CHAPTER II

LITERATURE REVIEW

***Centella Asiatica* (Linn.) Urban**

Synonyms: *Hydrocotyle asiatica*, Gotu Kala, Indian Pennywort, Indian Water Navelwort, Mandukaparni

Thai name: Bua Bok

Family: Apiaceae/Umbelliferae

Habitat: Asia and Africa

Part Used: Whole plant

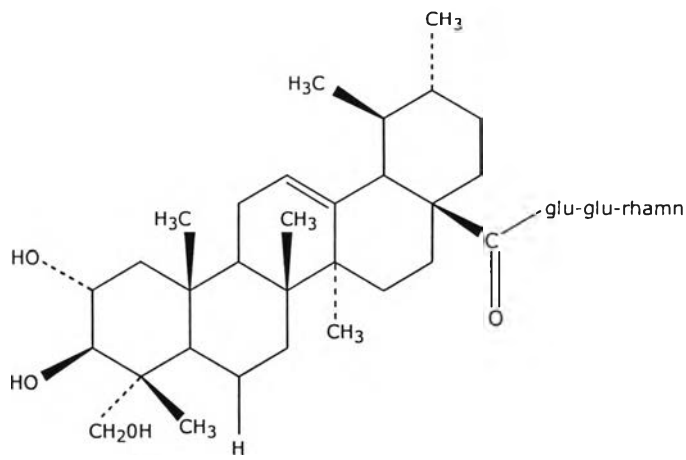

Figure 1 The chemical structure of asiaticoside

Chemical Components:

C. asiatica contains flavonoids (quercetin, kaempferol), various glycosides, terpenoids (asiaticoside, centelloside, madecasoside, brahmoside, brahminoside), madecassol, madecassic acid, asiatic acid, asiaticentoic acid, centellic acid, centoic acid, isothankuniside, fatty acids, amino acids, phytosterols, and tannin. The root region contains 14 different polyacetylenes.

Traditional Uses:

C. asiatica has been widely used in man for several decades. *C. asiatica* is used as a nourishing food and a valuable medicine in many cultures. In India, it is usually described under the name of "Mandukaparni" in the Ayurvedic system of medicine. The ancient Vedas used the plant either in poultries by direct application to the skin or by oral route to help heal wounds and ugly skin lesions of various origins. Fresh leaves were used for wound healing, gastritis, and anorexia in Nepal. In Madagascar and East Africa, this plant, dried and crushed, is used in the treatment of leprosy, bronchitis, asthma, syphilis, and as wound healing agent. Sometimes considered as a sedative, it is known to be tonic in Malaysia. In the Philippines, the leaves are either consumed raw in salads or as a tea for tonic and stimulant benefits to the body. The leaves have been employed medicinally in the French West Indies and Brazil to cure uterine cancer and leprosy. In the People's Republic of China, asiaticoside is used for fever, common cold influenza, sore throat and liver aliments such as cirrhosis and jaundice.

Nowadays, *C. asiatica* is the active ingredient of many drugs and cosmetic preparations in Europe, U.S.A. and Japan in the field of skin care for it as a revitalizing and healing herb. *C. asiatica* and its extracts were incorporated into the Indian Pharmacopeia in 19^{th} century with indications for inflammation and epidermal wound healing, e.g. leg ulcers and leprosy.

Medicinal Researches

Extracts of *C. asiatica* exhibit various pharmacological actions including:

1) Antipsoriatic

A cream formulation of *C. asiatica* was found to be successful in treatment of psoriasis in seven patients to whom it was applied (Natarajan and Paily, 1973).

2) Anticancer activity

Oral administration of the Centella extracted and its partially purified fractions retarded the development of solid and ascites tumors and increased the life span of these tumor bearing mice (Babu, Juttan and Padikkala, 1995).

3) Varicoses veins

Both asiaticoside and madecassoside are documented to be antiinflammatory, and the total saponin fraction is reported to be active in the

carrageenan rat paw edema test. Moreover, titrated extract of *Centella asiatica* (TECA) also showed significant improvement in symptoms, including edema, in patients with venous insufficiency of the lower limbs (Pointel et al., 1987).

4) Gastric ulcer

Studies in rats have shown that asiaticoside exhibits a protective action against stress-induced gastric ulcers, following subcutaneous administration, and accelerates the healing of chemical-induced duodenal ulcers, after oral administration (Ravokatra and Ratsimamanga, 1974a; Ravokatra et al., 1974b). It was thought that asiaticoside acts by increasing the ability of the rats to cope with a stressful situation rather than via a local effect on the mucosa. On the other hand, oral administration of Centella extract (0.05, 0.25, and 0.50 g/kg) prevented ethanol-induced gastric mucosal lesions in rats by strengthening action on the mucosal barrier and reducing the damaging effects of free radicals (Cheng and Koo, 2000).

5) Radiation ulcer

In another study, extracts of *C. asiatica* were able to reduce acute radiation reactions by its anti-inflammatory activity (Chen et al., 1999), and it could be useful in preventing radiation-induced behavioral changes during clinical radiotherapy (Shobi and Goel, 2001).

6) Wound healing and scar healing

C. asiatica extract was found to be useful for preventing and treating keloids and hypertrophic scars (Bossè et al., 1979). A Centella extract containing asiaticoside (40%), asiatic acid (29-30%), madecassic acid (29-30%), and madasiatic acid (1%), was stated to be successful as both a preventive and curative treatment, when given to 227 patients with keloids or hypertrophic scars. The effective dose in adults was reported to be between 60 and 90 mg. It was proposed that the triterpene constituents in the Centella extracted act in similar manner to cortisone, with respect to wound healing, and interfere with the metabolism of abnormal collagen.

The active ingredients of *C. asiatica* are claimed to possess wound healing properties. TECA (madecassic acid, asiaticoside and asiatic acid) acts on fibroblast cells and equilibrates collagen fiber synthesis. The overall effect contributes to the restoration of elastic connective tissue, a reduction in fibrosis, promotion of fibroblast proliferation and a shortening in the time necessary for wound healing (Maquart et al., 1990, 1999; Rosen, Blumenthal and McCallum, 1967; Shukla et al., 1999a;

Yoshinori, Reiko and Tsumematsu, 1982). Its wound healing effects may be due to its up-regulation of human collagen I expression (Bonte et al., 1994) and increase in tensile strength of wound (Suguna et al., 1996)

Since antioxidants have been reported to play a significant role in the wound healing process, the possible involvement of such a mechanism in wound healing promotion by asiaticoside was explored. The result showed that asiaticoside enhanced induction of antioxidant levels at initial stage of healing (Shukla et al., 1999b). Topical application of 0.2% asiaticoside twice daily for 7 days to excision-type cutaneous wounds in rats led to increased enzymatic and non-enzymatic antioxidants, namely SOD (35%), CAT (67%), GSH-Px (49%), vitamin E (77%) and ascorbic acid (36%) in newly formed tissues. It also induced a several fold decrease of lipid peroxide levels (69%)

7) Mental

Studies in mice and rats using brahmoside and brahminoside, by intraperitoneal injection, have shown a CNS-depressant effect. The compounds were found to decrease motor activity, increase hexobarbitone sleeping time, slightly decrease body temperature, and were thought to act via a cholinergic mechanism. A hypertensive effect in rats was also observed, but only following large doses.

In India, there was an impressive study dealt with the effect of *C. asiatica* on general mental ability of mentally retarded children. Whole plants were dried in the shade, powdered, and made into ½ gram tablets. Half of the thirty children studied were given one tablet and another half a placebo tablet daily. Apart from nutritional deficiencies, the children had no major illnesses. A Binet-Kamat test was administered and the children's intelligence quotients were recorded. Separate tests were also administered to record and changes in the children's co-operation, memory, concentration, attention, vocabulary and overall adjustment. After three months, the tests were repeated. The findings showed that youngsters taking *C. asiatica* had increased their power of concentration and attention.

It has also been reported that *C. asiatica* contains many antioxidant molecules like carotenoids, ascorbic acid, and terpenoids (Padma, Bhuvaneswari and Silambuchelvi, 1998). These antioxidants may scavenge free radicals, thus protecting the organism against the free radical-induced cytotoxic and genotoxic effects. Asiaticoside derivatives were tested for potential protective effects against Aβ-

induced cell death. Of the 28 asiaticoside derivatives tested, asiatic acid, asiaticoside 6, and SM2 showed strong inhibition of Aβ-induced death of B103 cells at 1 μM. The three asiaticoside derivatives were further tested for their effects on free radical injury and apoptosis. All three asiaticoside derivatives reduced H_2O_2-induced cell death and lowered intracellular free radical concentration. Three asiaticoside derivatives which protected neurons from Aβ toxicity did not alter physiological properties of the hippocampus at the concentration that blocks Aβ-induced cell death (Mook-Jung et al., 1999). In the recent years, various extracts from different parts of *C. asiatica*, leaves, petioles, and roots, were studied on antioxidative activity. Results showed that the extract of all parts exhibited high antioxidative activity as well as α-tocopherol; especially roots showed the highest activity of the parts tested (Abdul Hamid et al., 2002).

In the recent years, Centella extract was also found to improve learning and memory in rat, increase the levels of antioxidant enzyme and decrease brain lipid peroxidation (Gupta, Veeendra Kumar and Srivastava, 2003; Veerendra Kumar and Gupta, 2002).

Pharmacokinetics

A recent study in twelve healthy human volunteers investigated effects of single or repeated administration of *C. asiatica.* Thirty or sixty mg extract was administered orally to humans on a single occasion or once daily for 7 consecutive days. The assay method was only able to investigate levels of asiatic acid. The elimination half-life was 2 to 3 hours irrespective of the dose used. An extract containing asiatic acid, madecassic acid, madasiatic acid and asiaticoside, reached peak plasma concentrations in 2 to 4 hours. The peak plasma concentration, area under curve (0 to 24 hours) and the plasma half life were significantly increased following repeated administration of *C. asiatica.* These increases may in part be explained by the fact that asiaticoside is metabolized *in vivo* to asiatic acid (Grimaldi et al., 1990). The triterpene constituents, the active ingredients of *C. asiatica* extract, are reported to be excreted primarily in the feces in a period of 24 to 76 hours, with a small percentage excreted via the kidneys.

Toxicology:

In mice, a dose of 1 g/kg body weight of an extract of *C. asiatica* (in 50% ethanol) did not lead to any toxic effects. No mortalities were recorded.

Alzheimer's Disease

Alzheimer's disease, the most common cause of dementia, is a devastating illness characterized by cognitive deterioration as well as behavioral, affective, and psychotic disturbances. It is an age-related condition affecting 11% of the population over 65 years of age and 50% of that over age 85 (Hof et al., 1995). In 1907, Alois Alzheimer described a "peculiar disease of the cortex", stating that in sections which have been silver-stained, he found "strange alterations of the neurofibrils" and "foci" which are built up by a peculiar substance spread over the whole cortex. Ninety years later, the structures found by Alzheimer are well-known as neurofibrillary tangles (NFTs) and as senile plaques loaded mainly with Aβ.

AD is predominantly a disorder of the brain region that controls the human central nervous system (CNS). In a preclinical phase, already mild pathological alterations are detectable before the appearance of clear clinical symptoms. Clinically, AD is characterized by a progressive mental deterioration and significant changes in the personality with great individual differences with respect to the speed of the intellectual decay. While the episodic memory is lost very early, the short-term or working memory is preserved until very late in the course of AD development. Therefore, impairment of memory is an early feature of AD. This disease is currently an enormous health, social and economic burden to many countries. On the hopeful side, AD is also the most comprehensively studied degenerative disease of the nervous system, with major advances in the understanding of key pathological, epidemiological and molecular genetic factors in recent years.

Pathological hallmarks of Alzheimer's disease

There are specific abnormal structures that occur in the brains of individuals with AD that can be described as "hallmarks" of the condition.

1. β-Amyloid plaques

Masses of fine (7-10 nm) filaments form in the extracellular space within the brains of sufferers of AD. These filaments are comprised of an insoluble form of

the 4 kDa peptide, Aβ (Glenner and Wong, 1994), which is derived from the larger β-amyloid precursor protein (APP). In AD, these β-amyloid fibrils occur diffusely throughout the parenchyma as well as in more compact, spherical structures known as plaques, some of which may have a dense inner core. A variable proportion of β-amyloid plaques contain other proteinaceous and non-proteinaceous molecules and may also be associated with non-neuronal cells and abnormal (dystrophic) neuritic processes. The subset of β-amyloid deposits that are spatially localized with dystrophic neurites are referred to as "neuritic" plaques.

2. Neurofibrillary pathology

Profound cytoskeleton changes occur in neurons and their processes in AD. Abnormal filamentous structures, including paired helical filaments (PHFs) and straight filaments, contribute toward the formation of tangle-like inclusions in cell bodies, as well as neuritic structures near tangle-bearing neurons (neuropil threads) and the dystrophic neurites associated with plaques. The abnormal filaments that comprise neurofibrillary pathology are highly insoluble, such that "ghost" or "tombstone" neurofibrillary tangles (NFTs) remain in the extracellular space following the complete degeneration of the affected neurons.

Alzheimer's disease genes and risk factors

A) APP gene and Chromosome 21

The observation that patients with Down's syndrome (trisomy 21) develop cognitive deterioration and typical pathological features of AD by middle age led to the discovery of the APP gene on chromosome 21. Simultaneously, a locus segregating with a minority of early onset familial AD (FAD) kindred was mapped to this chromosome. Later, several missense mutations within the APP gene that resulted in amino acid substitutions in APP were identified in these FAD kindreds. It appears that such mutations alter the previously described proteolytic processing of APP, generating amyloidogenic forms of Aβ. Skin fibroblasts from subjects carrying APP mutations produced increased $A\beta_{42/43}$. Increased plasma concentration of $A\beta_{42/43}$ is also seen in these subjects regardless of age, sex, or clinical status.

B) Presenilin-1 and Presenilin-2

Fifty to seventy percent of early-onset autosomal dominant AD cases appear to be associated with a locus mapped by genetic linkage to the long arm of

chromosome 14. Numerous missense mutations have been identified on a strong candidate gene called presenilin-1 (PS1). At the same time, another autosomal dominant locus responsible for early-onset AD was localized to chromosome 1. Two mutations were identified on the candidate gene, designated presenilin-2 (PS2). The physiological role of presenilins and the pathogenic effects of their mutations are not yet well understood. Their two putative products, PS1 and PS2, share substantial amino-acid and structural similarities, suggesting that they may be functionally related. In addition, the expression patterns of PS1 and PS2 in the brain are very similar, if not identical. Both PS1 mRNA and PS2 mRNA are detectable only within neuronal populations. Mutations result in single amino-acid substitutions in PS1 and PS2. A selective increase in $A\beta_{1-42}$ was found in the plasma and skin fibroblast culture media of humans bearing PS mutations linked to FAD. Prominent deposition of $A\beta_{42/43}$ is found in many brain regions of patients with PS1 mutations. Molecular biological studies demonstrated that PS mutations alter APP processing, resulting in an increase of $A\beta_{1-42}$ (Thinakaran et al., 1996). On the other hand, mice deficient in PS1 showed markedly less γ-secretase processing of the C-terminal fragment of Aβ. In this regard, a recent molecular study has suggested that PS1 is directly involved in the cleavage of APP by γ-secretase (Wolfe et al., 1999).

C) Apolipoprotein E

The second gene to be specifically implicated in AD was the ApoE gene located on chromosome 19. Three major isoforms of ApoE (ApoE2, ApoE3, and ApoE4) are products of their respective alleles. The frequency of the ApoE4 allele is significantly higher in late-onset FAD than in the general population. ApoE4 allele increases the risk and lowers the age of onset distribution of AD (Corder et al., 1993), whereas ApoE2 allele lowers the risk and increases the age of onset distribution.

ApoE, a 34-kDa glycoprotein, is the major serum protein involved in cholesterol storage, transport, and metabolism, and is produced and secreted in the CNS by astrocytes. ApoE is accumulated in the senile plaques of AD patients. A neuropathological alteration in AD patients with ApoE4 allele is a significant increase in the number of amyloid plaque. ApoE binds to soluble Aβ *in vitro* and promotes amyloid fibril formation in an isoform-specific manner, with ApoE4 promoting Aβ fibrillogenesis more potently than ApoE3.

Table 1 Genetic factors associated with Alzheimer's disease

Chromosime	Gene Defect	Onset	Putative Mechanisms
21	APP	Early	Increased production of $A\beta_{1-42}$
19	APOE4	Late	Tau hyperphospherylation
			Impaired preduction / polymerization / clearance of Aβ
14	PS1	Early	Increased production of $A\beta_{1-42}$
1	PS2	Early	Altered Aβ metabolism

(modified form Felican and Sandson, 1999)

The β-Amyloid Protein

The AD-associated Aβ varies between 39 and 43 amino acid (aa) in length, and is derived from the larger precursor APP. APP is a transmembranous glycoprotein and three major isoforms can be found. While the 751 and 770 aa long isoforms, which contain a Kunitz protease inhibitor domain, can be found in neuronal as well as in non-neuronal tissues, the 695 aa long form of APP is expressed at high levels in neurons. Under normal conditions, APP is cleaved within the Aβ domain by a so-called α-secretase, leading to a 90-100 kDa soluble secreted form of the precursor (sAPP). An alternative processing driven by β- and γ-secretases cleaves APP at various sites in the extracellular and transmembrane domain that leads to the conversion of APP to the full-length soluble Aβ (Hass et al., 1992). Various factors can influence the processing pathway of APP cleavage, including metal ions. Interestingly, acetylcholine, the activation of protein kinase C, and also the female sex hormone estrogen, can promote the formation of the non-amyloidogenic sAPP. Consequently, the processing and, therefore, the routes that can lead to a "pathological" processing of APP are primary issues in AD research. Although the overall physiological role of APP in the brain is not well understood, it is known that sAPP has an autocrine function and stimulates the cell proliferation. In addition, it promotes the adhesion of cells to their substrate and protects neurons against excitotoxic and oxidative injury (Goodman and Mattson, 1994). Soluble APP can also interfere with intracellular signaling processes via G-proteins and increase MAP kinase activity. The overexpression of APP in transgenic mice leads to an overall increase in the number of cortical synapses. In conclusion, it

is intriguing to assume that a drop in the level of extracellular potentially neurotrophic sAPP could render neurons less protected and consequently, more vulnerable to exogenous neurotoxic insults. A striking correlation to the *in vivo* situation exists with the finding that the levels of sAPP are decreased in the cerebrospinal fluid of AD patients.

Aβ is a heterogeneous peptide in size due to differences in its C-terminus varying in the length. While so-called diffuse senile plaques almost exclusively consist of $A\beta_{1-42/43}$, classic senile plaques or neuritic plaques consists of $A\beta_{1-40}$, $A\beta_{1-42/43}$ and of shorter Aβ with truncated N-termini. The longer form of Aβ, $A\beta_{1-42}$, is less soluble and, therefore, forms fibrils much faster compared to shorter Aβs. For the formation of amyloid deposits, seeding and nucleation models are discussed. Therefore, it is believed that trace amounts of amyloid fibrils made up by $A\beta_{1-42}$ may be sufficient to promote the aggregation of shorter Aβ, such as $A\beta_{1-40}$. Based on these findings, the amyloid-cascade-hypothesis is modified and states that mainly $A\beta_{1-42}$ builds up so-called diffuse plaques (preamyloid lesions) and that these early lesions are thought to become more developed and compacted over decades, then leading to the typical senile plaque with deposited fibrillar Aβ and neuronal cell loss. Consistent with the fact that many preamyloid lesions are found in aged individuals with no neuropathology, and Down's syndrome patients with an extra chromosomal copy of the AβPP gene can have preamyloid lesions very early in their life. Then, preamyloid lesions could transform into neuritic amyloid lesions slowly overtime.

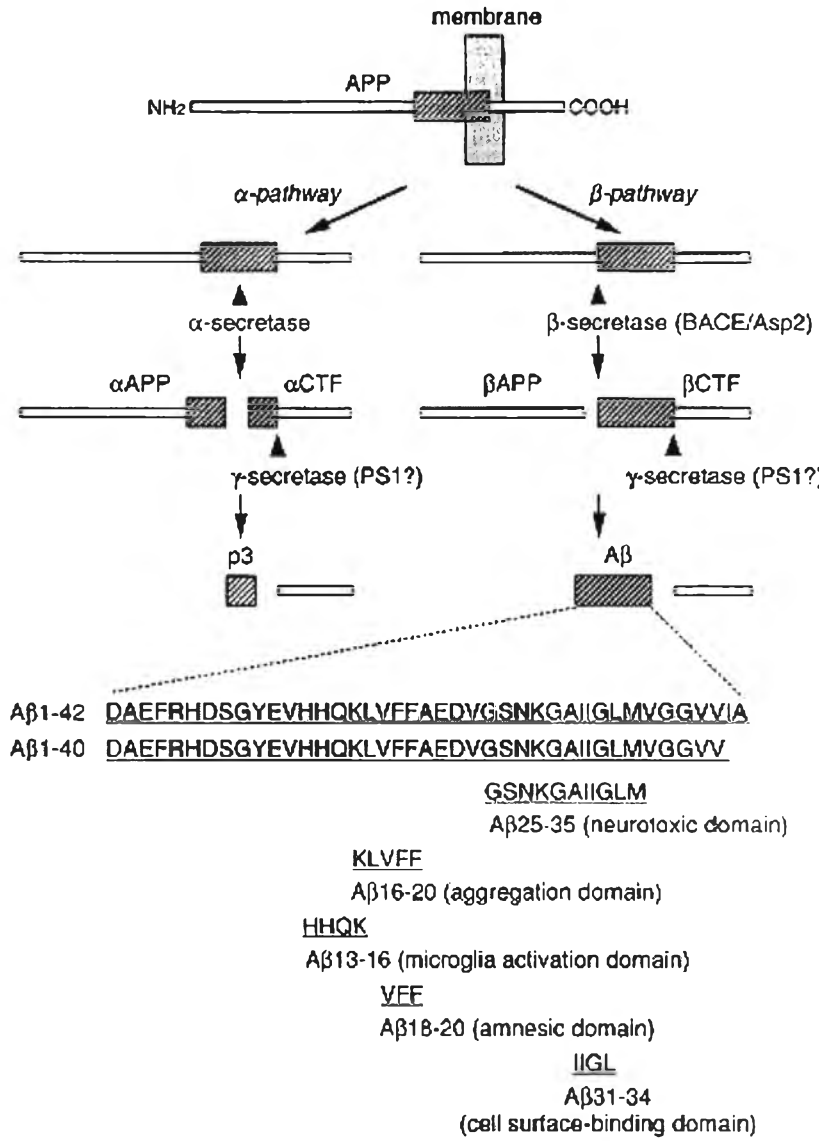

Figure 2 APP proteolysis and Aβ production. Processing of APP is divided into two pathways: the nonamyloidogenic α-pathway involves the cleavage of APP by α-secretase, and thereby precludes the formation of Aβ. In the amyloidogenic β-pathway, APP is cleaved by β-secretase, and then cut by γ-secretase. The β-secretase recently was identified and characterized as ACE/Asp2. PS1 plays a direct role in γ-secretase cleavage. Two major species of full-length Aβ defined by their C-terminus lengths are $A\beta_{1-40}$ and $A\beta_{1-42}$. Several functional domains in the Aβ sequence are proposed, including the neurotoxic, aggregation, microglial activation, amnesic, and cell-surface-binding domains. CTF, C-terminal fragment of APP; p3, the shorter APP fragment derived by α-secretase cleavage followed by β-secretase cleavage

(modified from Yamada and Nabeshima, 2000).

β-Amyloid Hypothesis

The Amyloid-Cascade-Hypothesis, Different lines of evidence support a causative role of Aβ and APP in the pathogenesis of AD, therefore, suggesting the deposition of Aβ as the central disease-causing and disease-promoting event:

1. Inherited mutations in the genes for APP and for PS are directly associated with AD;

2. The gene for APP is located on chromosome 21 which is overexpressed in Down's syndrome, where a typical AD pathology is found; and

3. The direct neurotoxicity of Aβ.

The amyloid-cascade-hypothesis is strongly supported by the genetic fact that every mutation isolated so far, which is linked to familial form of the disease, results in an abnormal APP processing and an increased Aβ production. This is a straight forward argument for the significance of Aβ in the AD process and indicates a final common pathological pathway (Haass, 1997). Besides this genetic evidence, a point of central importance in support of the amyloid-cascade-hypothesis is the intriguing fact that Aβ can directly act as a neurotoxin under certain.

β-Amyloid Cause Nerve Cell Degeneration

There is evidence that certain Aβs, particularly in an "aggregated" form, can cause nerve cell death or AD-like cellular changes *in vitro* and *in vivo* (Geula et al., 1998; Kowall et al., 1991; Weldon et al., 1998). One suggestion is that Aβ may be acting through a cell membrane receptor to stimulate the internal cellular changes leading to neuronal degeneration, including the abnormal phosphorylation of tau. In a similar fashion, it has been proposed that Aβ bind to a "receptor for advanced glycation end products (RAGE)", leading to increased intracellular OS. Alternatively, other studies have shown that Aβ by itself is capable of generating free radicals and reactive oxygen species that could cause neuronal degeneration by damage to membranes. Others have suggested that mitochondrial changes in AD result in aberrant oxidative phosphorylation. It has been postulated that Aβ may cause DNA damage and/or signal apoptotic pathways (Loo et al., 1993; Paradis et al., 1996). Markers for DNA damage have been shown to be present in the AD brain, including both neurons and glia (Sheng, Mark, and Griffin, 1998; Smale et al., 1995; Su et al.,

1994; Su, Deng, and Cotman, 1997). Interestingly, apoptotic markers have been found in a larger proportion of nerve cells than those affected by NFTs, indicating that there may be an alternate pathway to the degeneration of nerve cells. Subsets of apoptosis-related signaling proteins have also been demonstrated in glia and neurons, as well as select pathological hallmarks of the disease (Tortosa, Lopez and Ferrer et al., 1998). It has also been proposed that Aβ can cause a harmful elevation of intracellular calcium levels (Weiss, Pike and cotman, 1994), perhaps by the formation of calcium permeable channels. Alternatively, Aβ may potentiate the toxicity of other molecules, such as excitatory amino acids or TNF-alpha.

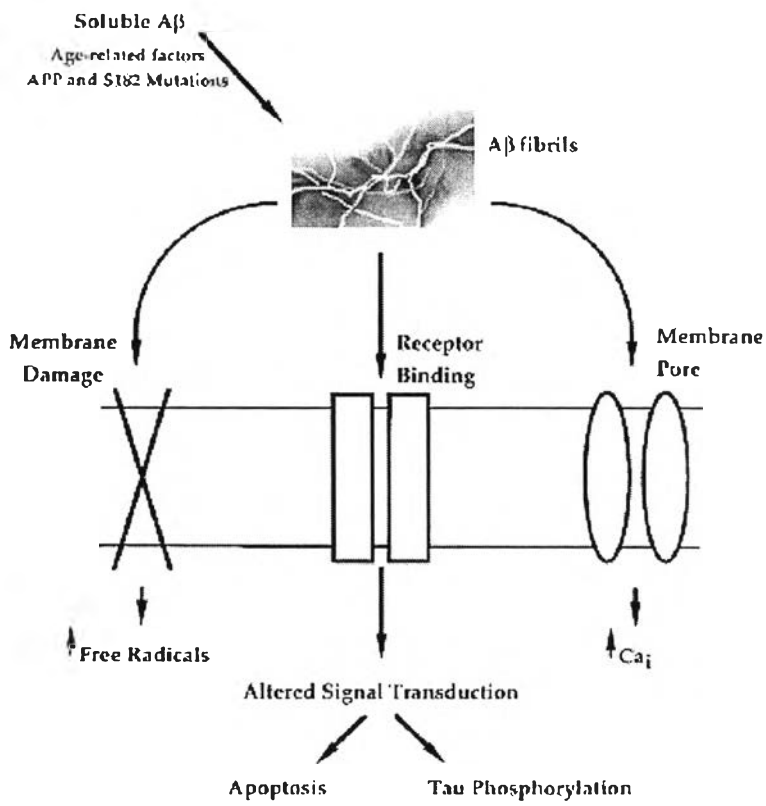

Figure 3 Aβ Fibril Formation and Potential Mechanisms of Neurotoxicity. Age-related factors or inherited mutations induce the formation of Aβ fibrils, which may cause neuronal degeneration through several potential cellular mechanisms

(modified from Yankner, 1996).

The Receptor for Aβ-Mediated Actions

A specific receptor could mediate the Aβ-mediated neurotoxicity and its characterization would be crucial to understand the neurodegeneration and apoptotic mechanisms reported to take place in AD brains. Several candidates have been proposed, including Aβ may induce its toxicity by engaging several binding sites on the membrane surface. The RAGE may be one of these receptors. RAGE is a member of the immunoglobulin superfamily of cell surface molecules known for its capacity to bind advanced glycation end products. This receptor is highly expressed by cortical neurons, especially in the hippocampus and cerebellum, during rat brain development. RAGE is also expressed in a variety of other cell types, including endothelial cells and mononuclear phagocytes. Activation of this receptor is believed to trigger cellular oxidative reactions. In addition, RAGE has been shown to mediate the interaction of Aβ protein with glial cells, which may be one of the first steps in the inflammatory cascade. Other receptors have also been implicated in AD, including the low density lipoprotein-related receptor protein (LRP), which binds multiple ligands including apolipoprotein E and α_2-macroglobulin, a suspected new gene for AD (Blacker et al., 1998). LRP can bind and internalize the secreted isoform of β-APP and an unprocessed amyloidogenic form of β-APP that can further lead to the formation of Aβ (Knauer, Orlando and Glabe, 1996). Recent evidence indicates that the LRP receptor of microglial cells may also play a role as an additional Aβ receptor (Marzolo, von Bernhardi, and Inestrosa, 2000). Since microglial cells can function as scavenger cells internalizing aggregates of Aβ and plaques the LRP receptor may play a role on the pathophysiology of the CNS during aging and AD. In addition, the distribution of LRP genotypes could be used as a genetic marker for the late onset of AD. Other candidate is the glycoprotein 330, (gp330)/megalin, a receptor for multiple ligands including apolipoprotein J, which has also been implicated in Aβ internalization and transport through the blood brain barrier (Zlokovic et al., 1996). Finally, the macrophage scavenger receptor expressed in microglia was found to internalize aggregated Aβ. In this way the scavenger receptor whose ligands include oxidized low density lipoproteins, could start immune mediated damage in AD (El Khoury et al., 1996; Paresce, Ghosh and Maxifield, 1996) a fact which is further supported by the strong expression of this receptor in association with senile plaques.

Oxidative Stress

A free radical is any chemical species that contains one or more unpaired electrons. Unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive than the corresponding non-radical, because they act as electron acceptors and essentially "steal" electrons from other molecules. This loss of electrons is called oxidation, and free radicals are referred to as oxidizing agents because they tend to cause other molecules to donate their electrons. The most common cellular free radicals are hydroxyl radical (OH•), superoxide radical (O_2-•), and nitric oxide (NO•) (Jenner and Olnaw, 1996; Simonian and Coyle, 1996). Other molecules, such as hydrogen peroxide (H_2O_2) and peroxynitrate ($ONOO^-$), are not free radicals, but can lead to their generation through various chemical reactions.

Free radicals and related molecules are often classified together as reactive oxygen species (ROS) to signify their ability to promote oxidative changes within the cell. Cells normally employ a number of defense mechanisms against damage induced by free radicals. Problems occur when production of ROS exceeds their elimination by the natural antioxidant defense system, or when the latter is damaged. This imbalance between cellular production of ROS and the ability of cells to efficiently defend against them is called OS (Ebadi, Srinivasan and Baxi, 1996). OS can cause cellular damage and subsequent cell death mainly by apoptosis in neurodegeneration because the ROS oxidize vital cellular components such as lipids, proteins, and DNA.

Oxidative Stress Involved in Alzheimer's Disease

OS has been implicated in the pathogenesis of AD by the finding of several characteristics, such as enhanced lipid peroxidation, in specific areas of the brain in postmortem studies. The suggestion that OS causes oxygen radical formation with resultant neurodegeneration and possibly plaque formation in the central nervous system was supported by the study of Frautschy, Baird and Cole (1991). Moreover Pappolla et al. (1997) provided evidence for the hypothesis that Aβ, the major constituent of the senile plaque, is neurotoxic and that such toxicity is mediated by free radicals *in vitro* and in a transgenic mouse model of AD.

Compared to all other tissues, which can potentially be damaged by ROS, the brain is particularly vulnerable to oxidative processes for several reasons:

1. The cells of the human brain utilize 20% of the oxygen consumed by the body but onstitute only 2% of the body weight, indicating the potential generation of a high quantity of ROS during oxidative phosphorylation in brain.

2. For the normal adult brain, glucose is the major nutrient and, therefore, the brain has a high glucose metabolism and respiratory turnover

3. Neuronal membranes of the brain consist of high concentrations of polyunsaturated fatty acids, which are potential substrates for the peroxidation by hydroxyl radicals (Halliwell, 1992; Porter, 1984).

4. A high content of iron has been reported for some brain areas (Gerlach et al., 1994), which can catalyze the generation of ROS.

5. The brain has only low levels of antioxidant defense enzymes compared to other tissues.

The oxidative phosphorylation in the mitochondria, numerous other enzymatic and non-enzymatic cellular mechanisms exist that can generate O_2-• or H_2O_2. Moreover, the production of ROS can result from the enzymatic conversion of catecholamines and indolamines by monoamine oxidase or from the non-enzymatic autooxidation of catecholamines. Superoxide can be produced from lipooxygenases, cyclooxygenases and various flavin oxidases. As the sources of ROS in nerve cells are numerous, the cells have to maintain an effective antioxidation defense system in order to protect themselves against free radical damage. Enzymatic and non-enzymatic antioxidants keep the fine-tuned balance between the physiological production of ROS and their detoxification. Because the oxidative phosphorylation, the main physiological source of ROS, is located within the defined micro-environment of the mitochondria, the free radicals are quickly reduced to H_2O. Leaking O_2-• can be dismutated by SOD to H_2O_2. The latter is a substrate for the intracellular antioxidant glutathione (GSH) and for the enzymes CAT and GSH-Px. Non-enzymatic and so-called chain breaking antioxidants, such as the lipophilic free radical scavenger α-tocopherol (vitamin E) and the hydrophillic ascorbate (vitamin C), the two most prominent antioxidants of their class, can directly react with ROS at the molecular level. In addition to its direct reaction with ROS, ascorbate is also necessary to regenerate vitamin E. An increased production of free radicals either

induced by an overdrive of endogeneous ROS generating systems or by exogenous oxidative insults challenges this intracellular balance maintained by the various antioxidants, ultimately, leading to a state of OS with massive cell damage and cell death (Behl, 1997).

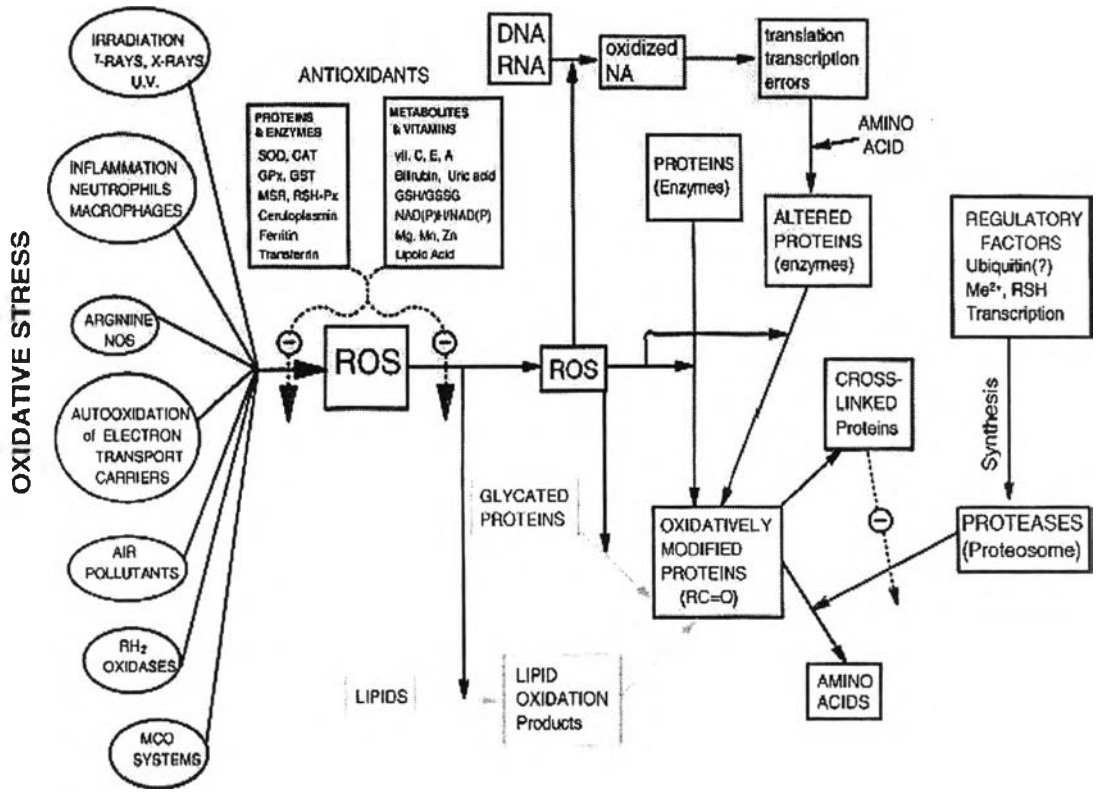

Figure 4 Accumulation of oxidized protein is dependent upon the balance between pro-oxidant, antioxidant, and proteolytic activities. MSH = methionine sulfoxide reductase; GPx = glutathione peroxidase; CAT = catalase; RSH-Px = thiol-specific peroxidase; NOS = nitric oxide synthetase; SOD = superoxide dismutase; GST = glutathione-s-transferase; NA = nucleic acid

(modified from Stadtman, 2002).

Oxidative Stress Induced by β-Amyloid

The mechanisms involved in the Aβ-mediated neurotoxicity are unclear, but there is evidence suggesting that *OS* plays a key role. The Aβ aggregation process is accelerated by transition metals via metal-catalyzed oxidation of Aβ peptide. Recently, it has been shown that Aβ peptide produces hydrogen peroxide (H_2O_2) through metal ion reduction, with concomitant release of thiobarbituric acid reactive

substances (TBARS), a process probably mediated by formation of hydroxyl radicals (Huang et al., 1999a, b). Free radicals peroxidize membrane lipids (Butterfield et al., 1997) and oxidize proteins (Stadtman, 1990, 2002) producing drastic cellular damages. The cytotoxicity of Aβ fibrils had been attributed to an oxidative mechanism and increased levels of H_2O_2 were detected.

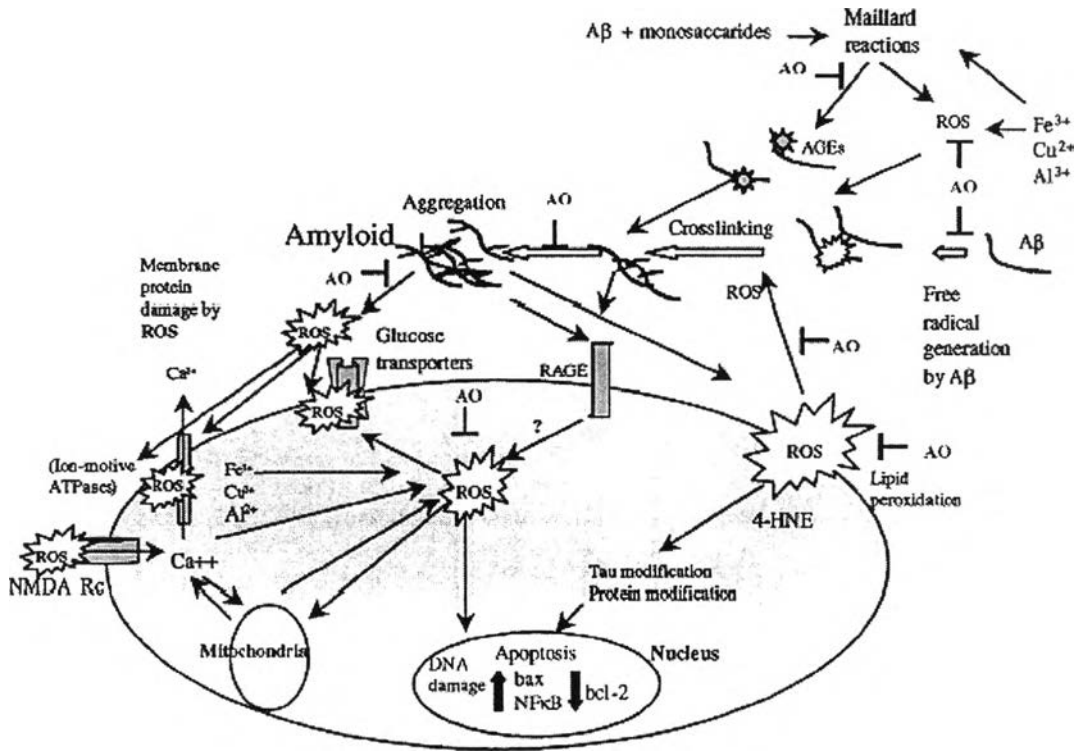

Figure 5 Sources of oxidative stress induced by Aβ and protection by antioxidants (AO). Aβ aggregation induces metal-catalyzed free radical generation which contributes to crosslinking of Aβ, increasing the production of Aβ fibrils. These oligomers formed by the crosslinking of reactive Aβ, could have themselves cytotoxicity activity by interacting with RAGE or other mechanism. RAGE activation causes generation of ROS, which induces the activation of transcription factors as NF-kB. ROS cause damage to membrane proteins, altering cell homeostasis by impairing ATPases and receptors. Moreover, the membrane protein damage produces the alteration of Ca^{2+} homeostasis. Lipoperoxidation leads to an increase in ROS and, by the action of 4-HNE, enhances the protein damage and contributes with triggering the apoptosis on neuronal cells

(modified from Marzolo et al., 2000).

Lipid Peroxidation

Lipid peroxidation is probably the most extensively investigated process induced by free radicals. The abundant presence of membrane phospholipids at sites where radicals in general and, more specifically, reactive oxygen species are formed render them easily accessible endogenous targets, rapidly affected by free radicals. Especially the group of polyunsaturated fatty acids is highly susceptible to reaction with free radicals. Peroxidation of lipids in fatty acids may lead to a radical chain reaction. Because of these chain reactions, one substrate radical (R•) may result in the formation of many equivalents of lipid peroxides (LOOH). These degenerative propagation reactions in lipid membranes are usually accompanied by the formation of a wide variety of products, including alkanes and carbonyl compounds. They may serve as second messengers for radical damage. Products resulting from lipid peroxidation are thus attractive parameters to monitor radical damage.

Malondialdehyde (MDA) Product; the Biomarker for Lipid Peroxidation

The most widely used index of lipid peroxidation is MDA formation, often assayed with the thiobarbituric acid (TBA) assay. Other AD is suspected etiology from radical-induced damage. Significant increases in GSH peroxidase activity, CAT activity and SOD activity were found in these patients. The data support the hypothesis that the brain in Alzheimer patients is under increased OS. Regardless whether the oxidative damage is an immediate cause of neurodegeneration or a result initiated by other factors; it could potentially accelerate the degenerative process, finally leading to neuronal death. Increased lipid peroxidation *in vivo*, as measured by TBARS, has been determined in brain tissue of Alzheimer's patients. Thus, MDA appears to be a promising non-invasive biomarker for free radical damage *in vivo*, an advantage of MDA over alkanes as products of lipid peroxidation is that MDA formation is little affected by oxygen concentration whereas alkane formation is decreased at high oxygen concentrations, as was observed in various *in vitro* systems.

Antioxidant Enzymes in AD

Continuous exposure of aerobic organisms to prooxidant challenges has endowed living cells with efficient and sophisticated antioxidant systems. These can be divided into enzymatic antioxidant and non-enzymatic antioxidant systems. As the most important members of the enzymatic defense systems against oxygen radicals

SOD, CAT and GSH-Px have been distinguished. Obviously, assaying these enzymes can offer an indication of the antioxidant status of an individual. Besides measuring the enzymatic antioxidant systems in blood samples, non-enzymatic antioxidants can be monitored as well, e.g., vitamin E and C, β-carotene, urate, retinyl esters and GSH. Antioxidant measurements have been applied in several human situations. Changes in antioxidant levels are likely to play a role in the induction of complications of the disease. Thus it was found that changes in antioxidant levels were correlated with the duration of the disease and with the development of complications. In patients suffering from AD, significantly increased GSH-Px, CAT and SOD activities were found in brain tissue, which were correlated with the increases in TBARS in these patients. This suggests a compensatory rise in antioxidant enzyme activities in response to increased free radical formation and oxidative damage in the brain.

Glutathione

The tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine) is the cellular thiol present in concentrations up to 12 mM in mammalian cells. It has important functions as antioxidant, is a reaction partner for the detoxification of xenobiotic, is a cofactor in isomerization reactions, and is a storage and transport form of cysteine (Meister and Anderson, 1983). In addition, GSH is essential for cell proliferation and maintains the thiol redox potential in cells keeping sulfhydryl groups of proteins in the reduced form (Cotgreave and Gerdes, 1998). In addition, recent results suggest that GSH plays a role in the regulation of apoptosis (Ghibelli et al., 1998; Hall, 1999). The GSH system is very important for the cellular defense against ROS. A high intracellular concentration of GSH protects against a variety of different ROS. GSH reacts directly with radicals in nonenzymatic reactions (Winterbourn and Metodiewa, 1994) and is also an electron donor in the reduction of peroxides catalyzed by GSH-Px (Chance, Sies and Boveris, 1979). It should be noted that the GSH system is only part of the cellular defense system against ROS.

During detoxification of ROS glutathione is involved in two types of reactions: (i) GSH reacts non-enzymatically with radicals such as the superoxide radical anion, nitric oxide or the hydroxyl radical (Winterbourn and Metodiewa, 1994; Singh et al., 1996) and (ii) GSH is the electron donor for the reduction of peroxides in the GSH-Px reaction (Chance et al., 1979). The final product of the oxidation of GSH

is glutathione disulfide (GSSG). Within cells GSH is regenerated from GSSG by the reaction catalyzed by glutathione reductase (GR).

Amyloid β-peptide-Related Nontransgenic Animal Models

The several numbers of studies have demonstrated that acute injection or continuous infusion of Aβ in to the brain causes brain dysfunction, as evidenced by neurodegeneration and an impairment of learning and memory (Nabeshima and Itoh, 1997; Pepeu et al., 1996; Sigurdsson et al., 1997; Yamada, Ren and Nabeshima, 1999). Freshly dissolved or 'aged' AP is injected into target area such as the hippocampus, cerebral cortex or basal forebrain nuclei. An *in vivo* model for the neurodegenerative effects of $A\beta_{1-40}$, including neuronal loss and degenerating neurons and neuritis, was first established by Kowall et al. (1991). Similarly, insoluble amyloid core from the AD brain produces neurotoxic effects when injected into the rat brain (Frautschy et al., 1991). The effects of Aβ fragments on learning and memory were first examined in mice (Flood, Morley and Roberts, 1991), and the results indicated the importance of the Val-Phe-Phe (VFF, $A\beta_{18-20}$) sequence for the amnesic effect. Subsequently, neurotoxicity of fibrillar Aβ fragments *in vivo* has been reported repeatedly in available animal test model, e.g. passive avoidance, Y-maze discrimination learning using an appetitive reinforcement and water maze performance. Many studies have demonstrated that various Aβ fragments, such as Aβ1-40, 1-42, and 25-35, cause learning and memory impairment in mice and rats (Chen, Wright and Barnes, 1996; Geula et al., 1998; Giovannelli et. al., 1995; Harkany et al., 1998; Maurice, Lockhart and Privat, 1996, 1998; McDonal, Dahl and Overmier, 1994; Nitta et. al., 1994; Pepeu et al., 1996; Sweeney et al., 1997).

Behavioral Tests

In years pharmacological techniques have been applied to the problem of learning and memory. Unfortunately, because of the complexity of nervous system and because of certain methodological problems to be described, definitive conclusions in this difficult area are not yet available. Nevertheless, some tentative conclusions can be drawn that are consistent with the available experimental evidence.

1. **Locomotor and movement tests:**

Locomotion is a complex behavior affected by many different brain systems, including the telencephalic dopaminergic system and the cerebellum, as well as by peripheral abnormalities (for example, muscle weakness or motorneuron degeneration). A change in locomotor activity could also result from general ill health of an animal. Because locomotor activity is required for many complex behavioral tasks, increases or decreases in locomotor activity can nonspecifically affect performance in many behavioral tests and should be measured before behavioral characterization.

2. **Learning and memory tests**

Learning is a complex phenomenon subserved by the activity of many brain regions. Learning usually refers to a change in behavior as a result of practice of observation. Typically, the term learning is reserved for the initial period during which a new habit is acquired. The term memory refers to the intervening variable, or underlying storage process, by which a change in behavior potentiality is preserved across time. Some aspects of learning that can be measured in rodent include attention, working memory (the short-term memory used while a task is being performed), memory consolidation, and long-term memory (lasting from 24 h to the lifetime of the animal).

The measurement of memory at a time after learning has occurred is usually considered to be a test of retention. Thus through learning the organism acquires the potential to exhibit modified behavior (retention) at a later time. It is useful to distinguish between changes in behavior resulting from alterations in learning or memory processes and changes in behavior that depend on performance variables, like motivation and motor dexterity.

The term retrieval is currently a somewhat controversial term. It is typically applied to the process required to make a learned response available during a retention test. A controversy can arise, however, when an organism fails to perform a learned response. Even when the influence of performance variables can be ruled out, failure to exhibit a learned response could conceivably arise from (1) an alteration in the memory storage process per se or (2) a defect in a hypothetical retrieval process. A final term requiring definition is consolidation. Consolidation refers to a hypothetical process occurring after training, during which time the memory storage

process becomes progressively invulnerable to disruption. In its most popular version consolidation refers to the gradual conversion of memory from a labile, "short-term" form to a more permanent, "long-term" form.

Various tests have been developed that evaluate preferentially one or another of these aspects of learning. For example:

2.1 The Y-maze, in which rodents are trained to visit a pattern of arms in the maze, is particularly geared toward measuring working memory. The animal must keeping mind which arms of the maze it has already visited during the course of the task to perform well.

2.2 The Morris water maze, in which an animal uses three-dimensional cues in the testing room to learn to find a hidden platform in a swimming pool, measures spatial learning which is critically dependent on hippocampal function. The test involves repeated trials in which the animal is placed in different parts of the pool, and the time taken to find the hidden platform is measured. Plotting the time to find the platform on successive trials generates a learning curve that can be used to compare the acquisition of the spatial learning task between animals. Long-term memory can also be measured 24 h or more after the final training trial in a task called the transfer test. In the task, the hidden platform is removed, and the time that the animal swims in the area where the platform used to be is record.

2.3 Aversive learning can be measured using the passive avoidance task. This task takes advantage of rodents on natural tendency to avoid well-light places in favor of dark places. On day 1, animal is allowed to explore apparatus. Once it enters dark chamber, a foot shock is administered. Next time when the animal is subsequently placed in the light area, it tends to remain there rather than to enter the area where it was previously shocked.