusion. The levels of Aß 42 remained more or less constant in both the EGb 761® treated group and the placebo group [figure 4-44D].

Even though the above results demonstrating the effects of EGb 761® on soluble Aß levels are quite perplexing, it has to be taken into account that Aß can be observed to self-assemble into fibrils, protofibrils, and oligomeric structures and not all of these Aß forms are neurotoxic. Recently, it has been highly suggested that an important neurotoxic form of Aß is an oligomer,

composed of approximately 12 subunits with a molecular weight of 54 kDa. Aß 42 is more prone to undergo self assembling into the ß-sheet structure, aggregating to form the neurotoxic oligomers.

4.7 Effects of EGb 761® on gene expression: RT-PCR

To find out the mechanism behind the effect of EGb 761® on Aß production in the EGb 761® treated mice, we quantified its effect on the expression of the genes which can play a role in Aß production.

Since the mice used in the treatment study were transgenic mice carrying a mutant human amyloid precursor protein [APP] gene which leads to over production of Aß, the influence of EGb 761® on the human APP gene expression after 21 days of treatment was determined.

As illustrated in the figure below the mutant human APP gene was down regulated after EGb 761® treatment.

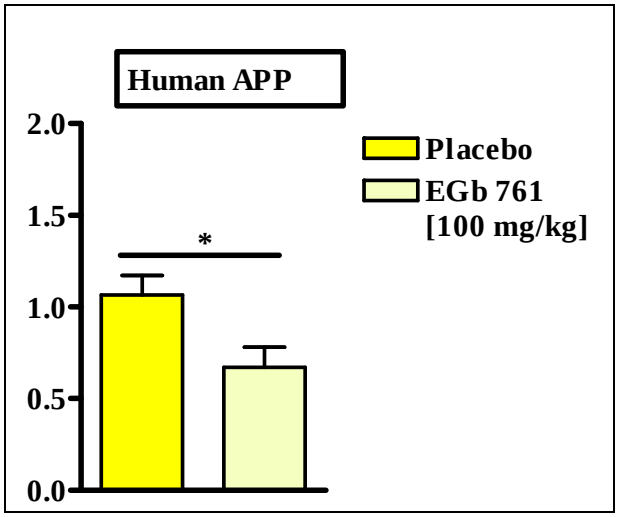

Figure 4-45

Effect of treatment with EGb 761® on human APP gene expression in tg-APP mice

Tg-APP mice were treated for 21 days with EGb 761® [100 mg/kg] or vehicle only. Relative mRNA levels of human APP gene were quantified as the ratio between the amount of target gene and the amount of the housekeeping gene beta-actin. Data are expressed as means ± S.E.M [n=8-9] [*p<0.05, student's unpaired t-test].

The levels of mRNA of murine APP and APP binding protein [APPbp1] were analyzed. Unpredictably, the Thy-1 APP transgenic mice showed an elevated amount of murine APP compared to the wild type mice.

EGb 761® treatment was able to decrease the levels of murine APP significantly [figure 4-46A], suggesting that EGb 761® is able to down-regulate not only the mutant human APP in the transgenic mice but also the unexpectedly elevated murine APP.

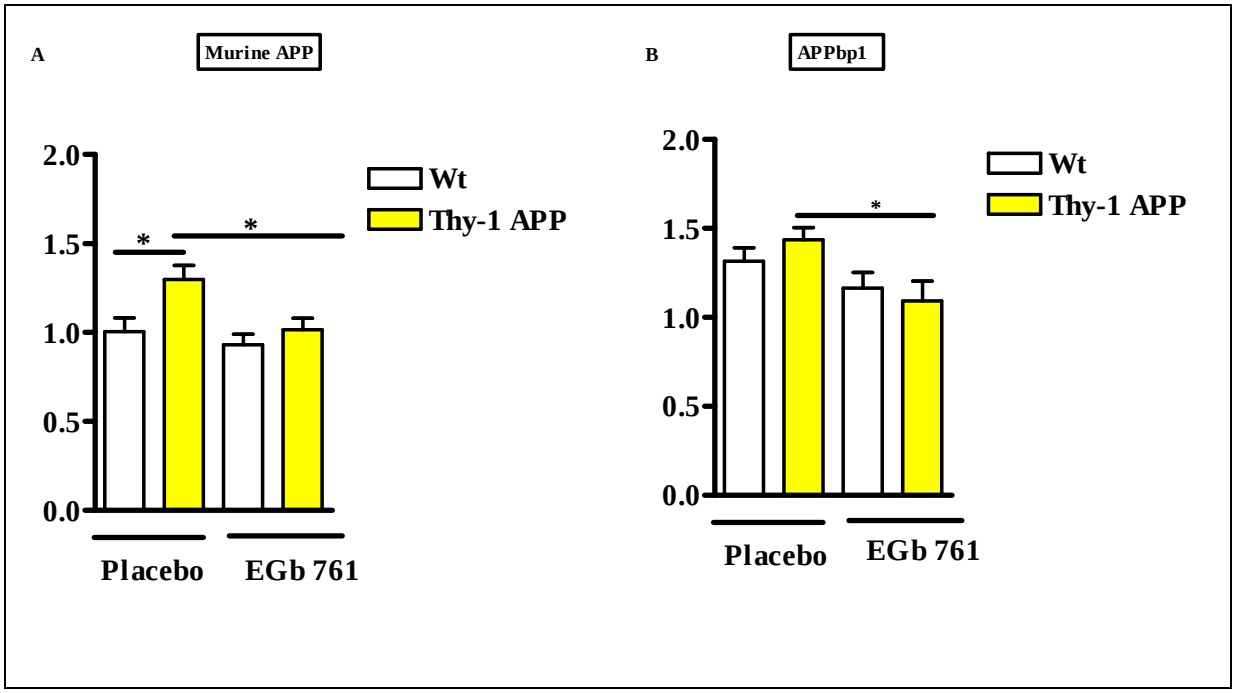

Figure 4-46

Effect of treatment with EGb 761® on murine APP and APPbp1 gene expression in non-tg and tg-APP mice

Non-tg and tg-APP mice were treated for 21 days with EGb 761® [100 mg/kg] or vehicle only. Relative mRNA levels of both APP [A] and APPbp1 [B] genes were quantified as the ratio between the amount of target gene and the amount of the housekeeping gene beta-actin. Data are expressed as means ± S.E.M [n=8-10] [*$p<0.05$, student's unpaired t-test].

The three major enzymes which are responsible for the cleavage of APP are the α-, ß- and γ- secretases. Cleavage of APP through α-secretase followed by γ- secretase is a non-amyloidogenic pathway and doesn't lead to Aß formation. On the contrary, cleavage through ß-secretase followed by γ-secretase leads to the amyloidogenic pathway forming both Aß 40 and Aß 42.

Consequently, the genetic expression of both α-secretase and ß-secretase in the placebo- and EGb 761®-treated mice was compared.

Although the exact identity of α-secretase is unclear, the most highly suggested candidate is the metalloprotease ADAM 10. Therefore, the

expression of ADAM-10 was quantified. As shown in figure 4-47A, comparing the placebo-treated and EGb 761®-treated groups there was no difference in the mRNA levels of ADAM-10.

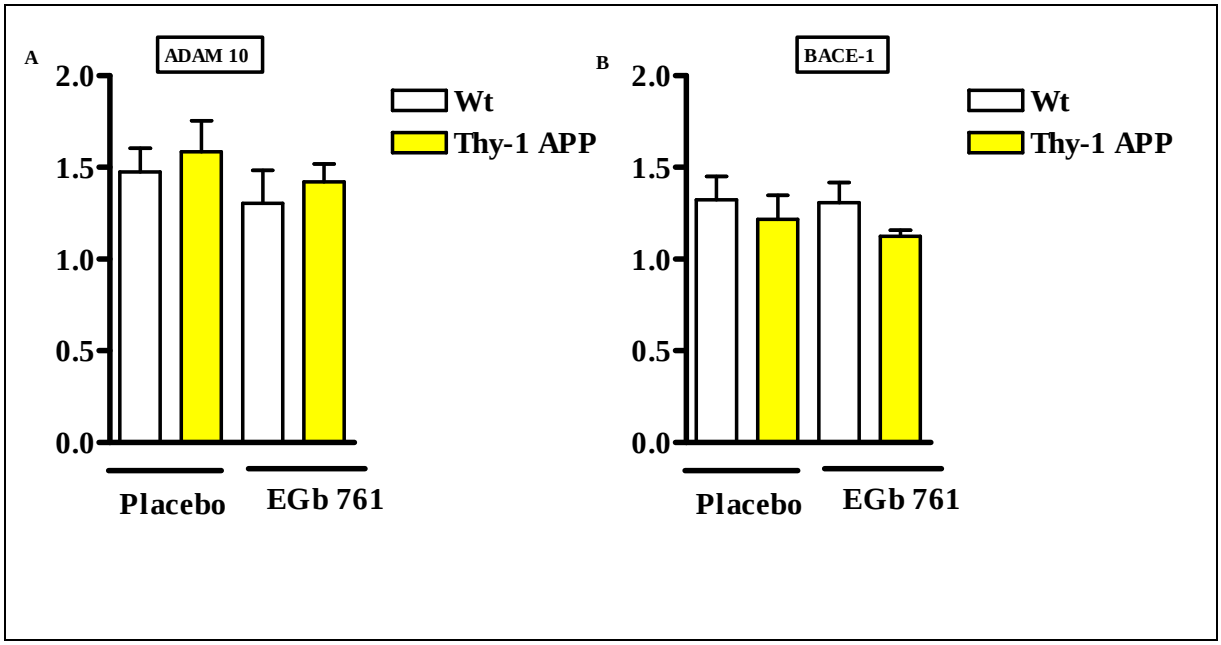

Figure 4-47

Effect of treatment with EGb 761® on ADAM-10 and BACE-1 gene expression in non-tg and tg-APP mice

Non-tg and tg-APP mice were treated for 21 days with EGb 761® [100 mg/kg] or vehicle only. Relative mRNA levels of both ADAM-10[A] and BACE-1[B] genes were quantified as the ratio between the amount of target gene and the amount of the housekeeping gene beta-actin. Data are expressed as mean ± S.E.M [n=8-9].

The membrane-bound aspartyl protease BACE-1[ß-site cleaving enzyme] encoded on chromosome 11 is recognized as ß-secretase. As represented above [figure 4-47B] there was no difference on the BACE-1 gene expression between the non-transgenic and the Thy-1 APP transgenic mice. Treatment with EGb 761® didn't alter the mRNA levels of BACE-1.

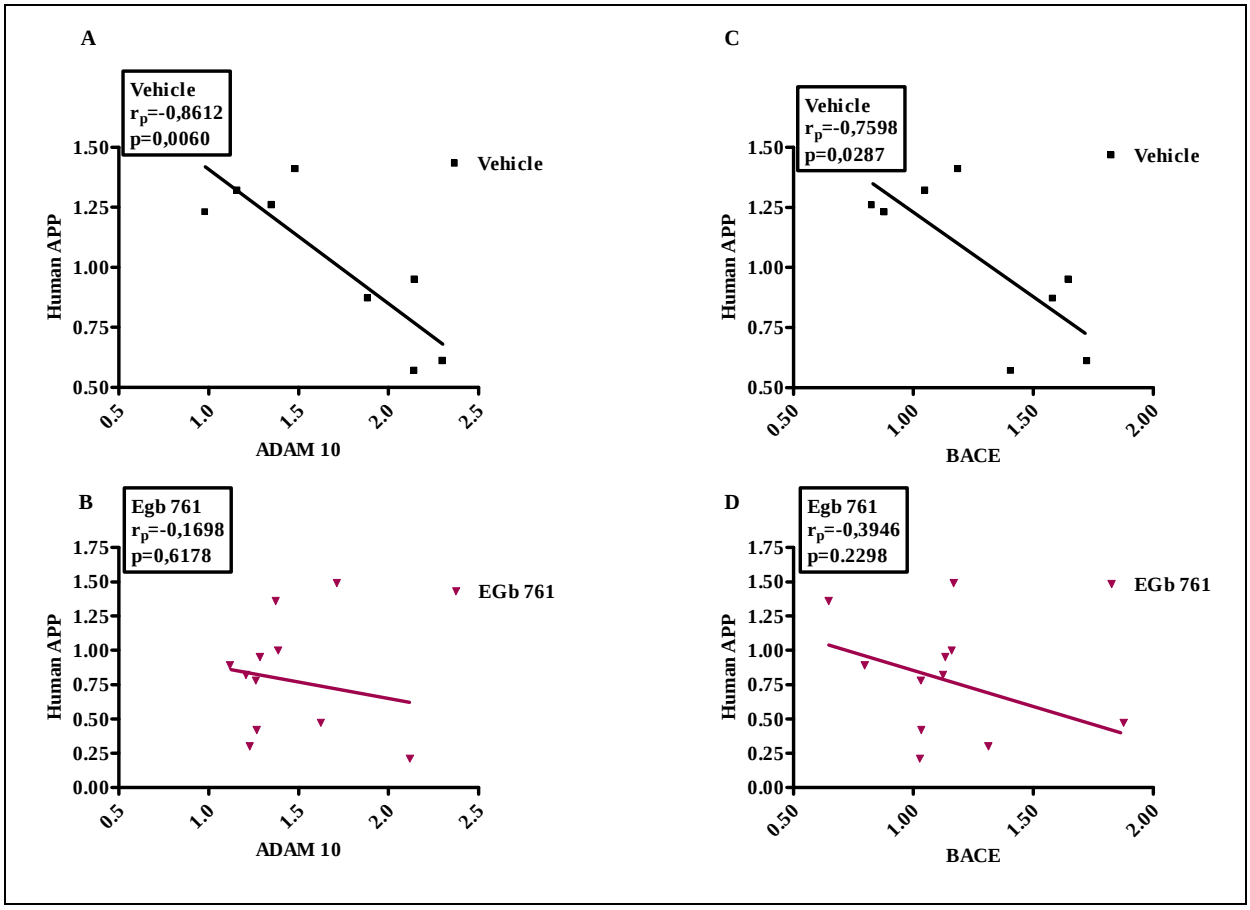

Figure 4-48

Correlation of Human APP expression with BACE and ADAM expression in transgenic mice

Transgenic mice were treated for 21 days either with Vehicle only or 100 mg/kg body weight EGb 761®. The Hu APP gene expression was plotted against ADAM expression for both placebo treated [A] and EGb 761®-treated[B] mice. Correlation r_p = -0.8612, P=0.006 for the placebo treated group, and correlation r_p =- 0.1698, P=0.6178 for the EGb 761® treated group. The expression of Hu APP was plotted against BACE gene expression for both placebo treated [C] and EGb 761®-treated [D] mice. Correlation r_p = - 0.7598, P= 0.0287 for the placebo treated group, and correlation r_p = -0.3946, P=0.2298 for the EGb 761® treated group.

The mRNA levels of HuAPP were correlated with BACE and ADAM as illustrated in figure 4-48. In the Vehicle treated group the higher the Hu APP expression the lower were the values of BACE and ADAM [figure 4-48A&C], suggesting less processing of APP. Treating the mice with EGb 761® decreased the expression of the mutant APP without altering the expression of its cleaving enzymes BACE and ADAM [figure 4-48B&D]. Accordingly, the cleavage of APP remained constant while the mutant Hu APP was down-regulated by using EGb 761®.

In view of the fact that the mRNAs of the enzymes which are responsible for the production of Aß were not altered, the Aß degrading enzymes were then taken into consideration.

Many proteases or peptidases have been reported with the capability of cleaving Aβ either *in vitro* or *in vivo*. The most important enzymes contributing to Aß degradation are Neprilysin [NEP], Insulin-degrading enzyme [IDE] and Endothelin-converting enzyme [ECE]. Two isoforms of ECE exist, ECE-1 and ECE-2. Expression of ECE-2 is only 1–2 % as much as the more abundant ECE-1 and studies have suggested that ECE-1, but not ECE-2, is a possible brain Aβ-degrading enzyme (Eckman *et al.* 2001).

Based on this knowledge the levels of mRNA of the aforementioned Aß-degrading enzymes, namely, NEP, IDE and ECE-1 were examined.

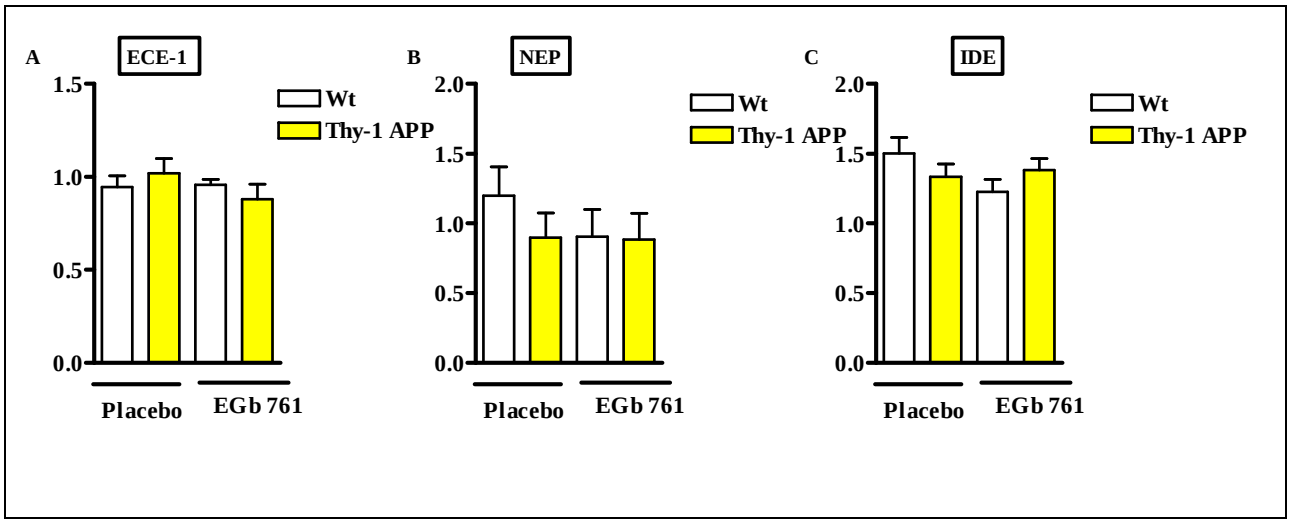

Figure 4-49

Effect of treatment with EGb 761® on ECE-1, NEP and IDE mRNA levels in non-tg and tg-APP mice

Non-tg and tg-APP mice were treated for 21 days with EGb 761® [100 mg/kg] or vehicle only. Relative mRNA levels of ECE-1[A], NEP [B] and IDE [C] genes were quantified as the ratio between the amount of target gene and the amount of the housekeeping gene beta-actin. Data are expressed as means ± S.E.M [n=8-9].

Observing the above graphs, one notices that NEP [figure 4-49A] and IDE [figure 4-49C] are slightly down regulated but not significantly in the transgenic mice compared to the non-transgenic mice; ECE-1 did not show the same trend.

EGb 761® treatment showed no significant effect on the expression levels of the 3 enzymes in both transgenic and non-transgenic mice [figure 4-49]. The levels of Aß were correlated with the expression of the Aß degrading enzymes in order to see if a relation does actually exist, and whether EGb 761® can influence this relation.

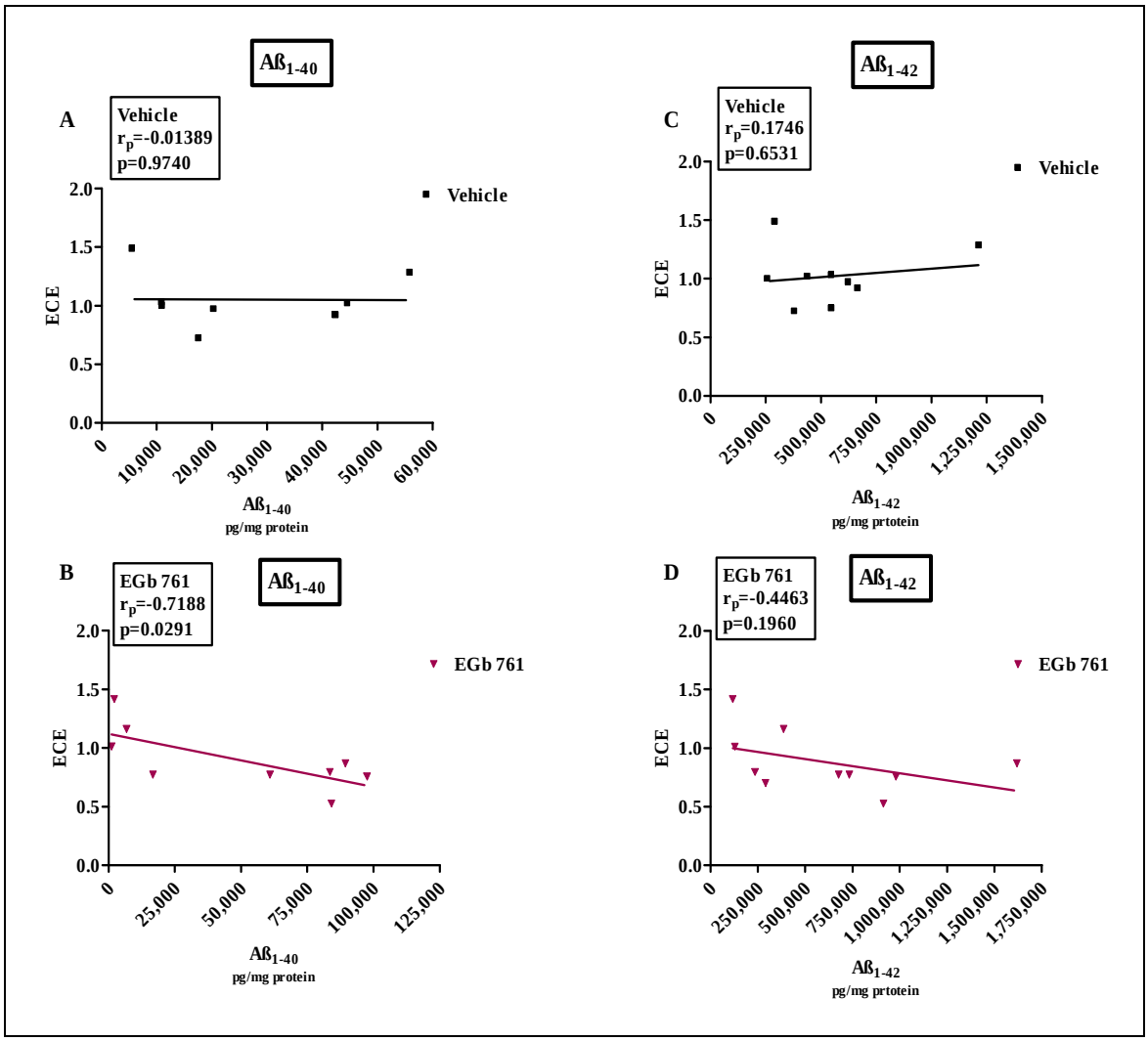

Figure 4-50

Correlation between ECE expression and Aß 1-40 or Aß 1-42 in transgenic mice

Transgenic mice were treated for 21 days either with Vehicle only or 100 mg/kg body weight EGb 761®.The amount of Aß 1-40 was plotted against ECE gene expression for both placebo treated[A] and EGb 761®-treated [B] mice . Correlation r_p = - 0.01389, P=0.9740 for the placebo treated group, and correlation r_p = -0.7188, P=0.0291 for the EGb 761® treated group. The amount of Aß 1-42 was plotted against ECE gene expression for both placebo treated[C] and EGb 761®-treated [D] mice. Correlation r_p = 0.1746, P=0.6531 for the placebo treated group, and correlation r_p =-0.4463, P=0.1960 for the EGb 761® treated group.

Although EGb 761® didn't alter the mRNA levels of ECE-1, it affected its correlation to Aß. The correlation between the ECE-1 expression and Aß 40 levels is a negative correlation, signifying that after EGb 761® treatment, mice with a high expression of ECE-1 exhibited high levels of Aß 40 [figure 4-50B]. There was no significant correlation seen between the levels of Aß 42 and ECE-1 expression [figure 4-50C&D].

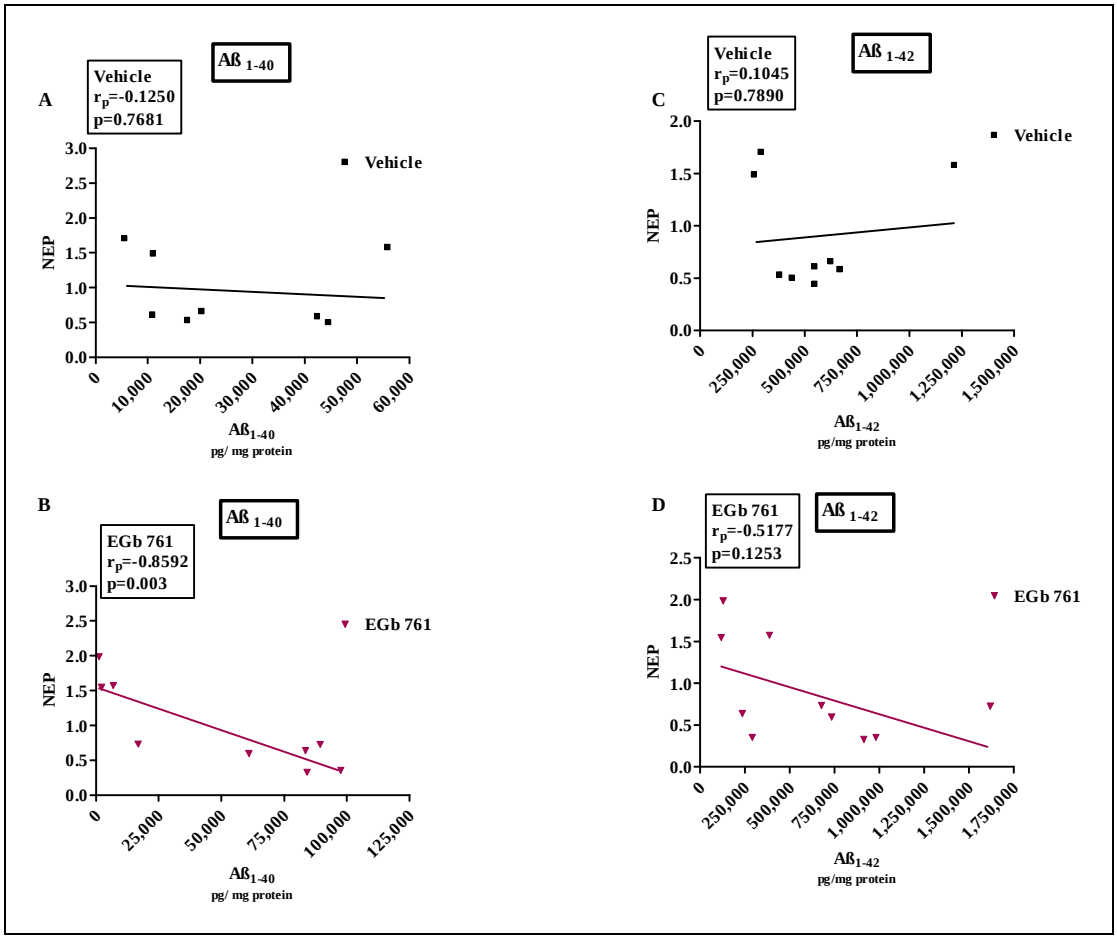

Figure 4-51

Correlation between NEP expression and Aß 1-40 or Aß 1-42 in transgenic mice

Transgenic mice were treated for 21 days either with Vehicle only or 100 mg/kg body weight EGb 761®.The amount of Aß $_{1-40}$ was plotted against NEP gene expression for both placebo treated[A] and EGb 761®treated mice [B]. Correlation r_p = - 0.7681, P=0.1250 for the placebo treated group, and correlation r_p = -0.8592, P=0.003 for the EGb 761® treated group. The amount of Aß $_{1-42}$ was plotted against NEP gene expression for both placebo treated [C] and EGb 761®-treated mice [D]. Correlation r_p = 0.1045, P=0.789 for the placebo treated group, and correlation r_p =- 0.5177, P=0.1253 for the EGb 761® treated group.

Interestingly, NEP and Aß 40 were also inversely proportional in the EGb 761® treated group, even more pronounced than ECE-1 [figure 4-51B]. The same trend was seen with Aß 42 but to a lower extent and was not significant [figure 4-51D].

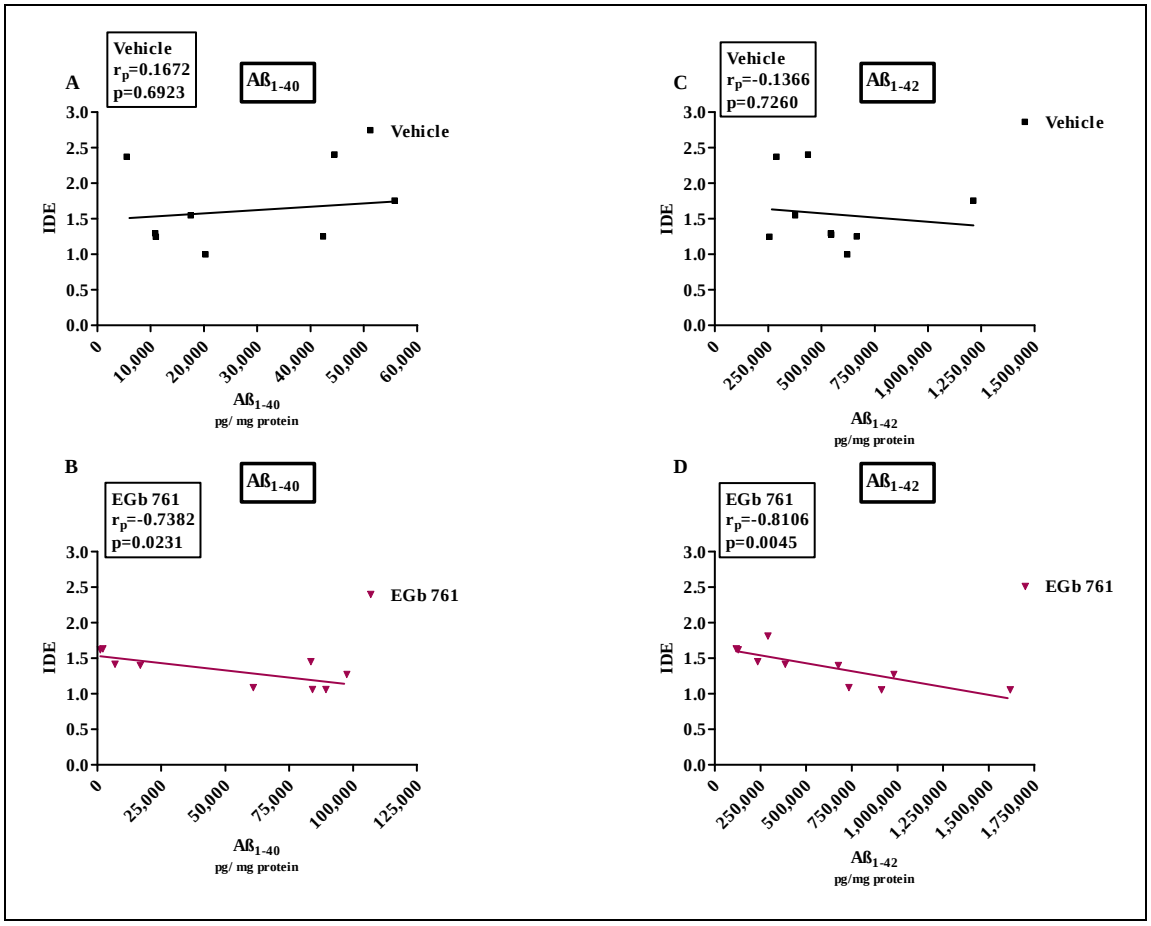

Figure 4-52

Correlation between IDE expression and Aß 1-40 or Aß 1-42 in transgenic mice

Transgenic mice were treated for 21 days either with Vehicle or 100 mg/kg body weight EGb 761®.The amount of Aß $_{1-40}$ was plotted against IDE gene expression for both placebo treated [A] and EGb 761®-treated mice[B] . Correlation r_p = 0.1672, P=0.6923 for the placebo treated group, and correlation r_p = -0.7382, P=0.0231 for the EGb 761® treated group. The amount of Aß $_{1-42}$ was plotted against IDE gene expression for both placebo treated [C] and EGb 761®-treated [D] mice. Correlation r_p = 0.1366, P=0.726 for the placebo treated group, and correlation r_p =- 0.8106, P=0.0045 for the EGb 761® treated group

Confirming the previous results the negative correlation seen between the Aß levels and the Aß degrading enzymes was also observed with IDE. However with IDE both Aß 40 and Aß 42 demonstrated a significant negative correlation.

5 Discussion

5.1 Mitochondrial protective properties of EGb 761®

Malfunction of the power house of the cell, that is to say the mitochondria, has been highly implicated to AD [see section 1.1.8]. This has been noticed in cell culture, transgenic models and most importantly AD brains. The role of EGb 761® as an anti-oxidant has been previously examined due to its high content of flavonoids, and definitely EGb 761® has proven its scavenging activities in *in vitro* acellular and cellular studies, as well as *in vivo* studies. However, the fact that other herbal extracts rich in flavonoids do not exert the same actions as Ginkgo, only confirms that other constituents present in the Ginkgo extract contribute profoundly to its actions. This implicates that the pharmacological effects are not only limited to the well known anti-oxidant property of EGb 761®. Since ROS is highly related to the mitochondria and mainly originates from it, some researchers directed their studies towards examining the effects of EGb 761® on the mitochondria. This was also encouraged as the link between not only AD but also Aß and the mitochondria started to emerge. Previously, our group observed mitochondrial protective actions on cell culture *in vitro* (Eckert *et al.* 2005). Therefore, these *in vitro* findings were extended on animal models *in vitro* and *ex vivo*, as well as in transgenic animal models for AD. Basic groundwork experiments were first carried out in order to find the most suitable concentrations, incubation times and schemes.

5.1.1 Protection against oxidative stress

H_2O_2 was applied here as an external source of oxidative stress, and for induction of mitochondrial dysfunction. H_2O_2 is able to inactivate, aconitase enzymes, succinate dehydrogenase [SDH] and alpha-ketoglutarate dehydrogenase [KGDH] of the tricarboxylic acid cycle (Nulton-Persson and Szweda 2001). Subsequently, this leads to reduction in the NADH level. The reduced NADH is insufficient to ensure an optimal rate of respiration and may consequently lead to inhibition of complex I. Under these circumstances the ATP levels are greatly reduced, which may be insufficient for F_0F_1 ATPase to work as a proton translocator, and thereby prevents the maintenance of mitochondrial membrane potential (Chinopoulos *et al.* 1999).

In addition, H_2O_2 is rapidly converted into the toxic hydroxyl radical, which represents the main ROS product responsible for lipid peroxidation. This leads to damage to some lipids such as cardiolipin. Cardiolipin is almost exclusively found in mitochondrial membranes where it serves specific roles in mitochondrial structure and function. Cardiolipins are associated with cytochrome c oxidase and other mitochondrial electron transport complexes and transporters that are required for full electron transport activity (Robinson 1993). Therefore, ROS induced lipid peroxidation and protein oxidation causes collapse of the respiratory chain, decreasing both the mitochondrial membrane potential and ATP levels [see diagram 5-1].

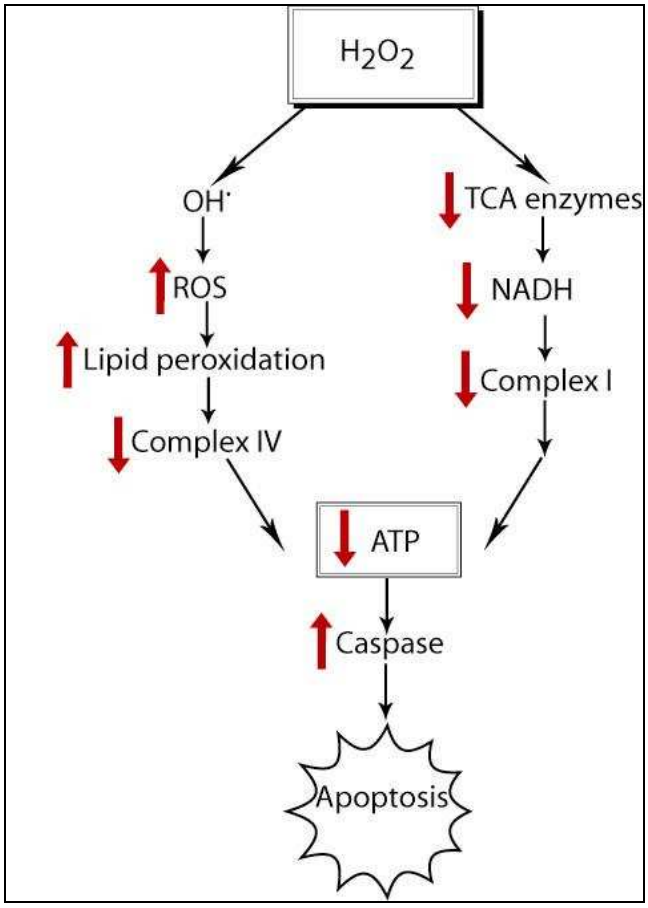

Figure 5-1

Scheme showing mitochondrial damage by H_2O_2

Incubating our DBCs with H_2O_2 led to a decrease of mitochondrial membrane potential and drop in ATP levels as expected. *in vitro* addition of EGb 761® after H_2O_2 insult, led to enhancement of mitochondrial membrane potential in both young and old mice with an enhancement of ATP levels only in the older mice. The *in vitro* radical scavenging activity and mitochondrial protective properties of EGb 761® was previously reported by several other groups. Formerly, PC12 cells were protected against H_2O_2-induced mitochondrial impairment by *in vitro* addition of EGb 761® (Eckert *et al.* 2003).

Moreover, treating the 2 age groups for 14 consecutive days with EGb 761® improved the mitochondrial membrane potential of the older mice only. Since decrease in mitochondrial membrane potential by H_2O_2 is secondary to reduction in ATP levels as explained above, it is not surprising that EGb 761®

protection is more pronounced on mitochondrial membrane potential than ATP levels.

In order to be able to explain the age-related sensitivity of EGb 761®, which was observed both *in vitro* and *ex vivo*, the differences between the mitochondrial characteristics of the 2 age groups should be discussed. Providentially, these were previously studied. DBCs prepared from 16 months old NMRI mice showed higher levels of lipid peroxidation than 2-3 months old mice. However, unexpectedly the 16 months old mice were less sensitive to *in vitro* H_2O_2 induced lipid peroxidation (Leutner *et al.* 2001). These results suggest that the brains of adult mice have an elevated capacity to sequester exogenous stimuli. This could be explained by the observation that several anti-oxidant enzymes in the older mice group show higher activities. Leutner *et al* reported that the activity of Cu/SOD was elevated in the 16 months old mice, GR slightly but not significantly and no difference was noticed in GPx levels.

Another disparity is the finding that both DBCs and isolated mitochondria prepared from NMRI mice exhibited lower mitochondrial membrane potential during aging, which became significant at an age of 24 months (Hauptmann 2008). Moreover a decline in other mitochondrial functions such as complex activity and respiratory control ratio was also described during aging of NMRI mice (Leuner *et al.* 2007).

One may argue that the difference in the mitochondrial properties of the 2 age groups, for example the elevated anti-oxidant enzyme activity in the older mice, is/are responsible for the observed age-related sensitivity. However, in the placebo-treated mice, the mitochondria of both young and old mice groups were equally damaged by H_2O_2. This observation calls off the assumption that the DBCs prepared from the older mice group are less sensitive to mitochondrial damage, and proves that EGb 761® is more effective in older mice.

In accordance to our observation, age related mitochondrial protection by EGb 761® has been previously reported. This goes back to 1995, when Holgado *et al* observed a decrease in the rates of O_2^- in both 4 and 33 months old rats after 3 months treatment. However, the scavenging activities of SOD, catalase and glutathione peroxidase were increased only in the 33 months old rats (Defeudis 1998). In addition, EGb 761® was able to protect isolated brain mitochondria from the influence of complexes I, IV and V inhibitors in aged mice with no effect in younger mice (Hauptmann 2008).

Additionally, lymphocytes from 24-month-old NMRI mice revealed a significantly higher protection by EGb 761® than from young mice. ROS-induced apoptosis triggered by d-ribose in isolated T-lymphocytes was significantly reduced after 14 days treatment (Schindowski *et al.* 2001).

Aging also affected mitochondrial size and structural complexity, this is partly responsible for mitochondrial dysfunction and mitochondrial membrane potential impairment. Sastre *et al*. were able to show that treating rats with EGb 761® prevented these age-associated impairments in mitochondrial morphology, providing a possible explanation for the observed enhancement in mitochondrial membrane potential in our aging mice (Sastre *et al.* 1998). They were also able to show that EGb 761® protects against the oxidative damage to mtDNA, the oxidation of mitochondrial glutathione, and the age-related increase in peroxide generation by mitochondria.

From these results it seems that EGb 761® efficacy is more pronounced on mitochondria that are impaired. Since many evidences link both morphological and functional mitochondrial alterations to AD, the next step was to examine the effect of EGb 761® on mitochondria of an AD model.

Thy-1 APP mice were utilized as a transgenic mouse model. These mice are double mutant carrying both the Swedish double mutation and the London mutation. Previous studies demonstrated the elevated Aβ levels in this mouse

model, and Aβ plaques were detected at an age of 6 months (Blanchard *et al.* 2003).

Interestingly, the Thy-1 APP transgenic mice were reported to exhibit mitochondrial dysfunction in previous studies. This makes them a more suitable AD model for testing mitochondrial impairment. Earlier results comparing mitochondrial properties of Thy-1 APP and wild type mice reported a decrease in mitochondrial membrane potential and ATP levels at an age of 6 months (Hauptmann *et al.* 2008). In addition, Thy-1 APP mice are characterized by exhibiting increased 4-hydroxy-2-nonenal [HNE] levels, reduced Cu/Zn-SOD activity (Schuessel *et al.* 2005) and decrease in COX activity at an age of 3 months.

Treating these mice at an age of 6 months with EGb 761® for 2 weeks didn't alter the basal levels of mitochondrial membrane potential significantly [Data not shown]. However the mitochondrial membrane potential of the EGb 761® treated mice in both wild type and transgenic mice were less susceptible than the placebo-treated group to H_2O_2-induced depolarization. A slightly higher protection was noticed in the transgenic mice group. These findings fit in our aforementioned observation that EGb 761® seems to be more effective in impaired or aged mitochondria as well as mitochondria of AD models. In agreement to these observations, EGb 761® was able to protect other AD models.

Depolarization of mitochondrial membrane potential leads to the release of cytochrome *c* into the cytoplasm, caspase activation, and finally apoptosis (Liu *et al.* 1996). Mitochondrial membrane potential was enhanced by EGb 761® in our AD transgenic mice model which improves mitochondrial functions. Consequently, this could prevent caspase activation and apoptosis. In fact Luo *et al* demonstrated that internally activated caspase 3 in AD mutant cells and mitochondrion-initiated apoptosis were attenuated by EGb 761® (Luo *et al.* 2002).

Additionally, ROS scavenging activity of EGb 761® in AD models was previously described. For example, treating APPswe/PS1d9 mice for 15 days reduced oxidative stress related to senile plaques by approximately 30 %. Smith *et al* demonstrated that treatment of cells expressing an AD-associated double mutation or *C.elegans* with EGb 761® significantly attenuated the basal as well as the induced levels of H_2O_2-related reactive oxygen species [ROS] (Smith and Luo 2003). A summary of EGb 761® intervention in the damage pathway of H_2O_2 is illustrated below.

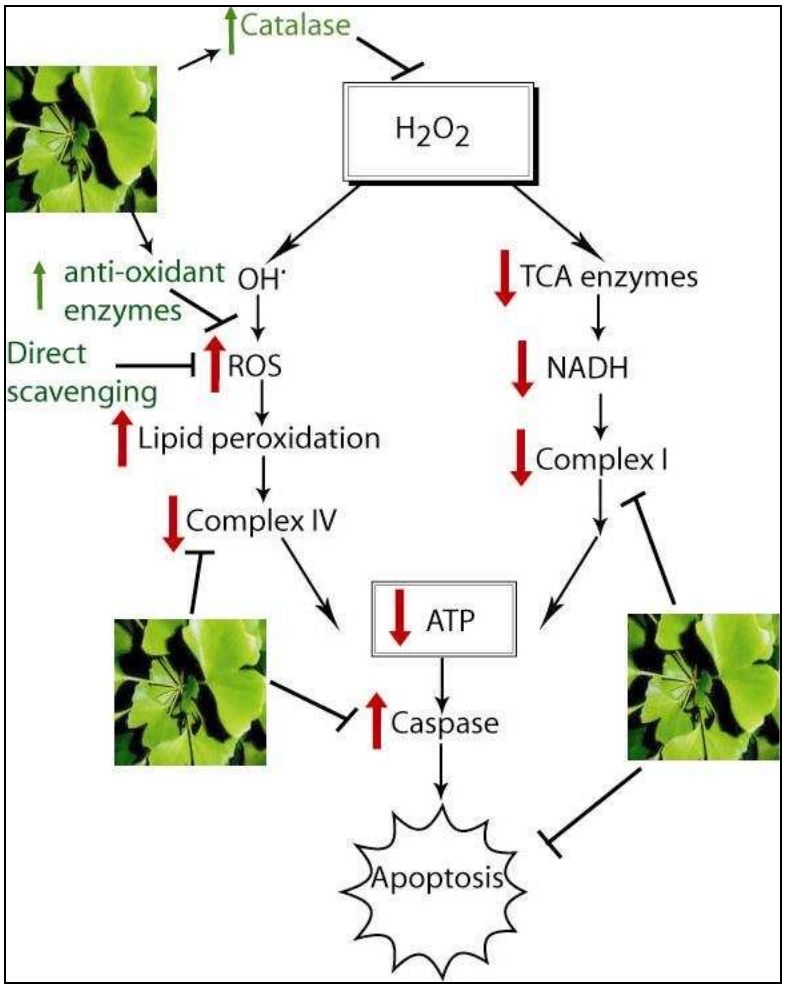

Figure 5-2

Summary of the actions of EGb 761® on H_2O_2-initiated damage

5.1.2 Protection against nitrosative stress

The association of NO in many cellular toxic effects has been previously reported. NO toxicity has been linked to both mitochondrial damage as well as certain neurodegenerative diseases such as AD [refer to section 1.1.7]. Therefore influence of EGb 761® on NO-initiated mitochondrial toxicity was tested. SNP was utilized as an NO donor. NO reacts with complex IV and causes reversible inhibition of the mitochondrial respiratory chain. Complex IV may transiently increase the leakage of superoxide anion from the electron transport chain. The toxicity of NO is more likely mediated by its oxidation products rather than NO itself. The superoxide formed could then react with NO to generate peroxynitrite which would cause irreversible injury to the mitochondria.

Mitochondrial enzymes are particularly vulnerable to attacks by peroxynitrite. It induces permeabilization of the inner mitochondrial membrane by the opening of the permeability transition pore. This decreases the mitochondrial membrane potential and consequently causes cessation of electron transfer and ATP production. Additionally peroxynitrite leads to an irreversible inhibition of all the respiratory chain complexes except complex IV, thereby decreasing ATP levels and enhancing cell death [refer to figure 5-2].

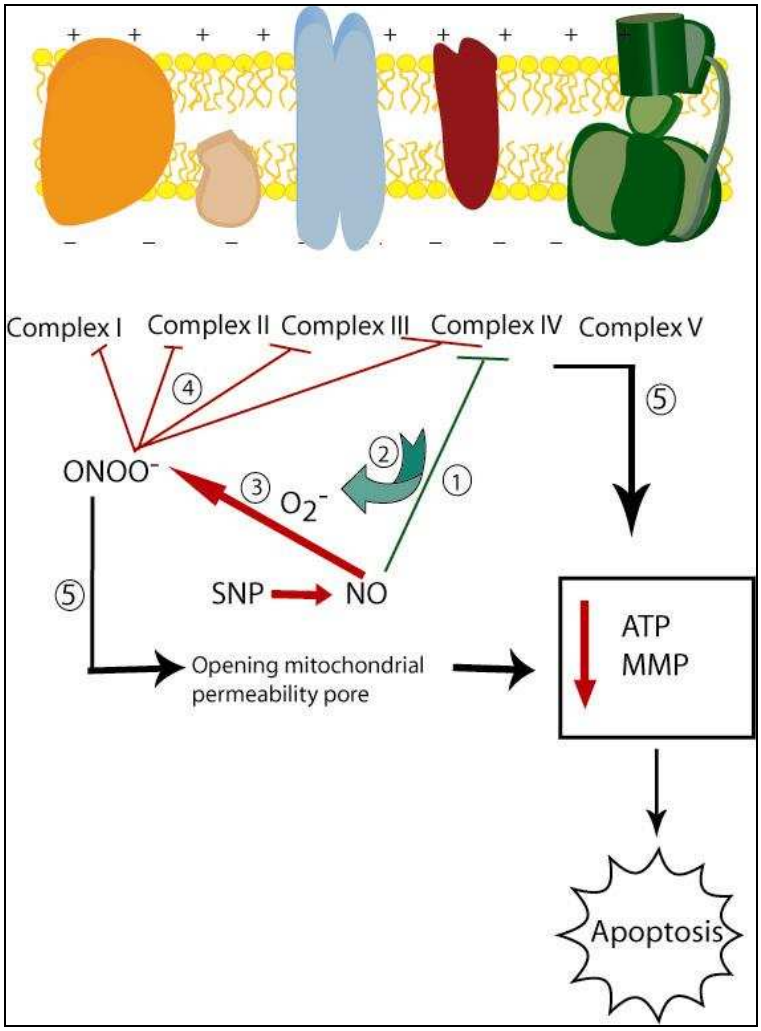

Figure 5-3

Scheme illustrating mitochondrial damage by SNP [NO]

To examine EGb 761®, DBCs were incubated with SNP for 4 hours. EGb 761® was added 30 minutes after the onset of SNP exposure, and mitochondrial membrane potential and ATP levels were determined. EGb 761® was able to enhance mitochondrial membrane potential at a concentration of 0.5 mg/ml significantly and ATP levels at a lower concentration [0.1 mg/ml] in both DBCs prepared from 3 and 15 months old mice.

These results are in accordance to previous outcomes reported by Eckert *et al* on PC12 cells. In this cell model ATP levels were stabilized at a concentration of 5 µg/ml and mitochondrial membrane potential at 10 µg/ml (Eckert *et al.* 2005) showing the same pattern as in DBCs but at lower concentrations as

expected. Since NO leads to inhibition of complex IV as mentioned above, a possible mechanism of EGb 761® could be the stabilization of the activity of this enzyme. This would then allow the respiratory chain to function normally preventing the decrease in ATP production and consequently mitochondrial membrane potential. Another possible explanation for the mitochondrial protection against NO damage is that EGb 761® blocks the SNP induced protein kinase C activation. This is supported by the findings that SNP activates protein kinase C in rat primary mixed hippocampal cell cultures and causes cell death. It was demonstrated that EGb 761® was able to prevent the SNP-induced events as well as the PKC activation (Bastianetto *et al.* 2000b).

After 2 weeks treatment of young and old mice with EGb 761®, SNP-induced mitochondrial dysfunction was partially protected in the older mice with no improvement in the younger mice.

Noteworthy was the observation that the 15 months old placebo-treated mice were less susceptible to SNP induced damage than the 2-3 months old placebo-treated mice. The ATP levels in the older mice were significantly higher than the ATP levels in 2-3 months old mice. A reason for this difference could be that in the 15 months old NMRI mice the activity of Cu/Zn SOD is higher (Leutner *et al.* 2001). This decreases the available superoxide for the reaction with NO, reducing the formation of the more deleterious peroxynitrite. DBCs of the older mice only, showed enhanced mitochondrial membrane potential after EGb 761® treatment. This is similar to our findings with H_2O_2 where EGb 761® treatment protected only the mitochondria from the aged mice with no alteration of mitochondrial properties in the younger mice. The enhancement of mitochondrial membrane potential by EGb 761® could be due to its ability to increase the scavenging activities of SOD, catalase and other anti-oxidant enzymes activities in all the brain regions (Naik *et al.* 2006). This decreases the available NO for further reaction with radicals and its conversion into higher nitrogen oxides, which

can react with thiols as well as primary and secondary amines causing mitochondrial and cellular damage (Pacher *et al.* 2007).

Another plausible mechanism could be the ability of EGb 761® to protect COX, which is one of the first targets of NO as mentioned above (Cleeter *et al.* 1994;Shiva *et al.* 2001). Either EGb 761® protects complex IV activity directly, or after the 2 weeks treatment it may be able to up-regulate it. Supporting this hypothesis, it was demonstrated that isolated brain mitochondria from EGb 761® treated mice were protected from sodium azide which is a specific complex IV inhibitor (Abdel-Kader *et al.* 2007). Interestingly this was again only a feature of the older mice and not the young ones. Also Chandrasekaran *et al* reported that a 7 days treatment with EGb 761® prevented ischemia-induced reductions in COX III mRNA (Chandrasekaran *et al.* 2001). Therefore up-regulation of mitochondrial complex IV gene expression is another possible mean of protection.

The question which naturally arises is what makes these mechanisms function only in the older mice and not in the young mice?

As mentioned before EGb 761® seems to function not on healthy but rather on impaired mitochondria. Chanderasekan observations were made on mice where ischemia was induced and as a consequence COX III mRNA was significantly decreased in their hippocampal CA1 cells. In contrast both young and old mice used in our experiments were healthy, but it is well known that mitochondrial respiratory chain enzymes are influenced by aging. Navarro and Boveris were able to show that the activities of complex I and complex IV were decreased by 28–30 % in the brains of 92 week old mice compared to 28 week old mice (Navarro and Boveris 2004). Therefore, the ability of EGb 761® to protect the complex in aged mice only, supports the assumption that EGb 761® functions only in damaged or impaired mitochondria.

Further on, the influence of a 14 days treatment with EGb 761® on an Alzheimer mouse model demonstrated again its efficacy against NO-induced mitochondrial damage.

Although mitochondrial functions of both wild type and transgenic mice models were improved, transgenic mice benefit more from the treatment. This finding supports the aforementioned observation that the mitochondrial properties of the EGb 761® treated transgenic Thy-1 APP mice were less susceptible to H_2O_2-initatied damage. In agreement with these results, EGb 761® also significantly reduced the SNP-induced decrease of mitochondrial membrane potential in transgenic PC12 cells bearing the Swedish mutation [APP-sw]. Eckert *et al* reported that the mitochondria of transgenic cells demonstrated better improvement from treatment with EGb 761® than the control cells (Eckert *et al.* 2005).

Concluding, knowing the importance of NO and its key role in aging and in the pathogenesis of AD, influence of EGb 761® on NO-initiated damage was examined. EGb 761® improved mitochondrial properties both *in vitro* and *ex vivo*. However, the *ex vivo* effects of EGb 761® were observed only in the aging mice and more pronounced in transgenic AD models.

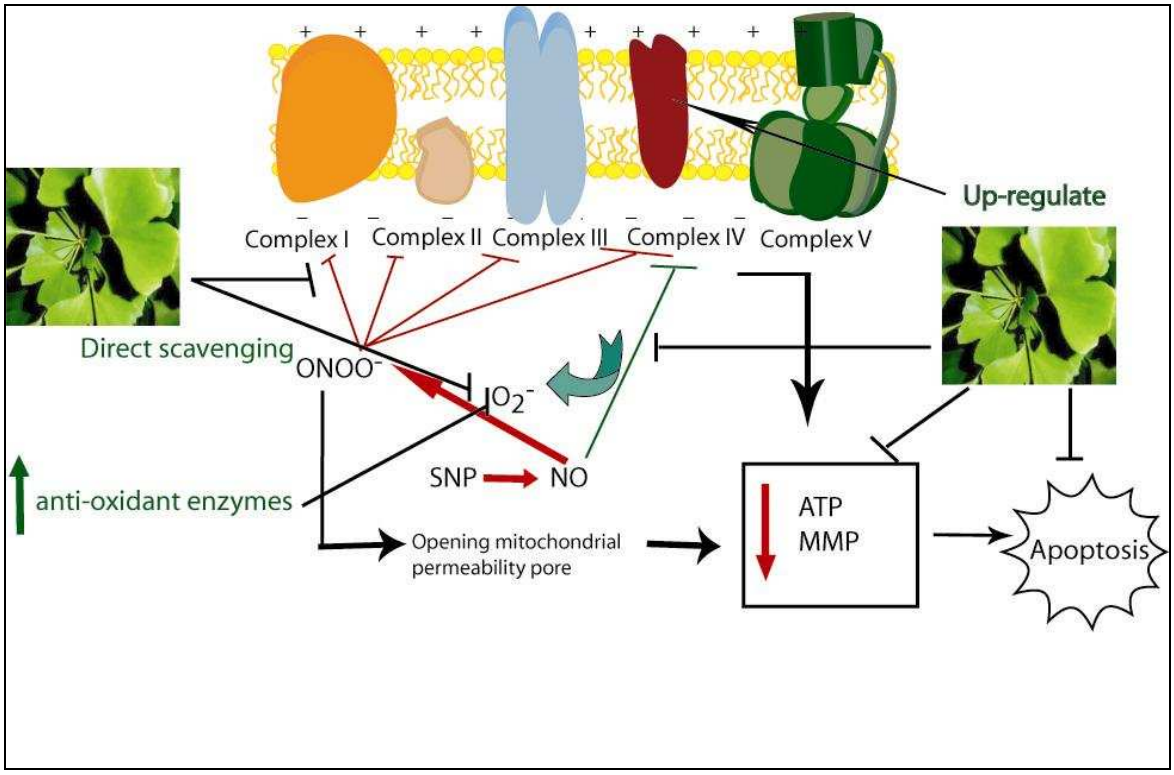

Figure 5-4

The actions of EGb 761® on NO induced mitochondrial damage

Summary

EGb 761® enhances mitochondrial properties in general, but its effect is pronounced in impaired or damaged mitochondria specifically. It has the ability to directly scavenge deleterious ROS *in vitro* and indirectly through enhancement of the activities of the scavenging enzymes *in vivo*. Additionally, it seems that EGb 761® protects the mitochondrial respiratory chain complexes, mainly complexes I and IV, which are the 2 major complexes that are reported to be impaired during aging and AD. Accordingly this would prevent further damage of the mitochondria and prevent the activation of the caspase cascade and induction of apoptosis [see diagram 5-4].

Knowing that EGb 761® is a plant extract composed of several components, the first question which crosses one's mind would be, which of its components is responsible for the observed mitochondrial effects?

5.2 Effects of various components of EGb 761® on mitochondrial function

To answer the previous question, mitochondrial functions of *in vitro* treated DBCs were assessed. Two different treatment methods were implemented, either post-treatment or pre-treatment. The rational behind this was not only to discover which of the EGb 761® component/s is/are more active but also to find out the mechanism of action. In pre-treatment the DBCs were incubated for 30 minutes with the single components, then NO was added for another 4 hours. The purpose of pre-treatment was to test the ability of the EGb 761® constituents to prevent the occurrence of mitochondrial damage.

For post-treatment studies, the efficacy of the constituents in regenerating the mitochondrial functions after NO-insult was addressed. The DBCs were first stressed with NO for 4 hours, 30 minutes after insult the constituents were added.

The idea of finding the most active constituent/s amongst the EGb 761® components is not novel. However, to our knowledge, examining the mitochondrial functions of all the components under the same conditions and using the same parameters was not previously investigated.

EGb 761® is a standardized extract composed of two major groups of substances, the flavonoid fraction [24 %] and the terpenoid fraction [6 %]. The terpenoid fraction consists of ginkgolides A, B, C, J and bilobalide.

GA, GB and GC account for about 3.1 % of EGb 761®. GA and GC are present in the highest amounts each representing ≥1 %, GB is ≤1 % and GJ≤0.5 %. As for BB, it constitutes around 2.9 % of the extract. Taking into account the abovementioned percentages and that EGb 761® enhanced mitochondrial properties *in vitro* in our cell model at concentrations starting from 0.1 mg/ml, concentrations ranging between 0.005 mg/ml and 0.1 mg/ml were tested.

Three different parameters were measured, cell viability using MTT assay, mitochondrial membrane potential and ATP levels. Pre-treating DBCs with the different constituents exemplified that the flavonoid fraction has the highest ability to prevent the occurrence of mitochondrial damage by NO in opposition to the other components. The presence of the flavonoids in the medium before addition of SNP could prevent the action of NO with free radicals and superoxide anions to form higher nitrogen oxides that cause immediate damage to the cells. This is most likely due to its radical scavenging activity which was previously reported by several other groups.

In accordance to these observations is that pre-treating cerebellar granule cells with the total flavonoid component of EGb 761® and a mixture of flavonoids and terpenes protected them from oxidative damage and apoptosis induced by hydroxyl radicals (Xin *et al.* 2000). However in the same study by Xin *et al*, total terpenes of EGb 761® did not protect against cytotoxicity. Nevertheless, this can't be directly compared to our findings due to the fact that the mechanism of mitochondrial dysfunction induced by H_2O_2 is quite different than that induced by NO. The major common injury to the mitochondria would be increased ROS. Moreover the total terpenes were not examined in our study, but every ginkgolide was tested alone.

In our pre-treatment assay, GC and GB showed moderate mitochondrial protection with no effects on ATP levels. BB demonstrated slight protection at the highest tested concentration, and GA and GJ had no effect at all in the pre-treatment study.

Similar to our results with BB and GB were the findings of Rapin *et al*. They demonstrated that pre-treatment of rat hippocampal neurons with GB and BB increased cell viability that was induced by AAPH, which is a peroxyl radical generator. GB was protective at a concentration as low as 0.2 µg/ml while BB was protective at a higher concentration of 1µg/ml (Rapin *et al.* 1998). The same tendency was noticed in our experiments, where GB was significantly

effective at a concentration of 0.01 mg/ml while BB at also 5x the concentration i.e. 0.05 mg/ml. Interestingly, they were also able to confirm the effects of GB after oral treatment. Unfortunately, the rest of the EGb 761® components were not tested by Rapin *et al*.

In cell culture system, Song *et al* were able to show that pre-treatment of PC12 cells with BB prevented NO-induced neurotoxicity. They also noticed an increase in the activities of SOD and catalase by BB. This drove them to draw the conclusion that the increase in the anti-oxidant enzymes activities is one of the contributing mechanisms to BB protective activity (Song *et al.* 2000).

Although only slight mitochondrial improvement was noticed on NO-induced damage by pre-treating DBCs with BB, it seems from other reports that BB indeed protects the mitochondria. As proposed by Song *et al* this could be due to enhancing the activities of the anti-oxidant enzymes and thereby scavenging the superoxide anion and preventing or decreasing the formation of higher nitrogen oxides. Another possible protective mechanism could be the protection of the activities of the respiratory chain. Since peroxynitrite causes deactivation of complexes I, II, III and IV it is probable that the actions of BB are due to enhancement of the activities of complexes I and III as observed earlier in isolated rat mitochondria (Janssens *et al.* 1999).

Due to the lack of data on the other terpenes, the improvement of cell viability and mitochondrial membrane potential by GC can't be supported by any previous reports. However, in an acellular model it was shown that BB, GB, GC and GJ but not GA possess radical scavenging activities and that the reaction of BB with superoxide is slow compared to GB and GC (Scholtyssek *et al.* 1997). In the present pre-treatment experiments, GC and GB were also more efficient than BB in preventing the NO-initiated mitochondrial damage; this could be explained by their scavenging activities as proposed by Scholtyssek *et al*. Moreover GA was also ineffective in our study. However in

contrast to Scholtysseks' findings no improvement at all was noticed by GJ. Yet again, the activity of the EGb 761® constituents against NO-induced damage is not only attributed to their radical scavenging activities and indeed other mechanisms play a role here.

Apparently, intrinsic differences between cell models, methodologies employed and incubation schemes seem to contribute to the efficacy of the single constituents of EGb 761®.

Therefore, in addition to the above treatment scheme we implemented another study design. In the second study DBCs were also utilized but they were first stressed with SNP and after 30 minutes incubation time, EGb 761® components were added. Here the presence of NO for 30 minutes before treatment is enough to cause substantial damage. To our surprise, the observations made in post-treatment were totally different than those made in the pre-treatment study. For example, mitochondrial membrane potential was enhanced by all terpenes and flavonoids, of course with different potencies. Although here GA was the least effective in increasing mitochondrial membrane potential, it improved cell viability at a very low concentration. GA also increased ATP levels being the only terpene beside the flavonoid fraction that affected the ATP levels.

These observations are in accordance with previous findings using PC12 cells. In this cell model mitochondrial membrane potential was improved after NO-induced depolarization by the post-treatment with every single constituent. Similar to our study in DBCs, GA showed the least enhancement on mitochondrial membrane potential (Abdel-Kader *et al.* 2007). The components of EGb 761® showed a more pronounced effect on the mitochondria in PC12 cells than in dissociated brain cells. This could be related to basic differences between the cell models and/or the fact that PC12 cells were incubated for longer time periods with the single components. Unfortunately, the other mitochondrial parameters were not measured in PC 12 cells, making it difficult for a direct comparison.

A previous study preformed by Bastianetto *et al* asserted the influence of EGb 761® constituents on cell viability in hippocampal cells after pre-exposure to SNP. Some but not all of their findings were in agreement with ours. They demonstrated that the flavonoid fraction CP 205 [25 µg/ml] rescues hippocampal cells when applied 2 h after a pre-exposure to SNP. These results are in consent to our observations, where 10 µg/ml flavonoids were

able to enhance mitochondrial membrane potential. Also ATP levels were significantly increased at a concentration of 50 µg/ml after pre-exposure to SNP. Bastianetto *et al* measured cell viability by MTT assay and another colorimetric assay known as the NR assay. However, in our experiments we noticed that the flavonoid fraction interferes with the MTT reagents. This prevented us from measuring cell viability for the flavonoid fraction to rule out any biased results.

In contrast to our findings they reported that the BB and GB [concentrations ranging between 1-5µg/ml] didn't enhance cell viability after SNP insult. In our experiment BB was effective at a higher concentration of 10µg/ml, which was not tested by Bastianetto *et al*. However we saw enhancement in cell viability using 5 µg/ml BB, which was not effective in Bastianettos' study. An explanation to this difference could be due to the fact that they stressed with 1 mM SNP and we exposed our cells to 0.5 mM SNP for MTT measurement. On the other hand, they incubated their cells for 2 hours only with SNP while in our study the cells were incubated for 4 hours. Alternative reasons would be either the cell models utilized or the solvents used for dissolving the GB; ethanol vs. DMSO (Bastianetto *et al.* 2000b).

It therefore seems that the activity of the EGb 761® constituents can be influenced by several factors, such as cell model, method for induction of mitochondrial damage and treatment schemes. These discrepancies were also noticed by others. Ahlemeyer *et al* detected these differences in an experiment to assert the anti-apoptotic properties of the ginkgolides. They reported that GJ reduced apoptotic damage of chick embryonic neurons, but not of neurons derived from neonatal rat hippocampus. Additionally, in mixed neuronal/glial cultures from neonatal rat hippocampus GB had protective effects after staurosporine treatment, but not after serum deprivation. Although both serum deprivation and staurosporine lead to increased ROS, the results were not similar. The ability of GB to enhance the activities of SOD and Catalase

(Ozturk *et al.* 2006) could explain the protective effect of GB in our experiment against NO-induced toxicity as well as against staurosporine induced apoptosis. These assumptions are based on the findings of Pong *et al* (Pong *et al.* 2001) that staurosporine induced apoptosis and mitochondrial dysfunction could be attenuated by synthetic SOD and Catalase mimetics.

Ahlemeyer *et al* also demonstrated that BB reduces apoptotic damage under both conditions, indicating that other mechanisms aside from its capability of increasing anti-oxidant enzyme activity are involved in its anti-apoptotic activity (Ahlemeyer *et al.* 1999). Another probable mechanism for enhancing cell survival by BB in the serum deprived medium could be the increase of neurotrophic factor and or growth factor as noted by Zheng *et al* in rat astrocytes (Zheng *et al.* 2000). Morover, chloride conductance has been observed to be affected by bilobalide and the ginkgolides through modulating of receptor-gated chloride channels, this may indirectly also improve mitochondrial function (Klein *et al.* 2003;Chatterjee *et al.* 2003)

The anti-apoptotic activity of BB was also demonstrated by several other mechanisms such as reduced ROS-induced elevation of Bax and activation of caspase 3 (Zhou and Zhu 2000).

Summary

Briefly, from the literature and our data one may conclude that the EGb 761® constituents exhibit different protective activities on the mitochondria. Not only the mechanism of action of the flavonoid fraction is different than that of the terpenes, but also there exists major differences in the actions of the ginkgolides. The observation that the single ginkgolides exhibit dissimilar influence on the 3 measured mitochondrial parameters under exactly the same conditions and using the same cellular model suggests that they act according to different mechanisms. Although radical scavenging activity and increasing anti-oxidant enzyme activities seem to play a big role here for the protective

effect of the constituents, it is clear that other anti-apoptotic and mitochondrial stabilization mechanisms contribute to the protective actions of EGb 761®.

Owing to the presence of flavonoids in several plant extracts and their early discovery, one has to acknowledge that their pharmacological actions and mechanisms are comparatively investigated. As for the EGb 761® terpenoids, they are exclusively found in Ginkgo and are recently gaining more attention. For this reason they are still not thoroughly investigated. However, the most researched are BB and GB, and as discussed above they both exhibit beneficial actions on mitochondria but under different conditions indicating different mechanisms of actions. As for the other EGb 761® terpenes, although most reveal promising results, they should be more extensively studied taking into consideration methodologies applied, cell models used and incubation schemes.

In this study all the EGb 761® components were studied under the same conditions and using 2 different incubation schemes in order to elucidate their efficacy. However one has to take into consideration during interpretation that DBCs were treated *in vitro* with the single constituents. Therefore, treating animals with the constituents *in vivo* could reveal other findings.

In light of our findings and the currently present literature we conceive that the EGb 761® constituents act in a complementary manner. That is to say, both the flavonoid and the terpenoid fractions are able to protect the mitochondria however in different approaches. Looking at EGb 761® effects on the mitochondria it seems that it works both anti-apoptotic and radical scavenging. However, each constituent seems to address a different target in the mitochondria, harmonizing together to achieve the full effect of the whole extract.

For example, the ability of BB to increase the activities of the respiratory chain complexes I and III, the enhancement of SOD, Catalase and GPx by GB, the anti-oxidant effect of flavonoids etc. all add to the mechanisms of action of EGb 761®. This gives EGb 761® its unique polyvalent action, which is buildup from the sum of the actions of every single component.

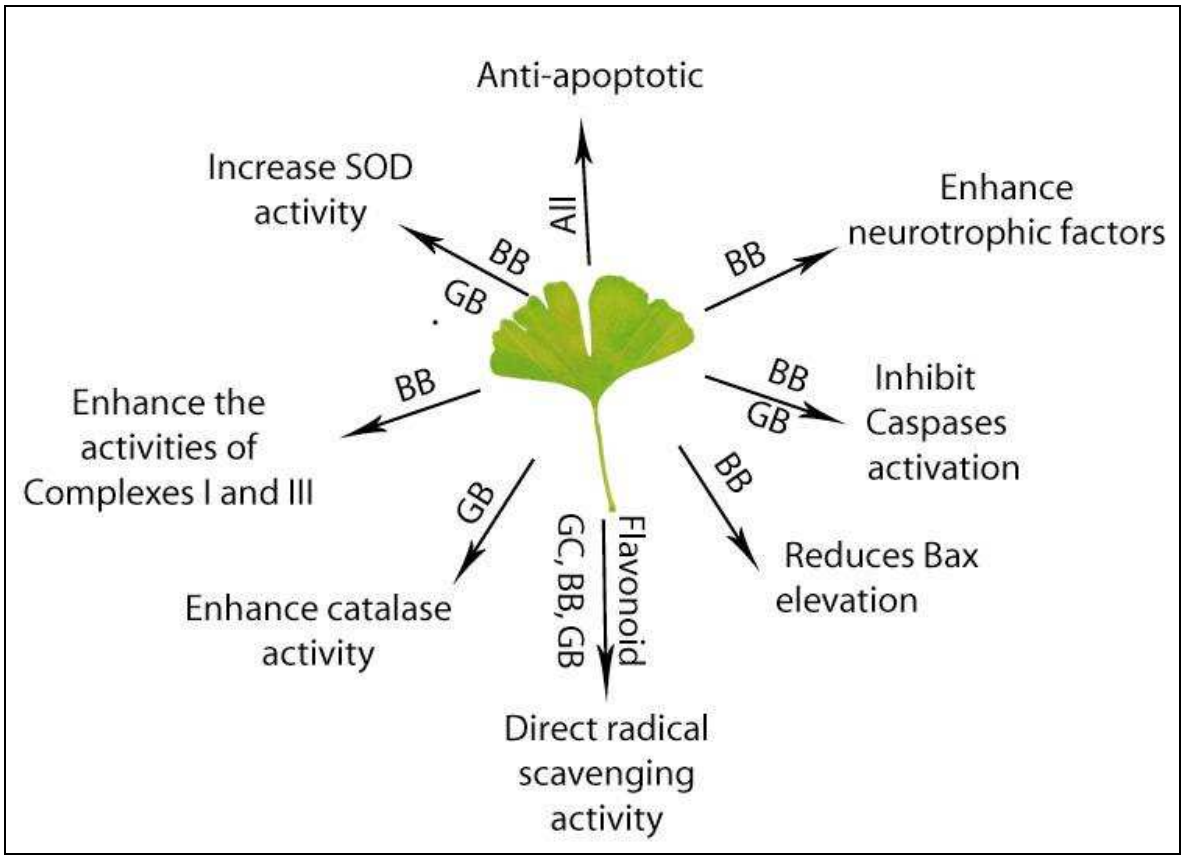

Figure 5-5

Targets and actions of the different components of EGb 761®

5.3 Long-term effects of EGb 761® in senescence accelerated mouse model

Since EGb 761® proved to be effective in impaired and aging mitochondria, its effect on mitochondria of the senescence accelerated mouse model [SAMP8] was tested. Not only the life span of these mice is much shorter than their controls [senescence accelerated resistant mice] SAMR1 but they also exhibit increased oxidative stress, age-related learning and memory deficits, anxiety, and age-dependent deposition of Aß (Butterfield and Poon 2005). It has been previously demonstrated that the abnormal expression of Aß contributes to the cognitive decline and the oxidative stress observed in SAMP8 mice (Kumar *et al.* 2000;Morley *et al.* 2002;Poon *et al.* 2004). These characteristics make SAMP8 mice a good model for studying age-related memory deficits with high relevance to AD.

In the current work, mitochondrial functions were observed to be lower in the SAMP8 mice compared to the SAMR1 mice. Both mitochondrial membrane potential and ATP levels were lower in the SAMP8 mice. In accordance to these findings Xu *et al* were recently able to observe that platelet mitochondrial membrane potential of SAMP8 was lower than SAMR1, moreover hippocampal and platelets ATP levels were significantly lower in SAMP8 mice. Several other work groups have also reported mitochondrial dysfunction in SAMP8 mice. Nakahara *et al* studied oxidative phosphorylation in the livers of SAMP8 and SAMR1 mice. They found that the respiratory control ratio decreased during aging in SAMP8 mice, and it was estimated that by 18 months of age, there was insufficient ATP synthesis for normal cell metabolism. They also observed that the amount of the apoptotic regulating protein Bcl-x in the liver mitochondria was slightly decreased in SAMP8 mice (Nakahara *et al.* 1998). Nishikawa *et al*. demonstrated a higher oxidation/reduction reaction [redox] state and higher activity of mitochondrial respiration with a lower respiration control ratio in

the mitochondrial fractions from the brains of 2-month-old SAMP8 males as compared to SAMR1 mice (Nishikawa *et al.* 1998). In the electron transport system, decreased activities of Complex I and Complex III were observed (Fujibayashi *et al.* 1998).They also observed a small but significantly greater amount of multiple mitochondrial DNA deletions in SAMP8 mice brains at 4 and 8 weeks of age. Moreover, the activities of Mn-SOD (Kurokawa *et al.* 2001), GPx (Okatani *et al.* 2002) and catalase (Sato *et al.* 1996) are all reduced in SAMP8 mice.

Long-term treatment with EGb 761® for 5 months enhanced mitochondrial membrane potential slightly and ATP levels significantly. Several characteristics of EGb 761® may contribute to this effect. For example the ability of EGb 761® to protect complexes I and III may play a role here. Another possible mechanism is the capability of EGb 761® to enhance the activities of anti-oxidant enzymes such as catalase or SOD which are known to be decreased in the SAMP8 mice.

Another factor which plays a role during aging is membrane fluidity. Reduced fluidity of brain membranes during aging has been previously described. Abnormal neural membrane phospholipids metabolism, increased cholesterol to phospholipids ratios and enhanced lipid peroxidation can all contribute to alterations in membrane fluidity. Interestingly, Stoll et al previously demonstrated that EGb 761® treatment improved short-term memory and increased membrane fluidity in aged mice, therefore this parameter was assessed in the SAM mouse model after EGb 761® treatment (Stoll *et al.* 1996).

Comparing the membrane fluidity of the SAMR1 and SAMP8 mice brains, no difference was noticed in the membrane fluidity at 6 months of age. However the fluidity of the membranes was increased in the EGb 761® fed group compared to the placebo group. Previous studies indicated memory enhancement with increased fluidity, and nootropics such as piracetam were

observed to increase membrane fluidity and cognition (Muller *et al.* 1997). For example reduced fluidity of rat hippocampal membranes impaired memory (Hong 1995;Clarke *et al.* 1999) whereas increased fluidity improved memory (Scheuer *et al.* 1999). Moreover, anisotropy studies have additionally demonstrated abnormal membrane fluidity in hippocampal synaptosomes of AD patients (Eckert *et al.* 2000), and it has been suggested that increasing membrane fluidity decreases the amyloidogenic processing of APP to form Aß.

The findings in the current work indicate that EGb 761® enhances mitochondrial functions in aged [SAMP8] and AD [Thy-1 APP] models. Recently, mitochondrial accumulation of Aβ and its precursor protein APP has been reported. Also, mitochondrial accumulation of APP directly correlates with mitochondrial dysfunction in various brain regions in AD. The data on mitochondrial APP and Aβ complement growing literature that mitochondria may interact with factors involved in Aβ metabolism and that mitochondria, APP, and Aβ metabolism might be interconnected in the cascade, leading to neuro-degeneration and dementia. Whether Aβ causes mitochondrial dysfunction, or aging leads to induction of mitochondrial dysfunction by increasing production of ROS and accumulation of Aβ, which in a viscous manner reinforces further mitochondrial impairment, is still a matter of debate. Taking into account the association of Aß and mitochondrial dysfunction, and that membrane fluidity plays a major role in APP processing, the first question which rationally arises, are the EGb 761® observed effects linked in any way to Aß formation, aggregation, and/or clearance?

5.4 The role of EGb 761® on Aß levels

Aβ is one of the major pathological hallmarks for AD, and recently it has been acknowledged that it plays an essential role in initiating mitochondrial dysfunction and apoptosis (Casley *et al.* 2002;Khan *et al.* 2000;Manczak *et al.* 2006).

Aβ is derived from its precursor protein APP [Amyloid Precursor Protein]. APP can be cleaved by 2 alternative pathways, either the non-amyloidogenic pathway by the action of alpha secretase or the amyloidogenic pathways by yielding Aß. The alpha secretase pathway forms an extracellular soluble N-terminus [APPα], and a membrane bound c- terminal fragment [C-83]. In the amyloidogenic pathway, APP is cleaved by two aspartyl proteases [β- and γ-secretases]. APP is first cleaved by β-secretase forming an extracellular soluble APPß, and leaving a membrane bound C-terminal stub [C-99]. According to the position where the γ-secretase cleaves the C-99 fragment either the Aß 40 is formed or the Aß 42. The longer form [Aβ 42] is far more prone to oligomerize and form fibrils than the more abundantly produced Aβ 40 peptide.

Recently, the precursor protein of Aβ [APP] was found to be associated in the mitochondrial compartment of the cortex and hippocampus of an AD transgenic mouse model [Tg 2576] that over-expresses Swedish APP (Anandatheerthavarada *et al.* 2003). Interestingly, APP was further associated with mitochondria in samples from the brains of individuals with AD, but not with mitochondria in samples from non-demented subjects (Devi *et al.* 2006).

In light of the above reported Aβ associated mitochondrial impairment and our recent findings that EGb 761® showed enhancement of mitochondrial functions during aging and more importantly in Alzheimer disease models, the influence of EGb 761® on Aβ was examined.

A cell line model for AD was utilized for the preliminary experiments. Human embryonic kidney cells [Hek-293] stably transfected with the Swedish double mutation were employed. APPsw mutation leads to an increase in β-secretase activity in cell culture (Haass *et al.* 1995), increasing vastly the amount of Aβ produced by these cells. This cellular model is therefore appropriate for mimicking familial AD, similar to our tg-APP mice. Expectedly, the Hek-sw cells show mitochondrial dysfunction such as decreased mitochondrial membrane potential and lower ATP levels compared to their untransfected controls (Keil *et al.* 2004).

Fortunately, the distribution of intracellular and secreted Aβ 40 and Aβ 42 in APPsw HEK cells were previously studied by Dr. Astrid Bonert. Hek-sw cells secrete large amounts of both Aβ 40 and Aβ 42 compared to their non-transgenic controls. However, the levels of secreted Aβ 40 are much higher than Aβ 42. The Intracellular, insoluble Aβ 40 and Aβ 42 are elevated in comparison to soluble Aβ 40 and Aβ 42 in APPsw HEK cells. The intracellular, insoluble Aβ 42 was only detected in the Swedish transfected Hek cells, neither in the Hek wild type nor in the Hek control cells (Bonert 2006). Concluding we may state that in APPsw HEK cells, the ratio of Aβ 40 and Aβ 42 is shifted in favor of intracellular, insoluble Aβ 42 and that the accumulation of insoluble Aβ species intracellularly is exclusively found in APPsw HEK cells.

In this study, extracellular, soluble Aβ 40 and Aβ 42 were assessed in the Hek-sw cells after EGb 761® treatment. The cells were treated for 24 hours with different concentrations of EGb 761® and the soluble Aβ was then determined. Unpredictably, extreme elevation in the Aβ 40 levels, reaching 200 %, was noticed already at the lowest applied EGb 761® concentration. Comparatively the extracellular soluble Aβ 42 also increased after treatment with EGb 761® but to a much lower extent.

Although the extracellular location of AD plaques initially led to the assumption that toxicity results from extracellular attack of neurons by Aβ, but Aβ has also been shown to exist intracellular in cell cultures and in rat brain tissue. There is evidence that intracellular, nonfibrillar Aβ oligomers cause cytotoxicity in human neurons that strongly exceeds that of extracellular Aβ species. Moreover, Kienlen-Campard *et al*. observed that although APP processing leads to production of extracellular Aβ 40 and soluble APP, these extracellular derivatives did not induce neuronal death. On the other hand, neurons underwent apoptosis as soon as they accumulated intracellular Aβ 42 (Kienlen-Campard *et al.* 2002).

This was also the case in the employed Hek-sw cell model. Where the findings that intracellular, insoluble Aβ 42 is present only in the Swedish transfected Hek cells which demonstrate decreased cell viability and ATP levels confirm that intracellular, insoluble Aβ 42 is a pathogenic and toxic form (Keil *et al.* 2004;Marques *et al.* 2003b).

A possible explanation for the vast increase in the extracellular soluble secreted Aß 40 could be that EGb 761® shifts the processing of APP towards the non-toxic Aß 40 rather than the Aß 42. However this does not explain the moderate increase in the extracellular soluble Aß 42 after EGb 761® treatment. Another possible clarification could be that EGb 761® prevents the accumulation of intracellular soluble Aß, leading to its secretion rather than aggregation to form deleterious forms. In accordance to this assumption Longpre *et al* were able to show that EGb 761® inhibits Aβ 40 fibril formation (Longpre *et al.* 2006) and Yao *et al* observed that EGb 761® inhibits Aβ 42 oligomers formation *in vitro*, presumably dimers and tetramers of Aβ 42 (Yao *et al.* 2001). This was confirmed in a neuroblastoma cellular system stably transfected with the Swedish mutation similar to our cell line. An aggregated Aβ band at a molecular mass between 7 and 21 kDa [dimers or higher oligomers] found in the control cells, was absent in the medium recovered

from cells treated with EGb 761® (Luo *et al.* 2002). A very important study in transgenic *C.elegans* reported not only a decrease in the Aβ oligomers by EGb 761® but concurs totally with our results and postulation by also observing an increase in the monomeric Aβ after EGb 761® treatment (Wu *et al.* 2006). Wu *et al* were also able to show that not only EGb 761® but also its terpenoid fraction, mainly GA, inhibited Aß oligomerization as well as Aß-induced paralysis in the transgenic *C.elegans*. Interestingly, in our observations, from the EGb 761® terpeniods, mainly GA enhanced cell viability and ATP levels after NO-induced mitochondrial insult. It was also previously reported that NO production is increased in transgenic models with high Aß secretion (Keil *et al.* 2004;Law *et al.* 2001).Therefore it seems that the mitochondrial protective properties of EGb 761® are indeed associated with both Aß production and/or oligomerization.

From the above mentioned literature findings and the observations on Aß production in Hek-sw cells, an assumption was made that EGb 761® inhibits Aβ oligomerization, leading to an increase in the non-toxic Aβ monomers and reduction in amyloid deposits. It also seems that EGb 761® directly secretes these small soluble Aβ, preventing them from accumulating and thereby aggregating intracellularly.

To confirm this hypothesis and to assess if these *in vitro* observations are consistent *in vivo*, Tg-APP mice were treated for 21 days with 100 mg/kg body weight with EGb 761® and the Aß levels in brain homogenates were evaluated after the treatment period.

As expected evaluating the transgenic mice model, they expressed very high amounts of human Aß both the 40 and the 42 forms. The total Aß 42 was around 20 times more than Aß 40. The transgenic mice showed an approximate of 26,000 pg/mg protein Aß 40 while the average amount of Aß 42 is 550,000 pg/mg protein. Correlating these results one notices that the correlation is highly significant; meaning a mouse with high levels of Aß 40

has also high levels of Aß 42. Assessing the amount of soluble Aß levels, the levels were much lower than the total Aß levels, with average amount of 11 pg/mg protein Aß 40 and soluble Aß 42 was undetectable at all. This is in agreement with the findings of Blanchard *et al*. Studying the Thy-1 APP 751 mice model, they stated that once plaques were present, the majority of Aß peptide was insoluble (Blanchard *et al.* 2003). In our study the tg-APP mice were 6- months old, which is the age, were they start developing Aß deposits. In the cortex of 6 months old transgenic Thy-1 APP 751 mouse model the total Aß 42 present is much greater than Aß 40 present. However, all the Aß 42 is in the insoluble form, and although the soluble Aß 40 was detectable, its amount can be considered minor compared to the total Aß 40 [11 pg/mg soluble vs. 26,000 pg/mg total].

Measuring the amount of soluble Aß 40 after 21 days treatment with EGb 761®, a significant increase was observed which concurs with our finding in Hek-sw cells. Since no soluble Aß 42 was quantifiable in the tg-APP mice model, it was not possible to measure the effect of EGb 761® on this parameter.

Looking at the total amounts of both Aß 40 and Aß 42, EGb 761® treatment did not alter their level in the brains of the Thy-1 APP mice. This supports that EGb 761® decreases the aggregation of Aβ, since the total amount of Aβ remains the same, meaning that the increase in the soluble Aβ form is compensated some how by a decrease in another form. However, in opposition to this hypothesis one may argue that the soluble Aβ 40 represents only a very small fraction of the total Aβ in our mice model, and a significant increase in its value would not influence the total level of Aβ 40.

The supposition that EGb 761® decreases Aß aggregation is in accordance with the recent findings that treating a transgenic mouse model for 1 month with EGb 761® reduced the level of the Aß oligomers (Tchantchou *et al.* 2007). On the contrary, Stackmann *et al* reported that neither the soluble nor

the insoluble Aß levels were changed after treating Tg2576 transgenic mice with EGb 761® although they noticed an enhancement in cognition in the EGb 761® treated group (Stackman *et al.* 2003). This may be due to the difference in the transgenic mice models used, or the feeding schemes implemented.

In Thy-1 APP mice it was previously reported that the C-99 fragment is highly expressed (Blanchard *et al.* 2003). Cleavage of this stub by the γ-secretase forms either the Aß 40 or the Aß 42 fragment. The London mutation present in Thy-1 APP mice increases the proportion of Aß 42 by 50–90 % (Suzuki *et al.* 1994), by shifting the balance of γ-secretase cleavage slightly toward the 42 over the 40 cleavage site. Since EGb 761® treatment increased the soluble Aß 40 and had no effect on the Aß 42 forms in the Thy-1 APP mice, another possible clarification for the observations made could be that EGb 761® treatment shifts the C-99 cleavage towards the formation of Aß 40.

There are several studies demonstrating that higher levels of Aß 40 directly interferes with Aß 42 aggregation by delaying the Aß 42- mediated nucleation step at an early stage in the fibrillogenesis process (Snyder *et al.* 1994;Hasegawa *et al.* 1999;Zou *et al.* 2003). Interestingly, mice expressing high levels of Aβ 40 in the absence of human APP over expression did not develop overt amyloid pathology. In contrast, mice expressing lower levels of Aβ 42 accumulate insoluble Aβ 42 and accumulate Aβ deposits.

Also the Aß 42/40 ratio plays a very important role and if EGb 761® is able to increase the soluble Aß 40 thereby lowering the ratio this is beneficial and could be a contributing mechanism of action of EGb 761®. The importance of the ratio of Aß 42/ Aß 40 has been demonstrated in animals. In transgenic mice where Aβ 40 production has been achieved using a viral vector, the increase in Aβ 40 inhibited Aβ 42-linked amyloidosis and death (Kim *et al.* 2007). This demonstrated that even in the presence of increased total Aβ, a shift in Aβ length distribution toward shorter lengths reduces amyloidosis and toxicity.

With regards to the observations in both transgenic models, EGb 761® treatment enhanced the levels of soluble Aß 40. This maybe due to a shift in the splicing of the C-99 fragment towards more production of soluble Aß 40 rather than the formation of deleterious Aß 42. In the Hek-sw cells EGb 761® not only enhanced the secreted soluble Aß 40 but also increased the secreted soluble Aß 42. From the current observations and literature findings it was concluded that EGb 761® enhances the clearance of the soluble Aß to prevent its intracellular accumulation and consequently its aggregation. This was not evident in our Tg-APP mouse model due to the absence of intracellular soluble Aß 42, besides the alteration on distribution of soluble Aß might not be detectable by ELISA on brain homogenates prepared without perfusion. However, recently it was reported that EGb 761® favors the clearance of Aß via regulating the expression of RAGE and LRP-1 during brain ischemia. RAGE is thought to be a primary transporter of Aß across BBB into the brain from the systemic circulation, while LRP-1 mediates the transport of Aß out of the brain (Yan *et al.* 2008).

Noticing the influence of EGb 761® on Aß peptide, its effect on Aß production and breakdown was studied on the genomic level.

5.5 Effects of EGb 761® on gene expression

The Thy-1 APP mice employed in our study are transgenic mice encoding human APP-751 with Swedish double mutation plus London mutation regulated by the neuronal murine Thy-1 promoter. This leads to an over expression of the mutated human APP and consequently enhanced Aß levels.

Measuring the mRNA levels of the mutant Hu APP after EGb 761® treatment, a significant down regulation was observed. Moreover, EGb 761® down regulated all forms of APP, not only the mutant human APP but also mouse APP and APP binding protein. The decrease in APP expression as well as protein level in rat brain after EGb 761® treatment was previously reported (Yao *et al.* 2004;Gong *et al.* 2005). On the other hand, Colciaghi *et al* demonstrated no change in APP levels after EGb 761® treatment in 2 months old rats (Colciaghi *et al.* 2004). This discrepancy could be due to the difference in treatment periods, in the last study where no effect was observed the rats were treated for 5 days only. As for the other reported studies treatment periods were 28 weeks and 2 months. Another possible reason could be that the effect of EGb 761® is observed only at an older age, where in the experiments by Yao and Gong the rats were older than the 2 months old rats used by Colciaghi *et al*.

In accordance to our finding, only very recently APP was reported to be down regulated in another transgenic mouse model. Augustin *et al* reported that human APP protein levels in Tg2576 mice were decreased after 16 months treatment with EGb 761®. However, in contrast to our observations Augustin *et al* noticed no down regulation in human APP in the mice treated only for 1 month. Additionally, no changes were detected in mouse APP or APPbp (Augustin *et al.* 2008). The variation to our findings could be due to the different transgenic mouse model implemented. This is also likely since Stackmann *et al* reported that neither the soluble nor the insoluble Aß levels

were changed after treating Tg 2576 transgenic mice [which is the same mouse model implemented by Augustin *et al*] with EGb 761® and in Thy-1 APP mice it was demonstrated that soluble Aβ 40 increased after EGb 761® treatment. Tg 2576 mice have the Swedish double mutation while Thy-1 APP mice implemented in our study have both the Swedish mutation as well as the London mutation. Comparing the forms of Aβ in both mice strains, one notices that in the Thy-1 APP mice the total amount of Aβ 42 is much higher than the Aβ 40 as expected, while in the Tg 2576 mice exactly the opposite is reported, that is to say the Aß 40 is greater than Aß 42 (Stackman *et al.* 2003).

Collectively, EGb 761® decreased the source of Aβ, namely APP, in Thy-1 APP mice as well as increased the soluble form of Aβ 40. Our earlier hypothesis before measuring APP levels was that EGb 761® may shift the cleavage of C-99 fragment towards Aβ 40 formation and may prevent the accumulation and hence aggregation of Aß. After noticing a decrease in the APP levels, another possible supposition could be that EGb 761® enhances the cleavage of APP by the β- secretase and γ-secretase which would increase the levels of Aβ. To rule out this assumption, the expressions of the secretases [BACE-1 and ADAM-10] were quantified in the EGb 761® treated Thy-1 APP mice. Due to the complexity of γ-secretase complex its expression was not quantified.

Confirming the previously reported data, the EGb 761® treatment did not change the expression of either BACE or ADAM-10. This is in accordance with the reported data that ADAM-10 and ADAM-17 protein levels were not altered in rats after EGb 761® treatment (Colciaghi *et al.* 2004). The recent findings of Augustin *et al* concur with our observations, where BACE-1 mRNA levels and enzyme activity as well as ADAM-10 mRNA and protein levels were unchanged in Tg2576 mice after EGb 761® treatment (Augustin *et al.* 2008;Augustin *et al.* 2009). Interestingly, there existed an inverse relationship between the Hu APP and the levels of both BACE and ADAM in

the placebo group, that didn't exist anymore after EGb 761® treatment. Indicating when the levels of BACE and ADAM in the mouse brain were low the amount of Hu APP present was high, obviously due to less processing and vice versa. In the EGb 761® treated mice this relationship did not exist any more since EGb 761® treatment decreases the levels of Hu APP and does not alter the levels of the β-secretase or α-secretase. Therefore, EGb 761® treatment decreases APP levels but does not increase its processing. Accordingly, the first assumption that EGb 761® may shift the cleavage of C-99 fragment towards Aβ 40 formation and prevents its accumulation and aggregation still seems the most reasonable justification. In addition EGb 761® decreases the availability of APP for further formation of Aß.

In order to form a complete picture of EGb 761® intervention in Aβ production and trafficking, the Aß degrading enzymes were then taken into consideration.

The 3 major enzymes that have been reported with Aß metabolizing activities are IDE, NEP and ECE. The expression levels of IDE, NEP and ECE were unaltered by EGb 761® treatment. Therefore it seems that EGb 761® does not influence the breakdown or cleavage of Aβ protein. There are no previous data in the literature addressing this issue, however very recently a study confirmed the observations with NEP, where Tg 2576 mice showed unaltered expression levels of NEP after both short-term and long-term treatment with EGb 761® (Augustin *et al.* 2008).

Associating the levels of Aβ with NEP, IDE and ECE-1, one notices that only in the EGb 761® treated group, NEP, ECE-1 and IDE correlate significantly and negatively with Aβ 40. Additionally, IDE is also negatively proportional to Aβ 42 in the EGb 761® treated group only. Although an inverse relationship between Aß levels and the degrading enzymes was previously reported, this was not the case in our placebo-treated mice. For example, an inverse relationship was observed between NEP mRNA levels and both Aß

plaques and Aß levels (Wang *et al.* 2006). In this study, the negative correlation was noticed only in the EGb 761® treated mice although the levels of the enzymes and the total Aβ levels were not altered by EGb 761® treatment. The correlation was significant and more pronounced between Aβ 40 and the enzymes in the EGb 761® treated mice. Although no correlation exists in the placebo treated group, a statistical comparison between the correlations of the placebo-treated and the EGb 761® treated mice does not yield significant difference. Nevertheless, the p values are 0.054, 0.065 and 0.075 for the correlations NEP/Aß 40, IDE/Aß 40 and IDE/ Aß 42 respectively. Owing to the small sample size, care must be taken in interpreting these correlations; however EGb 761® may possibly affect the relationship between some of the Aß degrading enzymes and Aß protein. A hypothesis which would require further verification is that although EGb 761® doesn't alter the mRNA levels of NEP, IDE or ECE in the transgenic mice, EGb 761® may alter their activities without altering their expression. Since it has been reported that these enzymes cleave mainly Aβ monomers, and EGb 761® either increases these monomers and/or prevents their aggregation. Therefore one may expect that the activities of these enzymes increase after EGb 761® treatment due to the presence of more monomers. Consequently, the mice which express more enzymes, their activities would be more enhanced by EGb 761® treatment than mice expressing lower RNA levels.

For example, a mouse expressing high levels of NEP would show higher NEP activity due to the increase in Aß monomers after EGb 761® treatment. This would increase the cleavage of Aß, decrease its value and therefore a negative correlation would exist. However this hypothesis needs to be verified by measuring the activities of these enzymes before and after EGb 761® treatment.

Conclusion:

EGb 761® decreases the availability of APP for processing into Aß. Further splicing of the already present C-99 fragment by γ-secretase is continued, however with a tendency of forming more Aß 40 than Aß 42. EGb 761® also seems to prevent the further aggregation of Aß monomers, and may enhance its clearance by enhancing its secretion. This maybe augmented by the regulation of both RAGE and LRP-1 expression levels (Yan *et al.* 2008). Enhancement of the activities of the Aß degrading enzymes could be another possible clearance mechanism which requires further verification.

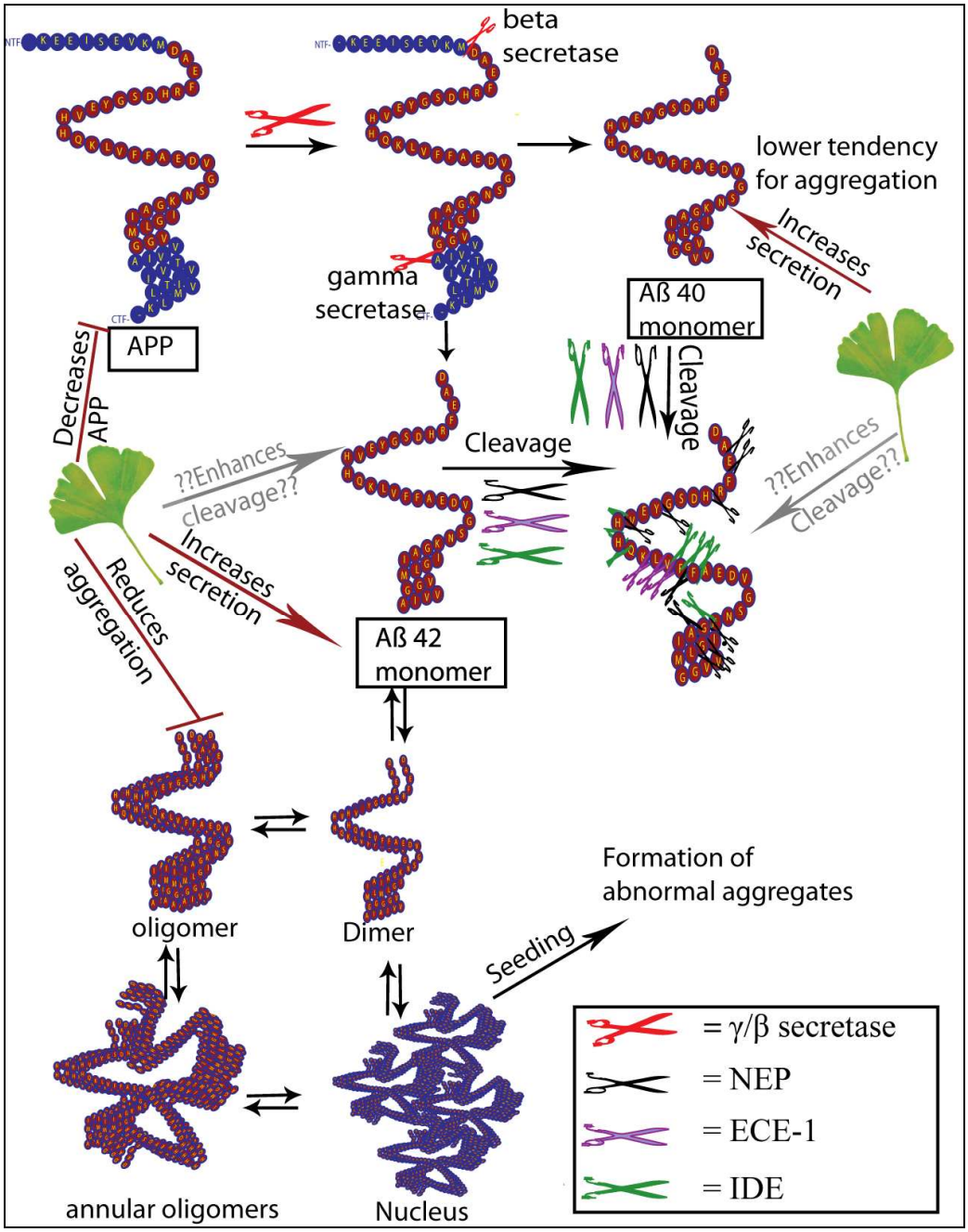

Figure 5-6

Summary of the effects of EGb 761® on Aß

6 Summary

The utilization of Ginkgo biloba in medicinal practice dates back to 1505 A.D. Ironically, the mechanisms of action of Ginkgo are not fully clarified till now. Nowadays, Ginkgo biloba leaf extracts are mainly indicated for mild to moderate cerebrovascular insufficiency and different forms of dementia. The fact that it is an herbal extract composed of several different components indeed adds to the intricacy of finding its mechanisms of actions. Indisputably, many scientists tried to elucidate the mechanisms of actions of Ginkgo. The first step to achieve this goal was to standardize the leaf extract. The standardized Ginkgo leaf extract contains 22-27 % flavonol glycosides, 2.8-3.4 % of ginkgolide A, B and C, as well as approximately 2.6-3.2 % bilobalide and below 5 ppm ginkgolic acids. A widespread standardized Ginkgo extract is the EGb 761, which was utilized in the current work.

One of the earliest proposed mechanisms is the ability of the Ginkgo extract to act as an anti-oxidant, which could be explained by its high flavonoid contents. However, without doubt EGb 761 encompasses other characteristics which distinguish it from other herbal extracts that are also rich in flavonoids. Since free radicals and reactive oxygen species are highly associated with the mitochondrial functions, examination of the effect of EGb 761 on mitochondrial functions was lately addressed. Moreover, this was encouraged as the link between Alzheimer's disease [AD] and the mitochondria started to emerge. Previously, our group observed mitochondrial protective actions of EGb 761 on cell culture *in vitro*. Furthermore, anti-apoptotic effects were previously described for EGb 761.

However, only very few studies addressed the single constituents and their effect on mitochondrial functions. Flavonoids were studied in several other plant extracts and their radical scavenging activity is unquestionable, but EGb 761 has anti-apoptotic actions which may be attributed to its terpenoid

fraction. Exclusively found in the Ginkgo plant, are the ginkgolides and therefore their actions are not yet fully elucidated. Moreover, those who attempted to address these constituents concentrated on one or two candidates, for example bilobalide or ginkgolide B and ignored the rest. Unfortunately, this led to incomplete results, and one couldn't compare the relative activities of all EGb 761 components in order to state whether all the components are effective or not.

Therefore, the first goal of the current work was to try to identify the relative mitochondrial activities of the single components of EGb 761 using the same model as well as identical parameters and conditions. Dissociated brain cells prepared from mice were chosen as a suitable *in vitro* model for this study and mitochondrial functions were assessed using MTT viability test, mitochondrial membrane potential and ATP levels. Moreover, two different treatment schemes as well as external stress were applied to the cells.

However, before testing the different constituents *in vitro*, the effect of the whole extract EGb 761 was examined as a reference. This was performed not only *in vitro* in DBCs prepared from young and old mice, but also *ex vivo* after a 14 days treatment with EGb 761 in young, old and APP transgenic mice. Here again external stress was applied and mitochondrial functions were evaluated by measuring the above mentioned parameters.

The current work was able to confirm that EGb 761 enhances mitochondrial properties in general, but its effect is pronounced in impaired, aged and/or damaged mitochondria specifically. Looking at its different components, it appears that they act in a complementary manner. That is to say, both the flavonoid and the terpenoid fractions are able to protect the mitochondria but in different approaches. Not only the mechanism of action of the flavonoid fraction is different than that of the terpenes, but also there exists major differences in the actions of the terpenes. Furthermore, according to the conditions and treatment schemes their relative activities differed

significantly. For example, in the first scheme pre-treatment with ginkgolide A did not alter any of the measured mitochondrial parameters. However, surprisingly in post-treatment ginkgolide A was able to enhance mitochondrial membrane potential, ATP levels and cell viability after nitric oxide [NO] induced insult. Both pre- and post-treatment studies gave the impression that the flavonoid fraction is the most protective fraction against NO induced mitochondrial insult. The ginkgolides appear to be more efficient in regenerating the mitochondria after damage than in scavenging the radicals. In the applied cell model ginkgolide J was the least effective, albeit its ability to increase mitochondrial membrane potential in post-treatment. In the present study the components of EGb 761 acted in a complementary matter, to achieve the observed effects of the EGb 761 extract.

Although these results are promising and give an indication of the relative efficacy of the single constituents in protecting the mitochondria, it should be taken into consideration that this work although carried on mice brain cells, was *in vitro*. An *in vivo* study could unveil even more promising and precise outcomes concerning the mechanisms of action of EGb 761 and its constituents.

In the current work, it was observed that EGb 761 is more effective in aged mice or transgenic mice. Therefore a long-term assessment of EGb 761 in aging mice was carried out. To evaluate the long-term effects of EGb 761 on mitochondrial function during aging, a special mouse model was chosen. Senescence accelerated prone mice [SAMP8] which age rapidly acquiring age related cognitive impairment, accumulation of oxidative stress and deposition of amyloid beta [Aß] as well as senescence accelerated resistant mice [SAMR1] were selected. SAMP8 mice were fed with EGb 761 for 5 months or placebo and membrane fluidity, which is another important parameter that plays a role in aging and cognition, was evaluated in addition to mitochondrial functions. The mitochondrial membrane potential and ATP levels were found

to be lower in the SAMP8 mice compared to the SAMR1 mice and EGb 761 treatment was able to alleviate the mitochondrial dysfunction. Moreover, membrane fluidity was increased after EGb 761 treatment. Increasing membrane fluidity was previously linked to memory enhancements and improving cognition. In addition, decreased membrane fluidity was reported in hippocampal synaptosomes in AD brains, which could be related to decreasing the amyloidogenic processing of APP and thereby the formation of Aß.

The focus of the project was then directed towards the link between the protective properties of EGb 761 in relation to AD and in particular to Aß. This was based on the findings that EGb 761 enhances mitochondrial properties and membrane fluidity and knowing that recent research relates both of these parameters to APP processing and Aß formation.

Consequently, Aß production in transgenic Hek-sw cells was assessed after *in vitro* treatment with EGb 761. Noticing an unexpected increase in the levels of extracellular soluble Aß after EGb 761 treatment, the *in vivo* influence of EGb 761 was examined in a transgenic mice model. Tg-APP mice over-express mutant human APP and accordingly develop high levels of Aß. Tg-APP mice were treated for 21 days with EGb 761 followed by evaluation of Aß levels in their brains. Moreover, the influence of EGb 761 on Aß production and breakdown was studied on the genomic level, by evaluating the mRNA levels of the genes involved in making and breaking Aß.

The increase in the levels of soluble Aß 40 was confirmed in the EGb 761 treated mice. However the total Aß level was not altered compared to the placebo group. Interestingly, the mRNa levels of APP decreased significantly after EGb 761 treatment, but the levels of the Aß degrading enzymes [NEP, IDE and ECE-1] were not altered after treatment. Nevertheless a negative relationship occurred between the Aß levels and the mRNA levels of the Aß degrading enzymes in the EGb 761 treated mice only.

From these observations together with present data in the literature one may presume that EGb 761 decreases the availability of APP for further processing into Aß. The C-99 fragment is cleaved with a tendency of forming more Aß 40. The clearance of Aß is enhanced by EGb 761 thereby preventing its accumulation and further aggregation. This may be either via regulating the transport of Aß in and out of the brain, through the RAGE and LRP. Another possible clearance mechanism which requires further confirmation could be the ability of EGb 761 to enhance the activities of the Aß degrading enzymes.

In conclusion, the current project contributed the following to research in the field of AD prevention and treatment with EGb 761: [1] EGb 761 improves mitochondrial abnormalities which occur during aging and AD development. [2] The constituents of EGb 761 act in a complementary manner achieving together the full effect of the extract. [3] Long-term treatment with EGb 761 is beneficial in preventing age-related mitochondrial abnormalities and increases membrane fluidity. [4] APP processing and Aß formation and aggregation, which play a major role in AD, are influenced by EGb 761 treatment in transgenic models.

7 Zusammenfassung

Die frühesten Aufzeichnungen über die medizinische Verwendung von Ginkgo biloba gehen auf das Jahr 1505 n.Chr. zurück. Trotzdem sind die Wirkmechanismen des Ginkgos bis heute noch nicht vollständig geklärt. Heutzutage werden Extrakte aus Ginkgo-biloba-Blättern hauptsächlich zur Behandlung von leichten bis moderaten Hirnleistungsstörungen und verschiedenen Formen der Demenz eingesetzt. Die Tatsache, dass es sich hierbei um einen pflanzlichen Extrakt, bestehend aus vielen verschiedenen Komponenten, handelt, erschwert die Aufklärung der Wirkmechanismen erheblich. Zweifelsohne wurden zahlreiche Versuche unternommen, diese Mechanismen wissenschaftlich zu ergründen. Eine wesentliche Voraussetzung hierfür war die Standardisierung des Extrakts aus den getrockneten Blättern. Der in dieser Arbeit verwendete, weit verbreitete standardisierte Ginkgoextrakt EGb 761 enthält 22-27 % Flavonolglykoside, 2.8-3.4 % Ginkgolide A, B und C, sowie 2.6-3.2 % Bilobalid und weniger als 5 ppm Ginkgolsäuren.

Einer der ersten Ansätze zur Aufklärung des Mechanismus des Ginkgoextrakts beschreibt seine anti-oxidativen Eigenschaften, was hauptsächlich dem hohen Flavonoidgehalt zugeschrieben werden kann. Allerdings weist EGb 761 zweifellos darüber hinaus weitere Eigenschaften auf, welche ihn von anderen pflanzlichen Extrakten mit ebenfalls hohem Flavonoidgehalt unterscheiden. Mitochondriale Funktionen stehen in engem Zusammenhang mit der Bildung von freien Radikalen und reaktiven Sauerstoffspezies. In diesem Zusammenhang deuten erste Erkenntnisse auf eine Schlüsselfunktion der Mitochondrien in der Pathogenese der Alzheimer-Demenz. Vor diesem Hintergrund rückte in letzter Zeit die Wirkung des EGb 761 auf die mitochondrialen Funktionen zunehmend in den Mittelpunkt des Interesses. Eine mitochondrien-protektive Wirkung von EGb 761 konnte von

unserer Arbeitsgruppe bereits in *in vitro* Zellversuchen gezeigt werden. Ebenso wurden anti-apoptotische Effekte für EGb 761 beschrieben.

Allerdings beschäftigten sich nur sehr wenige Studien mit der Wirkung der Einzelkomponenten auf die mitochondriale Funktion. Die Radikalfänger-Eigenschaften der Flavonoide, bereits in zahlreichen anderen pflanzlichen Extrakten untersucht, stehen außer Frage. EGb 761 weist jedoch auch anti-apoptotische Eigenschaften auf, die auf die Terpenfraktion zurückgeführt werden können. Ginkgolide, deren Wirkung noch nicht vollständig aufgeklärt ist, sind ausschließlich in Ginkgo vorzufinden. Bisherige Studien zu Ginkgoliden konzentrierten sich weitestgehend auf ein oder zwei Substanzen, wie beispielsweise das Bilobalid oder Ginkgolid B. Dies führte einerseits zu unvollständigen Ergebnissen und machte es auf der anderen Seite unmöglich die Wirkpotenz aller EGb 761 Komponenten eindeutig zu belegen.

Die hier vorliegende Arbeit hatte somit als primäres Ziel, die relativen mitochondrialen Schutzeffekte der Einzelkomponenten des EGb 761 Extrakts unter Verwendung eines einheitlichen Modells sowie einheitlicher Parameter und Bedingungen zu bestimmen. Dissoziierte Hirnzellen von Mäusen wurden als geeignetes *in vitro* Modell für diese Studie gewählt. Die mitochondrialen Funktionen wurden anhand des MTT-Viabilitätstests, des mitochondrialen Membranpotentials sowie des ATP-Spiegels ausgewertet. Hierzu wurden die Zellen mit den Einzelkomponenten in zwei unterschiedlichen Dosierungen inkubiert sowie externem Stress ausgesetzt.

Im Vorfeld zu den *in vitro* Tests der Einzelkomponenten wurde die Wirkung des Gesamtextrakts EGb 761 als Referenz bestimmt. Untersuchungen mit dem Gesamtextrakt wurden nicht nur *in vitro* an dissoziierten Hirnzellen von jungen und alten Mäusen sondern auch *ex vivo* nach einer 14-tägigen Behandlung mit EGb 761 von jungen und alten Mäusen sowie von transgenen Mäusen für mutierten humanen Amyloid Precursor Protein durchgeführt.

Auch hier wurde externer Stress angewandt und die mitochondrialen Funktionen anhand der oben beschriebenen Parameter erfasst.

Im Allgemeinen konnte die vorliegende Arbeit die positive Wirkung des EGb 761 auf die mitochondrialen Funktionen belegen, wobei der Effekt in gestörten und/oder älteren Mitochondrien deutlicher sichtbar war. Bei näherer Betrachtung der unterschiedlichen Komponenten scheint es sich hierbei um eine komplementäre Wirkung zu handeln. So vermögen sowohl die Flavonoid- als auch die Terpenfraktion eine protektive Wirkung auf Mitochondrien auszuüben, allerdings auf unterschiedlicher Weise. So unterscheiden sich nicht nur der Wirkungsmechanismus der Flavonoidfraktion von dem der Terpene, sondern auch die Wirkungen der Terpene untereinander. Darüber hinaus wurden signifikant unterschiedliche relative Aktivitäten in Abhängigkeit von Dosierung und Versuchsbedingungen festgestellt. So führte bei der ersten Dosierung eine Vorbehandlung mit ginkgolide A zu keiner Änderung der mitochondrialen Parameter, wohingegen eine Nachbehandlung mit ginkgolide A das mitochondriale Membranpotential, die ATP-Spiegel und die Zellviabilität nach nitrosativer Stress [NO] erhöhte. Sowohl die Vor- als auch Nachbehandlungsstudien weisen auf eine stärkere protektive Wirkung der Flavonoidfraktion bei NO induziertem, mitochondrialem Sress hin. Ginkgolide hingegen erweisen sich in der Regenerierung von Mitochondrien als effizienter als in der Neutralisation von freien Radikalen. In dem angewandten Zellmodell war ginkgolide J, trotz seiner Fähigkeit das mitochondriale Membranpotential bei Nachbehandlung zu erhöhen, am schwächsten wirksam. Die in der vorliegenden Studie beobachteten Effekte des EGb 761 Extrakts sind daher auf eine komplementäre Wirkung der Einzelkomponenten zurückzuführen.

Obwohl es sich hierbei um vielversprechende Ergebnisse handelt, die auf eine relative Wirksamkeit der Einzelkomponenten bei der Protektion von Mitochondrien hindeuten, sollte man bedenken, dass diese Versuche *in vitro*

an Mäusehirnzellen durchgeführt wurden. Eine *in vivo* Studie könnte noch vielversprechendere und präzisere Ergebnisse hinsichtlich der Wirkmechanismen von EGb 761 und seiner Einzelkomponeten liefern.

Im Rahmen der vorliegenden Arbeit wurde eine höhere Wirksamkeit von EGb 761 in älteren Mäusen oder den transgenen Mäusen beobachtet. Vor diesem Hintergrund wurde eine Langzeitstudie mit EGb 761 an alternden Mäusen durchgeführt. Um die Langzeitwirkung des EGb 761 auf die mitochondriale Funktion während des Alterungsprozesses zu evaluieren, wurde ein spezielles Mausmodell ausgewählt. SAMP-8 Mäuse [senescence accelerated prone mice], die eine schnelle Alterung mit entsprechend altersbedingten kognitiven Beeinträchtigungen, erhöhtem oxidativem Stress und Ablagerungen von Aß zeigen, sowie Seneszenz resistente SAMR1-Mäuse [senescence accelerated resistant mice] wurden in die Studie mit einbezogen. SAMP-8 Mäuse wurden für 5 Monate mit EGb 761 oder Placebo gefüttert. Zusätzlich zu den mitochondrialen Funktionen wurde auch die Membranfluidität bewertet, die als weiterer wichtiger Parameter eine große Rolle bei Alterungs- und Wahrnehmungsprozessen spielt. Im Vergleich zu SAMR-1 Mäusen waren in SAMP-8 Mäusen das mitochondriale Membranpotential und die ATP-Spiegel niedriger. Die Behandlung mit EGb 761 konnte diese mitochondrialen Funktionsstörungen lindern. Des Weiteren wurde eine höhere Membranfluidität nach EGb 761 Behandlung festgestellt, was entsprechend früherer Erkenntnisse in direktem Zusammenhang mit einer verbesserten Gedächtnis- und Wahrnehmungsleistung steht. Eine erniedrigte Membranfluidität in hippocampalen Synaptosomen von AD Hirnen wurde von unsere Arbeitsgruppe vor einiger Zeit berichtet, die auf eine reduzierte amyloidogene Prozessierung von APP und eine damit erhöhte Bildung von Aß zurückzuführen ist. Der weitere Fokus der vorliegenden Arbeit richtete sich daher auf den Zusammenhang zwischen den protektiven Eigenschaften des EGb 761 unter besonderer Berücksichtigung von Aß. Als Basis dienten

die Erkenntnisse über die positiven Wirkungen des EGb 761 auf die Membranfluidität und die mitochondrialen Funktionen sowie bereits vorliegende Forschungsergebnisse, die diese beiden Parameter mit der APP-Prozessierung und Aß Bildung korrelierten.

Infolgedessen wurde die Aß Bildung in transgenen Hek-sw Zellen nach Inkubation mit EGb 761 *in vitro* evaluiert. Basierend auf dem unerwarteten Anstieg des extrazellulären löslichen Aß nach Inkubation mit EGb 761, wurde der *in vivo* Einfluss von EGb 761 im transgenen Mausmodel untersucht. Tg-APP Mäuse exprimieren mutantes humanes APP im Überschuss und entwickeln damit hohe Spiegel an Aß. Im Anschluss an eine 21-tägige Behandlung von Tg-APP Mäusen mit EGb 761 wurden die Aß Spiegel im Hirn bestimmt. Zusätzlich wurde der Einfluss von EGb 761 auf die Produktion und den Abbau von Aß auf Gen-Ebene untersucht. Hierzu wurden die mRNA-Spiegel der Gene, die an dessen Bildung und Beseitigung beteiligt sind, evaluiert.

Die Erhöhung des löslichen Aß 40-Spiegels konnte in EGb 761 behandelten Mäusen bestätigt werden. Hingegen wurde der Gesamt-Aß-Spiegel im Vergleich zur Placebogruppe nicht verändert. Interessanterweise wurden die mRNA-Spiegel von APP erheblich reduziert, während der Spiegel der Aß abbauenden Enzyme [NEP, IDE and ECE-1] nach der Behandlung unverändert blieb. Nichtsdestotrotz wurde lediglich in EGb 761 behandelten Mäusen ein umgekehrt proportionaler Zusammenhang zwischen den Aß- und mRNA-Spiegeln festgestellt.

Auf der Basis dieser Beobachtungen und vorhandener Literaturdaten kann man argumentieren, dass EGb 761 die Verfügbarkeit von APP für die weitere Prozessierung zu Aß verringert. Nach Abspaltung des C-99 Fragment kommt es tendenziell zu einer verstärkten Bildung von Aß 40. Die Eliminierung von Aß und die damit einhergehende Verringerung seiner Akkumulation und weiteren Aggregation werden durch EGb 761 begünstigt. Dies kann einmal

durch die Regulierung des Transports von Aß in und aus dem Gehirn mittels RAGE und LRP erreicht werden. Ein weiterer Eliminierungsmechanismus, der allerdings noch bestätigt werden muss, könnte auf der Fähigkeit von EGb 761 beruhen, die Aktivität von Aß abbauenden Enzymen zu stimulieren.

Zusammenfassend hat die vorliegende Arbeit mit folgenden Erkenntnissen für die Forschung auf dem Gebiet der AD Prävention und Behandlung mit EGb 761 beigetragen: [1] EGb 761 verbessert mitochondriale Funktionsstörungen, die während des Alterungsprozesses und einer möglichen Alzheimer Erkrankung auftreten. [2] Die Gesamtwirkung des Extrakts ist auf eine komplementäre Wirkung der Einzelkomponenten zurückzuführen. [3] Eine Langzeit-Behandlung mit EGb 761 ist für die Prävention von altersbedingten mitochondrialen Abnormalitäten sowie für die Erhöhung der Membranfluidität vorteilhaft. [4] APP-Prozessierung sowie Aß Bildung und Aggregation, welche eine wesentliche Rolle in der Alzheimer Demenz spielen, können durch Behandlung mit EGb 761 in transgenen Maus-und Zellmodellen beeinflusst werden.

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9 Appendix

9.1 Publications and Presentations

9.1.1 Original publications and Reviews

Leuner, K., Hauptmann, S., **Abdel-Kader, R**., Scherping, I., Keil, U., Strosznajder, J. B., Eckert, A. and Muller, W. E. (2007)
Mitochondrial dysfunction: the first domino in brain aging and Alzheimer's disease? *Antioxid.Redox.Signal.*, **9,** 1659-1675.

Abdel-Kader, R., Hauptmann, S., Keil, U., Scherping, I., Leuner, K., Eckert, A. and Muller, W. E. (2007)
Stabilization of mitochondrial function by Ginkgo biloba extract (EGb 761®). *Pharmacol.Res.*, **56,** 493-502.

9.1.2 Other Publications

Franke, C., Noldner, M., **Abdel-Kader, R**., Johnson-Anuna, L. N., Gibson, W. W., Muller, W. E. and Eckert, G. P. (2007) Bcl-2 upregulation and neuroprotection in guinea pig brain following chronic simvastatin treatment. *Neurobiol.Dis.*, **25,** 438-445.

9.1.3 Oral Presentations

Ginkgo biloba extract (EGb 761®) protects mitochondrial functions *in vitro* and *ex vivo*
Young Researcher Meeting (2007), Muenster

9.1.4 Poster Presentations

R. Abdel-Kader, I. Scherping, S. Hauptmann, U. Keil, W. E. Müller
Mitochondrial protection using Ginkgo biloba extract (EGb 761®) *in vitro* and *in vivo* using several animal models.
4th Mitochondrial physiology conference [MiP] 2005, Schroecken, Austria

Abdel-Kader, R., Scherping, I., Hauptmann, S., Keil, U. and Müller, W. E.
Protective effects of Ginkgo biloba extract in different animal models.
47th Spring Meeting Deutsche Gesellschaft für Experimentelle und Klinische Pharmakologie und Toxikologie [DGPT] 2006, Mainz.

R. Abdel-Kader, I. Scherping, S. Hauptmann, U. Keil, W. E. Müller
Stabilization of mitochondrial function by Ginkgo biloba extract (EGb 761®)
Neuroscience society's 36th annual meeting 2006, Atlanta, Ga

R. Abdel-Kader, U. Keil, K. Leuner and W. E. Müller
Ginkgo biloba extract (EGb 761®) protects mitochondrial functions in both non-transgenic and APP transgenic mice.
ISAO Workshop 2007 (Current aspects in the field of Alzheimer's Research), Bad Honnef, Bonn 2007

9.2 Resume

Reham Mahmoud Abdel-Kader
509 Choueifat school str.
5th District, New Cairo, Helwan, Egypt
Cell Phone: +20163333000

E-mail: rora20@hotmail.com

Education

6/2004 - PhD student in Pharmacology, Institute of Pharmacology, Johann Wolfgang Goethe-University, Biocenter, Frankfurt, Germany

2001 Graduated from Faculty of Pharmacy, Cairo University, Egypt

1996 International General Certificate of Secondary Education (IGCSE)

Work Experience

12/2003-6/2004 Teaching assistant in the German University in Cairo, New Cairo, Egypt

9/2001-12/2003 Research assistant at the National Institute of Laser Enhanced Science, Cairo University, Egypt

Conferences and Workshops

- **DAAD workshop for young scientists from Arabic speaking countries**

Participated in the DAAD-workshop in Ulm-Germany (12/2002)

- **Cairo International Model United Nations (CIMUN-1999)**

Member in CIMUN in the American University in Cairo as a delegate in the commission on organized crime, Cairo, Egypt.

- **Cairo International Model United Nations (CIMUN-1998)**

Member in CIMUN in the American university in Cairo as a delegate in the commission of human rights, Cairo, Egypt.

Internships

- **8/2001** Internship in a private pharmacy in Cairo, Egypt
- **7/2000** Internship in a hospital pharmacy in Ulm, Germany
- **7/2000** Internship in the military hospital in Ulm, Germany
- **8/1999** Internship in Sanofi Aventis, Cairo Egypt

Computer Skills

- **Office applications**: Microsoft PowerPoint, Excel and Word
- **Design and drawing**: Adobe illustrator.

Language Proficiency

- **Arabic:** Mother tongue
- **English**: Fluent
- **German:** Fluent spoken and good written
- **French:** Fair

Personal Interests

- Traveling
- Hiking
- Gym
- Reading
- Badminton

Personal Information

- **Date of Birth**: 2/4/1980
- **Nationality:** Egyptian
- **Place of Birth**: Cairo, Egypt

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Appendix

My Family, my parents, my brother Wael and my sister Sarah, thanks for the support you gave me and believing in me. Special thanks to my father who gave me the first push and then supported me all the way. I am proud to have such a family, I love you. Oh I nearly forgot Koki and Kitty (our cats), thanks for…for being our cats!

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Leaving Germany is hard enough, but leaving you guys is devastating. Whenever I asked you for something I got it, so I'll ask you for one last thing, promise me not to disappear and to always stay in my life. Ihr fehlt mir!