CHAPTER II

LITERATURE REVIEW

Neuropathology of Alzheimer's disease

1. Morphological characteristic of AD

Microscopically, the AD brain is smaller than average. The atrophy is usually symmetrical between the hemispheres, thought the degree of atrophy between lobes sometimes varies. Typically, the temporal lobes are more severely affected. Conversely, the primary motor and sensory regions of the brain are relatively spared (Kuo et al., 2001).

2. Histopathological feature of AD

It was almost a century ago when Alois Alzheimer first described the clinico-pathological findings from a patient he followed a few years after the onset of dementia in her late 40s until her death at age. Using newly developed silver stains, Alzheimer observed the two defining neuropathological lesions later associated with all forms of AD, neurofibrillary tangles and senile plaques which have been considered the major pathological hallmark of AD. Neurofibrillary tangles consist of intraneuronal bundles of abnormal filaments, composed a highly phosphorylated form of the microtubules-associated protein tau which has first twisted together in pairs to form paired helical filaments (PHF). Senile plaques are composed of extracellular deposits of various types of proteins, especially β-amyloid peptides, which are intimately associated with dystrophic axons and dendrites as well as activated microglia and reactive astrocytes. Large numbers of both senile plaques and neurofibrillary tangles are found in the memory and intellectual function portion of the brains of AD patients. Plaques and tangles accumulate in highest numbers in the hippocampus and the neocortex. Functionally, these regions play important roles in memory and cognitive function. These functions are particularly compromised in Alzheimer patients and represent the hallmark symptoms in the earlier stages of the disease (Kuo et al., 2001).

Components of theories of causality

There are many scientific theories concerning the cause of Alzheimer's disease. The simple fact in the cause of Alzheimer's disease is that the nerve cells in several regions of the brain are attacked and eventually destroyed. Why and how these events occur in Alzheimer's are the basis of many controversial scientific theories. A definite cause of AD is unknown. Current wisdom suggests that there may be a number of factors that must occur in sequence or combination to bring about the condition. The following list presents many of the prevalent theories on cause today.

1. The neurotransmitter imbalance

Memory processes in the hippocampus and cortex are mediated through the neurotransmitter, acetylcholine (ACh), which becomes depleted as Alzheimer's disease progresses. The resultant decrease in ACh may be due to either a decrease in the activity of the enzyme responsible for producing ACh (choline acetyl transferase), or an increase in the enzyme that destroys ACh (acetylcholinesterase). In fact, the extent of the deficit in cholinergic neurochemical parameters also correlates well with the numbers of plaques and tangles present (Capsoni et al., 2000). Excitatory amino-acids (EAAs) (glutamate and aspartate) may also play a role in the cause of AD. Changes in levels of these have been measured in AD. They may be not only directly neurotoxic but also by indirect pathways as they increase reactive oxygen species and thus oxidative stress (Behl et al., 1994; Nakajima et al., 2001). Nitric oxide hypothesis, NO is an important component of brain function; however, increased amounts can cause brain cell death. Increased NO is released in response to various stresses, and toxins released by infective agents (Parks et al., 2001).

2. The genetic factor

Studies done on families with many cases of Alzheimer's occurring in late life have implicated a gene on chromosome 19. The gene codes for a protein called apolipoproteinE (apoE) that can bind to β-amyloid. Once researchers discovered that 40% of Alzheimer's patients had, called apoE4, they began to suspect that it may

latch onto β-amyloid and make it more likely to form plaques. Other genes on other chromosomes have also been implicated. The genetic risk factor explains the idea that family history can be an element in AD. Loci on chromosomes 1, 14, 19, and 21 have been associated with AD. Subsequently it has been found that certain genetic defects result in a higher prevalence of AD (Plassman and Breitner, 1996).

3. Oxidative stress

Free radicals are produced as by-products of oxidative metabolism and can cause oxidative damage to proteins, lipids and nucleic acids, which might be relevant to AD pathogenesis. Supplementation with the antioxidant vitamin E has been associated with reduced disease progression in AD (Sano et al., 1997). Altered oxidative metabolism has been reported in AD postmortem brain (Valla et al., 2001). Studies of cultured primary neurons revealed increases especially in intracellular $A\beta_{1-42}$ upon oxidative stress (i.e. hydrogen peroxide treatment), and protection against this with concomitant antioxidant treatment (Behl et al., 1994).

4. Inflammation or immune response

Retrospective epidemiological studies have suggested that intake of anti-inflammatory medications is protective against the development of AD (McGeer et al., 1990). Inflammatory processes occur in the brain in AD, including gliosis and recruitment of microglia to sites of AD pathology. Interestingly, markers of inflammation have been shown even to precede Aβ deposits in transgenic Familial Alzheimer's disease (FAD) mutant amyloid precursor protein (APP) mice. Although inflammatory processes are general features of most types of brain disease, a role for inflammatory processes received support from recent reports indicating protection against and reversal of Aβ plaque deposition in transgenic FAD mutant APP mice immunised with Aβ intravenous infusion of Aβ led to reductions in Aβ plaques (Monsonego et al., 2001).

5. Glucose metabolism

When the metabolism, or breakdown, of glucose is disturbed, the cells may not be able to manufacture neurotransmitters like acetylcholine or they may react

abnormally to such chemical messengers. Eventually they die. Researchers are trying to find out whether the decline in glucose metabolism they see in AD's patients is due to the disease or causes the disease (Arnaiz et al., 2001).

6. Cardiovascular and cholesterol hypotheses

Converging epidemiological and biological evidence have also implicated cardiovascular factors in the development of AD. Risk factors for atherosclerosis, such as hypertension, high cholesterol, diabetes mellitus and especially apoE genotype, increase the risk of developing AD (Skoog et al., 1999). Moreover, intake of cholesterol-lowering statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) has been reported in retrospective studies to be associated with a lower prevalence of probable AD (Fassbender et al., 2001).

7. The environment

Many theories have examined the influence of environmental factors on AD. The brains of Alzheimer's patients have aluminum levels up to 30 times higher than other, age-matched people. However, it is not clear whether the aluminum resulted in Alzheimer's, or whether Alzheimer's resulted in aluminum buildup. Support of this theory is partially based on the observation that the injection of aluminum salts into animals leads to brain changes similar to the tangles and plaques found in Alzheimer's. Scientists are also searching in the areas of foodborne poisons and viruses for answers to the Alzheimer's cause (Abreo et al., 1999).

8. Tau hypothesis

One hypothesis is that in neurodegenerative diseases, tau function is disrupted through phosphorylation or through the binding of effector molecules which leads to its polymerization. Tau is a normal cell protein used in the construction of neuron microtubules that helps distribute nutrients. In a cell affected by Alzheimer's, something causes the tau molecules to twist, collapsing the scaffolding, and killing the cell. Research has found many enzymes that will phosphorylate tau and in AD the tau in the PHF is often abnormally phosphorylated. The pathological significance of this is

unknown. It has been reported that phosphorylated tau will not bind well to tubulin and this would destabilize the formed microtubles, which would degenerate. Whatever the cause or process, the PHF form masses of tangles which grow to occupy the cytoplasm and pushes the nucleus to one side. The tangles remain intracellular until they eventually cause cell death. This may be disrupting the function of the mocrotubules, or they could simply destroy the cystoplasmic functions (Alonso et al., 2001).

9. Amyloid cascade hypothesis

β-amyloid (Aβ) is also normally found in healthy individuals but something makes it accumulate abnormally in Alzheimer's patients. The amyloid cascade hypothesis predicts that β-amyloid plaque development in the brain has an early and essential role in the neuronal degeneration that leads to dementia. A causative role for Aβ in the neuropathology associated with AD is supported by several lines of evidence. In the brains of AD patients, amyloid plaques containing β-amyloid are surrounded by dystrophic neurons and areas of gliosis. The current prevailing version of the 'amyloid cascade hypothesis' is based on the neurotoxic properties of Aβ, and increased secretion of Aβ leads to elevated extracellular levels of Aβ as senile plaques, which in turn are toxic to surrounding neurons (Hsiao et al., 1996).

The amyloid cascade hypothesis for AD received major support with the discoveries of autosomal dominant forms of early-onset familial AD (FAD) with specific pathogenic mutations in APP and these mutations appear to promote increased production of β-amyloid (Selkoe, 1996). In addition, transgenic mice expressing mutant forms of APP show a brain region specific increase in β-amyloid deposition which increases with age and results in AD-like neurodegeneration in those brain areas containing β-amyloid (Tong et al., 2001). The presence of AD pathology with trisomy of chromosome 21 (Down's syndrome), the chromosome where APP is located, had previously suggested such a genetic link, since an extra copy of APP is thought to increase amounts of Aβ. Subsequently it was found that genes encoding the presenilins 1 and 2, located on chromosomes 14 and 1 respectively, are associated with forms of early-onset autosomal dominant FAD, and also cause increased amounts of Aβ.

Furthermore, a major genetic risk factor for the more-common, late-onset, 'sporadic' AD, is the apoEe4 allele, which correlates with increased Aβ burden. Moreover, the generation of transgenic mice bearing human FAD mutations led to remarkable AD-like plaque pathology. Thus, cumulative data supported the concept that Aβ is central to AD, and resulted in the hypothesis that all forms of AD might have increased Aβ as an underlying and unifying pathogenic mechanism (Selkoe, 1996).

Aβ accumulation and APP processing in Alzheimer 'disease

Aβ, a 39-43 residue peptide, is produced through proteolytic of the large membrane-anchored APP by enzymes known as β- and γ- secretases. It is now known that Aβ is present in human plasma and cerebrospinal fluid and it is secreted by cultured cells during normal metabolism (Haass and De Strooper, 1999). Thus, Aβ is produced by a normal processing pathway, which does not involve cleavage within the β-amyloid sequence. The secreted peptide appears to comprise mainly residues $Aβ_{1-40}$, although the proportion of the peptide comprising residues $Aβ_{1-42}$ increases when production is accelerated. Previously, it was believed that the key to plaque formation was an abnormal processing of APP resulting in the β-amyloid peptide which had a propensity to self aggregate to form insoluble fibrils. There is, however, uncertainty about the pathways responsible for APP processing and their cellular location. Two major competing pathways exist that are capable of processing APP. Each pathway involves the actions of protease that cleave the protein at certain sites to produce fragment that may be either non-amyloidogenic, do not contain the full Aβ sequence or amyloidogenic, contains the complete Aβ sequence.

The non-amyloidogenic pathway, which is the constitutive secretory pathway, processes the majority of APP. In this setting an α-secretase cleaves within β-amyloid at positions 16-17 that precludes the formation of Aβ by cleaving within the Aβ sequence. Therefore this pathway is non-amyloidogenic and does not contribute to the pathological lesion of AD. The resulting large soluble amino-terminal fragment of APP is found in human CSF. Little is known of the identity of this α-secretase although it does

appear to cleave APP at a fixed distance from the cell surface rather than recognizing specific sequences.

Alternatively, APP may be proteolytically cleaved by the competing amyloidogenic pathway that result in the release of fragments which include intact Aβ. Endocytosis of cell surface APP generates C-terminal fragments of 8-12 kDa that are degraded in the lysosomes, some of these generated by β-secretase cleavage at the N-terminus of the Aβ sequence are amyloidogenic. A second protease activity called γ-secretase cleaves these C-terminal fragments of APP to release the full-length Aβ protein. Thus, Aβ is produced normally at low levels by the cell. Most of the Aβ that is generated is secreted, although small amounts can be detected intracellularly. The normal proteolytic processing of APP involved cleavage of a central region of β-amyloid by α-secretase to give rise to a large secreted N-terminal fragment, and a smaller C-terminal component. The crucial pathological change that leads to plaque formation may, therefore, involve a relative change in the processing of APP by α-secretase relative to the β- and γ-secretases (Haass and de Strooper, 1999).

In recent years, it has been revealed that an alternate proteolytic pathway gives rise to an insoluble form of the entire β-amyloid peptide through the action of putative β- and γ-secretases (Gouras et al., 1998; Golde et al., 2000). However, it has also been determined that Aβ can occur in alternate forms, which differ only C-terminus, including shorter, $A\beta_{1-40}$ and longer $A\beta_{1-42}$ peptides, which have been proposed to contribute differentially either to the formation of insoluble filaments or the relative toxicity of plaques. $A\beta_{1-42}$ shows a higher propensity for fibrillogenesis (Lorenzo and Yankner, 1996) and a shift to a higher proportion of this isoform over $A\beta_{1-40}$ may be crucial to the earliest stages of fibril deposition into plaques.

Two variants, $A\beta_{1-40}$ and $A\beta_{1-42}$, which differ by truncation at the carboxyl terminus are the predominant plaque proteins. Both are found in the blood and cerebrospinal fluid at nanomolar levels. Experimental data indicate that different molecular forms of Aβ affect a wide variety of neuronal function and thereby may lead to neuronal death in the nervous system. Biochemical analysis of the amyloid peptides

isolated form AD brain indicates that $A\beta_{1-42}$ is the principle species associated with senile plaque amyloid, while $A\beta_{1-40}$ is more abundant in a soluble form in cerebrospinal fluid and more abundantly generated, and produced by different cell types and normally present in the circulation (DeMattos et al., 2001). It is capable of forming stable amyloid fibres but the rate of aggregation is a very slow process in the absence of nucleating agents (Westlind-Danielsson and Arnerup, 2001). On the contrary, the rate of aggregation is increased for the longer $A\beta_{1-42}$ that is the major constituent of insoluble amyloid fibres of senile plaques which comprise $A\beta$ peptides of vary lengths, attention has focused on $A\beta_{1-42}$, as being particularly important and, is more specifically linked to AD. Since the first plaques in AD are composed of $A\beta_{1-42}$, all forms of FAD cause an increase in the $A\beta_{1-42}$, which is particularly neurotoxic that is thought to be central in AD pathogenesis.

The four genes known to either cause or be major risk factors for AD have been shown to increase the production of $A\beta_{1-42}$ including mutations in trisomy 21 (Down's syndrome), chromosome 21 (APP), chromosome 14 (presenillin 1), chromosome 1 (presenillin 2). All mutations increase amyloid production, when mutations used to create transgenic mice lead to increase amyloid and neuritic plaques. The plasma concentration of variant $A\beta_{1-42}$ increases in FAD patients bearing an APP mutation, and also, strikingly, in FAD patients bearing mutations on presenilin 1 or presenilin 2 (Scheuner et al., 1996; Calhound et al, 1998; Iversen et al., 1995). Analyses of $A\beta$ in genetically engineered cell lines expressing FAD mutations in both APP and the presenilins have shown that all of the mutations cause either increased overall secretion of $A\beta$ or secretion of the longer form, $A\beta_{1-42}$ relative to the shorter form, $A\beta_{1-40}$ (Ancolio et al., 1997; Siman et al., 2000). In vitro toxicity of $A\beta$ peptide fragments (i.e. $A\beta_{1-40}$, $A\beta_{1-42}$, $A\beta_{1-28}$, and $A\beta_{25-35}$) were examined in hippocampal neuron cultures. Of these fragments, $A\beta_{1-42}$ was the most potent to affect cell survival (Dore et al., 1997).

Polymerization of β-amyloid peptide

The basic hypothesis in AD has been that enzymatic cleavage of APP generates the release of monomers of Aβ, ultimately into the extracellular millieau. It was found that AD is associated with the overproduction of an Aβ that either misfolds or polymerizes into aggregates within or around nerve cells. The formation of Aβ as aggregates and its deposition in senile plaques are believed to be a central step in the pathogenesis of AD. Under certain circumstances, Aβ aggregates into long filaments that can not be cleared by the body's usual scavenger mechanisms. The aggregates for the Aβ, which make up the neuritic plaque in Alzheimer patients (Stephan et al., 2001). The toxic Aβ-amyloid fragments can build up outside of the cell. Conformation changes, which change α-helices to β-sheets, can occur. β-sheets are less soluble than α-helices, causing them to fall out of the solution, forming toxic plaques in the brain and which, at some point during the fibril formation process, acquire neurodegenerative properties.

Since Aβ has the ability to aggregate into insoluble fibers, it has been proposed that the precipitates resulting from the aggregation status of Aβ may induce neuronal cell death. The correlation of neurotoxicity with direct measurements of secondary structure confirmed the previous observations that aggregation of Aβ was necessary to impart direct neurotoxic properties to the peptide (Pike et al., 1993; Lorenzo and Yankner, 1994; Ray et al., 2000). Thus, in many cases, freshly dissolved synthetic Aβ was lacking in such activity while peptide that had been incubated at 37°C for hours or even days was neurotoxic, a property associated with the formation of β-sheet folded peptide. Whereas freshly made solutions of β-amyloid 1-40 and 1-42 are not toxic to cultured neurones, "aging" of the solutions by incubation for many days produces material that kill neurons. When synthetic β-amyloid dissolves readily in water to yield monomers, but after several hours at 37°C in buffer at pH 7.4, soluble oligomers of increased molecular weight can be identified by gel filtration, polyacrylamide gel electrophoresis, or by specific ELISAs. Circular dichroism measurements show that oligomerisation is followed by a change in structure from a random and partly α-helical secondary structure to β-sheet. In time, fibrils come out of solution. Dropping the pH to

that found in some cellular compartments (pH 5.5), which balances the positive and negative charges in the peptide, enhances β-sheet formation. Zinc, aluminium, or the presence of proteins like α-1-antichymotrypsin or apolipoprotein E4 which have been implicated in Alzheimer's disease, also increase the rate of β-sheet formation (Pappolla et al., 2000). Initially, it was thought that only the fibrillar form of Aβ was toxic to neuron but through characterization of Aβ structure demonstrated that dimers and small aggregates of Aβ are also neurotoxic (Matsuoka et al., 2001; Yoshiike et al., 2001) and, in cell culture systems, can be formed intracellularly (Podlisny et al.,1998). Recently, it has been demonstrated that small oligomers of Aβ can form intracellularly (Walsh et al., 2000; Sudoh et al., 2002). There is evidence that certain β-amyloid peptides, particularly in an aggregated form, can cause nerve cell death or AD-like cellular changes in vitro (Pike et al., 1993; Lorenzo and Yankner, 1994; Lambert et al., 2000).

Fibril form of Aβ is remarkably stable in the CNS in vivo, which suggest that Aβ fibril neurotoxicity is mediated in large part by factors released from activated microglia and astrocytes, as opposed to direct interaction between Aβ fibrils and neurons (Weldon et al., 1998). Although neurodegenerative properties have been demonstrated for a variety of multimeric forms of Aβ, the precise identity of the neurotoxic species has been the topic of much debate. Analysis of AD brain tissue suggested the presence of dimers, trimers and oligomers of Aβ (Kawarabayashi, et al., 2001). Aβ has been shown to form small toxic oligomers or protofibrils in vitro (Hartley et al., 1999; Walsh et al., 1999).

Toxicity of Aβ in vitro is clearly correlated with aggregation into cross- β-pleated sheet fibrils. Several groups have proposed that Aβ associates specifically with cells via membrane-bound receptors (Dineley et al., 2001). An alternative hypothesis is that Aβ aggregates are toxic via nonspecific association with cell membrane. There is growing evidence indicating that Aβ-membrane interactions occur that affect both peptide and membrane properties. For example, Aβ binds to rat cortical homogenates in vitro in an aggregation-dependent manner (Good and Merphy, 1995). Membrane components promoted changes in Aβ secondary structure and/or aggregation propensity (Koppaka and Axelsen, 2000). Aβ or its fragments reportedly cause the

formation of large ion channels in phospholipid planar bilayers (Terzi et al., 1997; Del Mar Martinez-Senac et al., 1999).

Mechanisms of Aβ-induced cellular perturbation

There are several different ways that these plaques can damage the brain. Although most neurodegenerative events are of multifactorial origin, the mechanisms by which aggregates produce neurotoxicity are being elucidated. It has been suggested that Aβ is either directly or indirectly cytotoxic to neurons. Aβ cascade leading to neuron death is the central process and causes the loss of synapses, which is earliest detectable change and the synaptic density correlates with dementia severity (Hsia et al., 1999). While it seems certain that Aβ plays a role in neurotoxicity associated with AD, the molecular mechanism of Aβ neurotoxicity remains unclear.

Aβ induces membrane blebbing and cell shrinkage followed by DNA damage, the generation of nuclear apoptotic bodies, a DNA ladder, and other classic hallmarks of apoptosis. Neuronal apoptosis was readily initiated by oxidative insults and oxidative damage is known to occur in the aging and AD brain. Under some conditions, excitotoxic damage initiated by glutamate and other excitatory amino acids can initiate apoptosis and it has been suggested by many researchers that excitotoxic damage plays a role in the development of AD. The biochemical cascades that lead to apoptotic cell death are being elucidated and involve activation of one or more members of a family of cysteine proteases called caspases, mitochondrial Ca^{2+} uptake and membrane-permeability transition, and release of factors from mitochondria (for example cytochrome c) that ultimately induce nuclear DNA condensation and fragmentation. Alterations of ER-mediated Ca^{2+} homeostasis are sufficient to induce apoptosis.

Different studies reported both necrotic and apoptotic mechanisms for Aβ-mediated neurotoxicity (Mook-Jung et al., 1997; Troy et al., 2001). In particular, oxidative mediated DNA damage, with a pattern indicative of apoptosis, was found in AD brain (Smith et al., 1994), which is consistent with several lines of experimental evidence linking oxidative stress and neuronal apoptosis. Apoptosis is induced by micromolar concentrations of Aβ in cultured CNS neurons, however, physiological

nanomolar concentrations of $A\beta_{1-40}$ and $A\beta_{1-42}$ are insufficient to initiate significant apoptosis in cultures of human fetal neurons (Loo et al., 1993). In fact, both $A\beta$ peptides downregulate Bcl-2, a key anti-apoptotic protein, while only $A\beta_{1-42}$ upregulates ``bax'', a protein known to promote apoptotic cell death (Paradis et al., 1996). Bcl-2 is an anti-apoptotic protein that can prevent neuronal apoptosis in experimental models of developmental cell death and neurodegenerative disorders. It associates with the ER and mitochondrial membranes and such associations might stabilize Ca^{2+} homeostasis and suppress oxidative stress. In addition, agents that disrupt ER-mediated Ca^{2+} regulation cause mitochondrial dysfunction and caspase activation.

There are numerous hypotheses including, but not limited to, $A\beta$-induced alterations in calcium homeostasis, oxidative stress, induction of apoptosis and mitochondrial dysfunction. None of these mechanisms are mutually exclusive as fairly short pathways can be drawn to interconnect them. There is considerable in vitro support that the major mechanisms proposed for $A\beta$-induced cytotoxicity involve the loss of Ca^{2+} homeostasis, oxidative dysfunction and the generation of reactive oxygen species (ROS) (Behl et al., 1994; Mark et al., 1997; Mattson 1997; Varadarajan et al., 2001).

1. Oxidative metabolism dysfunction

Although numerous mechanisms have been implicated as initial triggers of $A\beta$ toxicity, such as activation of surface receptors, generation of ROS, or interfere with cell adhesion to the extracellular matrix. Several lines of evidence point to an $A\beta$-generated ROS as the responsible factor for energy metabolism alterations in AD brain. Metabolic compromise, indicated by inhibition of cell redox activity, may be a primary step in a cascade of degenerative events. It has been reported that $A\beta$ neurotoxicity includes the mediation of ROS production, probably by hydrogen peroxide (H_2O_2) which induces lipid peroxidation on the cell membrane, perhaps generated by the peptide itself (Behl et al, 1994; Richardson et al., 1996). Aggregates can also stimulate free radical production via specific receptor-mediated pathways. It has been proposed that $A\beta$ bind to a receptor for advanced glycation end products (RAGE) or

type 2–scavenger receptors, can induce free radical production by stimulating the activity of the reduced form of nicotinamide adenine dinucleotide oxidases leading to increased intracellular oxidative stress (Yan et al., 1996). Aβ also can enhance metal-catalysed oxidation reactions and stimulate increases in intracellular ROS in several cell models (Pratico et al., 2001). Alternatively, other studies have shown that Aβ itself is capable of generating free radicals and ROS (Mark et al., 1999) that could cause neuronal degeneration by damage to membranes (Butterfield et al., 1994). Others have suggested that mitochondrial changes in AD result in aberrant oxidative phosphorylation (Wallace et al., 1995). In addition, micromolar concentrations of Aβ generate membrane lipid peroxidation in synaptosomes, as measured by the production of malondialdehyde and 4-hydroxynonenal, the subsequent conjugation of Aβ with cellular proteins impairs several functions including mitochondrial function, glutamate and glucose transport, changes which are prevented by several antioxidants (Keller et al., 1997). The in vitro protective effect against Aβ neurotoxicity exhibited by antioxidant compounds such as vitamin E and its derivatives, Trolox (Huang et al., 2000b), supports the hypothesis that Aβ toxicity is mediated by the oxidative pathway.

However, despite the apparent evidence for Aβ-induced oxidative stress, some investigators have been unable to block Aβ neurotoxicity with antioxidants or free radical scavengers that afford neuroprotection against other oxidative stressors (Glazner et al., 1999; Yatin et al., 1999; Yan et al., 2001). There are multiple mechanisms by which cellular alterations may be induced by oxidative stress, including production of ROS in the cell membrane. Free radical-mediated toxicity can account for the multiple metabolic and functional impairments demonstrated when cells are exposed to Aβ. An increased vulnerability to excitotoxicity, disruption of calcium homeostasis, and an impaired glucose uptake have been reported in cells treated with Aβ. Due to their destructive effects on lipid membranes and proteins, ROS can induce these cellular dysfunctions. Finally, Aβ peptide also disrupts ion homeostasis, changing the function of ion motive ATPases, and promoting the activation of N-methyl-D-aspartate (NMDA) receptors which determine an increase in intracellular calcium levels and also contributes to the toxic action of Aβ (Mark et al., 1995a). ROS would target the

proteins important in transporting calcium to the extracellular space and disrupt lipid membranes making cells more permeable to calcium. This leads to Na^+/K^+-ATPase and Ca^{2+} ATPases impairment thus creating membrane depolarization and calcium influx.

2. Disruption of calcium homeostasis

Hypothesis propose that in AD, Aβ alters cell regulation by modifying several ion transport systems and also by forming heterogeneous ion channels. The changes in membrane transport systems are proposed as early steps in impairing neuronal function preceding plaque formation which conclude that these changes damage the membrane by compromising its integrity and increasing its ion permeability. This mechanism of membrane damage is not only central for AD but also may explain other malfunctioned protein-processing-related pathologies. Perturbation of neuronal Ca^{2+} homeostasis has been hypothesized to be a primary factor underlying Aβ toxicity (Schneider et al., 2001). The Aβ has been shown to disrupt neuronal intracellular calcium homeostasis. The calcium ion is normally found in the brain and is important in long-term potentiation as well as releasing transmitter substances, however calcium becomes toxic to the cell if it is in excessive amounts (Misonou, et al., 2000). Several lines of evidence have shown the involvement a Ca^{2+}-mediated process in toxic effects of Aβ. The presence of extracellular Ca^{2+} is required for Aβ toxicity. Administration of Aβ to neuronal cultures disrupts calcium homeostasis with a significantly increased intracellular free calcium concentration ($[Ca^{2+}]_i$) (Mattson, et al., 1992; Weiss, et al., 1994; Mogensen, et al., 1998; Huang, et al., 2000 (a)).

The role of neuronal $[Ca^{2+}]_i$ overload in initiating $[Ca^{2+}]_i$-dependent neurotoxicity and death is well documented, and thus a link between $[Ca^{2+}]_i$ and morphological or functional loss in AD has been proposed. The harmful elevation of intracellular calcium levels results from the flow of calcium ions from the extracellular component, perhaps by the result of (1) changes in endogenous ion transport systems, e.g. Ca^{2+} and K^+ channels and Na^+/K^+-ATPase, and membrane receptor proteins, such as ligand-driven ion channels, NMDA receptor and G-protein-driven releases of second

messengers (Mattson et al., 1992), and (2) formation of heterogeneous ion channels, calcium permeable channels (Arispe, et al., 1993a; 1993b; Rhee et al., 1998).

Electrophysiological studies have shown that Aβ potentiates L-type Ca^{2+} currents (Ueda et al., 1997). Calcium channel have been suggested as playing a role in mediated Aβ acting on neurons. The removal of the calcium ion and its channel blockers from the extracellular fluid of cultured neurons protected the neurons from Aβ toxicity. L-type calcium channel blocker, nimodipine, was found to protect neuron against Aβ toxicity (Weiss et al., 1994). Protective effects of phenothiazines, a class of neuroleptic agent, against Aβ toxicity in primary cultures of rat cortical neurons and PC12 cells. These results suggest that phenothiazines attenuate $Aβ_{25-35}$ toxicity possibly by reducing of Ca^{2+} influx through L-type calcium channels (Keiichi et al., 1997). The mechanism of Aβ on Ca^{2+} channels proposed Aβ-induced calcium channel phosphorylation via the activation of mitogen-activated protein (MAP) kinase that cause calcium influx (Ekinci et al., 1999).

Amyloid β-molecules link up into little tubes in a synthetic membrane that become channels for calcium to rush from outside to inside the cell. The cause of the calcium influx is also Aβ and it is the excess of calcium causing neuronal degeneration, however, rather than the peptide affecting the existing calcium channels it is suggested that the peptides actually form calcium channels that pass 10 times more calcium than normal channels. Synthetic Aβ when packed in phospholipid vesicles and then applied to phospholipid bilayers in vitro induced the formation of channels conduction calcium and other cations (Arispe et al., 1993a). The toxic effects may be derived from the formation of ion channels within the cell membrane by Aβ, fostering direct leakage of calcium into cells (Lin et al., 2001). Several studies have demonstrated an ability of Aβ to form in planar lipid bilayer membranes. The Aβ-induced channels are able to conduct calcium current. However, these channel are formed at levels which are five times the physiological concentration of Aβ.

Sustained elevation of $[Ca^{2+}]_i$ is known to have many deleterious consequences, such as the activation of cellular proteases, the disruption of protein

phosphorylation/dephosphorylation cycles, and increased formation of free radical and ROS. A consequence of disruption in calcium homeostasis is the induction of oxidative stress and accumulation of ROS (Kuperstein et al., 2001). This increased intracellular calcium further potentiates oxidative stress in a number of way. Firstly, a calcium-induced increase in phospholipase activity leads to increased arachidonic acid levels and production of oxygen radicals via fatty acid metabolism. The mitochondria, in response to excessive calcium, develop problems with electron transport, leading to mitochondrial leakage of electrons generating superoxide anions. Thus, a cycle of ROS-induced calcium influx with further ROS generation can ensure. In this respect, the maintenance of cellular calcium gradients represents a major energetic expense, which links alterations in intracellular calcium levels to ATP utilization and the associated generation of ROS through respiratory control mechanisms. The selective oxidation or nitration of the calcium regulatory proteins calmodulin and Ca^{2+}-ATPase that occurs under conditions of oxidative stress may represent an adaptive response to oxidative stress that functions to down-regulate energy metabolism and the associated generation of ROS. Since these calcium regulatory proteins are also preferentially oxidized or nitrated under in vitro conditions, these results suggest an enhanced sensitivity of these critical calcium regulatory proteins, which modulate signal transduction processes and intracellular energy metabolism, to conditions of oxidative stress. Thus, the selective oxidation of critical signal transduction proteins probably represents a regulatory mechanism that functions to minimize the generation of ROS through respiratory control mechanisms.

The concerted action of Ca^{2+} antagonists might prevent an excitotoxin-induced Ca^{2+} overload, sustained membrane depolarization and action potential generation or propagation leading to cell death.

3. Increased vulnerability to excitotoxicity

Reports to date have implicated Aβ as increasing neuronal vulnerability to excitotoxicity-mediated death. Excitotoxicity is a mechanism of neuronal death that involves overactivation of glutamate receptors, particularly under conditions

of metabolic and oxidative stress, resulting in cellular Ca^{2+} overload; the ER contributes to the excitotoxic process by releasing Ca^{2+}(Mody and MacDonald, 1995). Excitotoxicity causes an increase in intracellular calcium, likewise the disruption of calcium homeostasis increase the susceptibility of neurons to excitotoxicity. Furthermore, both excitotoxicity and increased intracellular calcium can lead to the generation of more free radicals and perpetuation of Aβ–initiated oxidative stress. Elevation of resting intracellular calcium concentration ($[Ca^{2+}]_i$) and increase in $[Ca^{2+}]_i$ responses to membrane depolarization and excitatory amino acids have been described also response to Aβ in mature primary cortical neurons (Mattson et al., 1992).

In vitro studies indicate that Aβ peptide fragments interact with extracellular glutamate regulation and thereby potentiate glutamate-induced neurotoxicity (Mattson et al., 1992) and augment NMDA receptor mediated transmission (Wu et al., 1995; Nakajima et al., 2001). Recent studies revealed that Aβ peptides exert their neurotoxic properties via an excitotoxic pathway that involves the activation of NMDA receptors and subsequent pathological increases in the $[Ca^{2+}]_i$, which consisted the report showed that Aβ-induced excitotoxicity has effectively been blocked by NMDA receptor antagonists, such as MK-801 or ifenprodil (Calligaro et al., 1993; Hasegawa et al., 1995; Harkany et al., 2000). It has been well documented that NMDA receptor stimulation induced either by NMDA or Aβ fragments increases the production of free radicals, particularly those of superoxide anions (Huang et al., 2000b). $Aβ_{25-35}$ is found to inhibit [^{3}H]glycine binding and stimulates [^{3}H]MK-801 binding which implies that this β-amyloid fragment might enhance NMDA receptor function acting as glycine agonist or partial agonist (Cowburn et al., 1997). Recently, in vivo evidences support this idea and show that neurotoxic Aβ peptide can increase oxidative stress through mechanism involving NMDA receptors and nitric oxide synthase (Parks et al., 2001).

Neuroprotective effect of valproic acid

Valproic acid (VPA, 2-propylpentanoic acid), a short-chain branched fatty acid is widely used in humans as an anticonvulsant and as a mood stabilizer. VPA has been used to control a variety of seizures, including generalized and partial

seizures. The mechanism of action in both seizure and mood disorders of VPA is unclear but seems to involve in the increase of turnover of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) with potentiation of GABAergic functions, blockage of cell firing induced by NMDA-type glutamate receptors, and attenuation of protein kinase C isoenzymes (Watson et al., 1998; Urbanska et al., 1999). Several hypotheses have been put forth to explain the anticonvulsant activity of VPA, and given the efficacy of VPA in diverse forms of epilepsy, it may act through more than one target. Since inhibition of GABAergic signaling can cause seizures and potentiation of GABA signaling can prevent seizures, this effect of VPA on GABA levels has been proposed as a mechanism for the anticonvulsant activity of VPA. Acute administration of VPA increases the level of GABA in the rodent brains. VPA also reversibly inhibits neuronal calcium currents or calcium influx by blocking different types of voltage-dependent calcium channel e.g. L-type, T-type, N-type and P-type channels (Otoom and Alkadhi, 1999). VPA is also found to protect cultured rat hippocampal neurons against Aβ and glutamate-induced injury possibly through the stabilization of intracellular calcium levels (Hashimoto et al., 2002). Additionally, SH-SY5Y cells were incubated with valproate 0.6 mM for 3 days. Cells were then exposed to two different toxins either thapsigargin (which mobilizes intracellular calcium; 0.5 mM for 16 hours) or MPP$^+$ (25 μM for 16 hours). It was found that lithium and valproate treatment exerted significant protective effects against both toxins (Manji et al., 2000).

Although the anticonvulsant effects of VPA can be observed quite rapidly, its therapeutic effects in the treatment of manic-depressive illness generally requires chronic administration with a lag time of action for several days to weeks. As the therapeutic effects require a lag period for onset of action, it has suggested that it induce alterations at the genomic level. These changes in gene expression after treatment with mood stabilizers suggest that these drugs may also regulate the levels or function of specific transcription factors. VPA has been reported to regulate neuronal signal transduction such as the G-protein-coupled cyclic AMP pathway and polyphosphoinositide generated second messengers. These pathways couple to specific transcription factors such as the cyclic AMP responsive element binding protein

(CREB), c-Fos and c-Jun which further regulate gene expression (Chen et al., 1997). VPA has been shown to decrease myristoylated alanine-rich C kinase substrate (MARCKS) expression (Watson et al., 1998). VPA increases AP-1 binding in cultured cells, suggesting that genes containing this consensus sequence in their promoter may be targets of this drug (Chen et al., 1997; Chen, et al., 1999b; Asghari et al., 1998). Mitogen-activated protein (MAP) kinases play a key role in the regulation of the AP-1 family of transcription factors (Gutkind, 1998). MAP kinases play important physiologic roles in the mature CNS and have been postulated to represent important targets for the actions of CNS-active agents (Yuan et al., 1999).

Several agents have been shown to be neuroprotective in an in vitro system by targeting to specific pathway responsible for Aβ-induced toxicity. These agents include antioxidants or free radical scavengers calcium ion channel blockers, growth factors and caspase inhibitors. Recent evidence shows that besides the anticonvulsant activity, several other mechanisms are considered to be involved in neuroprotection mediated by VPA, including, VPA's effects on Bcl-2 and GSK-3β suggest that this mood stabilizer may also possess neuroprotective and/or neurotrophic properties (Chen, et al., 1999c). Exposure of SH-SY5Y cells to VPA (1 mM) for 5 days not only resulted in robust neurite outgrowth cell survival, but also prominent growth cone formation, and marked increase in the levels of both GAP-43 and Bcl-2. VPA also activated the ERK mitogen-activated protein kinase pathway, a signaling pathway utilized by many endogenous neurotrophic factors that are consistent with its preclinical neuroprotective effects (Yuan et al., 2001). A variety of neurotrophins activate the ERK pathway via cell surface tyrosine kinase receptors (e.g., trkB), and ERK pathways are known to play a major role in neurotrophin-induced cell differentiation and neurite growth (Segal and Greenberg, 1996). Interestingly, recent studies have demonstrated that the chronic administration of a variety of antidepressants increases the expression of BDNF, neurotrophin-3, and trkB (Smith et al 1995). It is thus noteworthy that valproate activates ERKs and promotes neurite growth in SH-SY5Y cells effects that are characteristic of neurotrophic factors. These results, as well as the dramatic prolongation of the survival of SH-SY5Y cells grown in the absence of other trophic factors, suggest that valproate

possesses neurotrophic properties and may thus have utility in the treatment of other neuropsychiatric disorders. Additionally, chronic treatment with VPA increased expression of the molecular chaperone, glucose regulated protein (GRP78) in rat brain and rat C6 glioma cells, which suggests that it may have a neuroprotective effect. Because GRP78 is a protein that binds Ca^{2+} in the endoplasmic reticulum, and protects cells from the deleterious effects of damaged proteins (Wang et al., 1999).

Because of the ability of VPA to promote neuronal survival and to protect neurons against various insults, VPA has been put forward as potential therapeutic agents in neurodegenerative disorders. Valproyl urea (VPU; n-propylpentanoyl urea), a urea derivative of VPA, possesses more potent anticonvulsant activity than VPA, but less CNS depression (Tantisira et al., 1997). It is interesting to examine another potential role for VPU as neuroprotectants in neuronal cultures. To date, a potential role of VPU as a neuroprotectant in neuronal culture has completely remained unknown. Furthermore, VPU might be considered to have a neuroprotective activity similar to VPA.
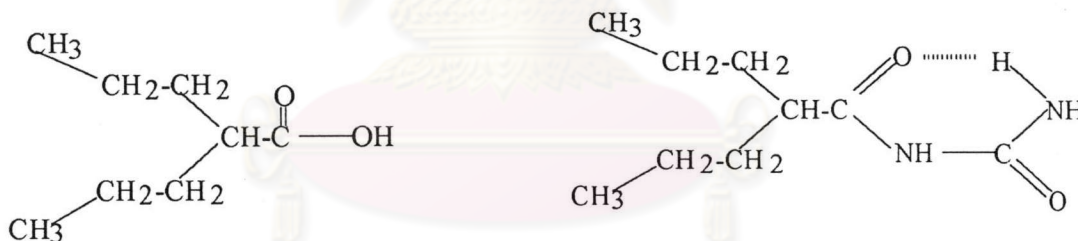
2-Propylpentanoic acid (Valproic acid; VPA)   N-(2-Propylpentanoyl) urea (Valproyl urea; VPU)

P19 embryonal carcinoma cell culture model

While there are obvious benefits in studying intact nervous systems in vivo, there are some major drawbacks, which limit the use of these methods in certain kinds of molecular studies. Therefore, various in vitro models have been developed to study molecular events, for example, those involved in neuronal growth, survival and apoptosis. If used in an appropriate manner and in a sensible context, these models mimic the naturally occurring phenomena of biological events, and the metabolic

conditions and biological events can still be manipulated in a meaningful and controlled way.

P19 cells are a mouse teratocarcinoma cell line with a normal karyotype (McBurney et al., 1982). Which are pluripotent stem cells and may differentiate into a broad spectrum of cell types. They can be cultured continuously as a cell line without loss of pluripotency (Martin, 1981). Teratocarcinoma cell lines bear remarkable resemblance to normal early embryo cells, including ultra-structure, biochemical properties, cell surface antigens, and their ability to form embryoid bodies (EBs). P19 cells were produced by grafting an embryo at 7 days of gestation to the testes of an adult male C3H/He mouse (McBurney et al., 1982). P19 cells are capable of bypassing early embryonic induction and differentiating directly into neurons as long as the downstream signal molecules are properly activated. This cell line can be differentiated in vitro, and the derivatives of all three embryonic germ layers (ectoderm, mesoderm, and endoderm) can be obtained depending on the culture condition. There are two key elements involved in regulating P19 cellular differentiation in vitro, the chemical inducers and the formation of aggregates (Bain and Gottlieb,1994). All the pluripotent P19 cells can be induced to differentiate if they are aggregated. Aggregates form when they are culturing in a suspension medium, in a tube, or in a bacterial grade culture dish for 4-5 days. The aggregates, also called EBs, are classified into two types, simple and cystic. Simple EBs resemble the embryonic portions of 5-day mouse embryos in that the pluripotent cells are surrounded by endoderm. Cystic EBs arise from simple ones and are similar to older mouse embryos, but clearly disorganized. If simple EBs are allowed to reattach to the substratum after aggregation, the pluripotent cells will grow out of the aggregates and start extensive differentiation (Jones-Villeneuve et al., 1982; Martin, 1980). It has also been known that P19 cell can differentiate into neuronal cells or muscle cells by proper chemical inducers such as retinoic acid (RA) or dimethylsulfoxide (DMSO), respectively (McBurney et al.,1982). The P19 cell line can be induced in vitro to differentiate only along the neuroectoderm by culturing EBs with > 5×10^{-8} M retinoic acid. When replated into tissue culture dishes, the RA-treated aggregates adhere and develop into neurons, glia, and fibroblast-like cells in manner

similar to that observed in vivo. High concentrations of RA resulted in efficient neuronal differentiation paralleled by the expression of tissue-specific genes, proteins, ion channels, and receptors in a developmentally controlled manner. Both aggregation and RA are necessary to induce neuronal differentiation of P19 cells, but the continued presence of the inducer is not required. Culture containing up to 90% neurons can be obtained by treating differentiating P19 cells with mitotic inhibitors which eliminates the glia and fibroblast dividing cells.

Mechanism of retinoic acid-induced neuronal differentiation in P19 cells

RA, the biologically active derivative of vitamin A, induces a variety of embryonal carcinoma and neuroblastoma cell lines to differentiate into neurons. Retinoids, the biologically active metabolites of vitamin A, are potent effectors of cellular proliferation, differentiation and developmental formation. Retinoids, play fundamental roles in vertebrate development and homeostasis, and function in signaling by entering cells and binding to specific receptors in the nucleus. These receptors are in fact transcription factors, that upon binding the ligand, become activated and induce the expression of specific genes of interest. RA regulates the growth and differentiation of many cell types, such as the P19 cells. Depending on the concentration of RA and the culture conditions, P19 cells can differentiate into all three embryonic germ layers, that is, endoderm, mesoderm, and ectoderm (McBurney et al., 1982).

The two most important retinoids in vivo, namely all-*trans* retinoic acid and 9-*cis* retinoic acid, exert their functions by binding to cellular RA-binding proteins (CRABP) that interact with the nuclear RA receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). All-*trans* RA binds to RAR and 9-*cis*-RA binds to RXR and RAR. Homodimers of RXR or heterodimers of RAR/RXR bind via a DNA-binding domain to specific sites of target genes, so-called RA response elements (RARE) in the promoters of target genes. and then result in transcriptional initiation of the target genes. During in vitro differentiation of EC cells into the neuronal lineage, RA might act in the same way as in vivo. After formation of endodermal, mesodermal, and ectodermal cells in EBs, neuroectodermal cells were selectively differentiated by serum

depletion and functional neuronal and glial cells were induced and maintained by addition of neural differentiation factors.

The molecular mechanism by which this is thought to occur is through the activity of ligand-inducible RARs, of which there are three known subtypes α, β, γ and numerous isoforms. These nuclear receptors bind to and, in the presence of RA, transactive promoters associated with RARE. In some cell types the resulting gene products participate in events which culminate in cellular differentiation. The regulatory regions of the RARβ gene contain a direct repeat of the consensus RARE. However, the mechanisms whereby RA induces neural differentiation of EC cells are not fully understood. The hypothesis that in vitro and in vivo pathways are comparable is supported by findings of a significant upregulation of RARα and RARβ mRNA, but a rapid downregulation of RARγ and retinoid X receptor-γ (RXRγ) mRNA during RA-induced neuronal differentiation of mouse EC cells. This suggests a role for RARα, β, γ and RXR during neuroectodermal differentiation (Jonk et al., 1992). During early nervous system development, RARβ is expressed after neural tube closure, while RARγ is only expressed in the open neural folds. RXRs are similarly differentially expressed during development (Krezel et al., 1999). RXRβ is expressed in a general fashion, perhaps at slightly higher levels in the anterior regions of the spinal cord and hindbrain. RXRα is abundantly expressed in liver, kidney, spleen and intestine. RXRα is also abundant in epidermis, together with RARγ. RXRγ expression is highly restricted, mostly to the developing ear, muscle, retina, pituitary and thyroid gland. In addition, strong expression is found in the developing striatum and spinal motor neurons where it is coexpressed with RARβ. A number of neural-specific genes encoding transcription factors and signaling molecules were induced by RA during neural differentiation of EC cells in vitro, for example, mash-1, neuroD (Boudjelal et al., 1997) in mouse EC cells. In parallel, the expression of mesodermal genes such as cardiac actin and γ-globin was downregulated (Bain et al., 1996). This suggests that RA-induced neuronal differentiation is accompanied by repression of mesodermal differentiation, while activation of neuroectoderm differentiation.

Biochemical and physiological properties of neuron-like cells derived from P19 cells

The cells acquire certain traits of mature neurons only after several days of differentiation which have the key physiological, morphological and molecular properties of primary cultured neurons derived from the central nervous system such as the ability to release neurotransmitter; the segregation between axons and dendrites; the formation of morphologically mature synapse among neighboring cells. Aggregation of P19 cells into EBs followed by stimulation with all-*trans* retinoic acid leads approximately 35-40 % of the cells to acquire a neuronal phenotype, as judged by their expression of neuronal specific markers and characteristic electrophysiological properties (Finley et al., 1996).

RA-induced neuronal cells expressed neuron-specific genes in a developmentally controlled manner and were characterized by complex electrophysiological and immunocytochemical properties of postmitotic nerve cells. Genes encoding 68-kDa (NFL) and 160-kDa (NFM) neurofilament proteins and the synaptic vesicle protein, synaptophysin were expressed at an early stage in parallel to the expression of voltage-gated Ca^{2+}, Na^+, and K^+ ion channels (Fraichard et al., 1995; Strübing et al. 1995). Further differentiation was achieved by an increase in the density of voltage-gated ion channels and expression of receptor-operated $GABA_A$, glycine, kainate, and NMDA ion channels, in parallel to the expression of proteins characteristic of mature neurons such as NFL, NFM, NFH, neurocan, synaptophysin, syntaxin, synaptobrevin, neuronal cellular adhesion molecules (NCAM), class III β-tubulin, and microtubule-associated proteins MAP2 and MAP5 (Bain et al., 1996; Fraichard et al., 1995; Strübing et al., 1995; Finley et al., 1996). In addition to the expression of specific neuronal receptors, neuronal cells generated Na^+-driven action potentials and were functionally coupled by inhibitory GABAergic and excitatory glutamatergic synapses as revealed by measurements of postsynaptic currents (Strübing et al. 1995; Finley et al. 1996).

The embryonal carcinoma cell line, P19 is establishing its place as flexible model system for CNS neurons. The cell differentiate into neuron, astrocytes and fibroblast-like cells following induction with retinoic acid. The P19 cells were chosen as a model system to study neuronal function since, these cells are among the

few neuronal cell line that can be differentiated in culture into polarized cells with defined axons and dendrites (Finley et al., 1996). Differentiated P19 cells were shown to express a number of neuronal markers in a programmed manner. GAP-43 (neuromodulin) is a nervous-system specific phosphoprotein, that is a component of diifferentiated P19 cells (Mani et al., 2000). P19-derived neurons form a mixed population of cells producing a spectrum of neurotransmitters and neuropeptides resembling that of neurons within the striatum, hippocampus, and cerebral cortex. In addition, they are mature to functional cells since they become responsive to electro-physiological stimuli, competent in releasing neurotransmitters in a Ca^{2+} and depolarization-dependent manner, and are able to form functional synapses.

The P19-derived neurons contain high levels of acetylcholine transferase (ChAT; E.C. 2.3.1.6) and acetylcholinesterase (AChE; E.C. 3.1.1.7), indicating that at least some of neurons are cholinergic. The neuronal function and feature of differentiated P19 cell have been well characterized, e.g. the presence of wide range of voltage-dependent membrane current, expression of numerous cell membrane receptors for various neurotransmitters, and formation of cholinergic synapse. Moreover, P19 cells posses a primarily cholinergic phenotypes. The neurotransmitter profile of P19-derived neurons resembles that of the mammalian neocortex (Staines et al., 1994) making this cell line an attractive system for studies pertaining to CNS-type-cells. In addition, the cholinergic nervous system is a primary site of insoluble Aβ accumulation, a hallmark of Alzheimer's disease.

AChE has been suggested not only as an important catalytic molecule for CNS functioning, but also to have effects upon cell proliferation and differentiation along nervous system development. AChE appears early in the vertebrate nervous system and it seems to be responsible for some mechanisms of synaptogenesis. For example, embryonic Xenopus brain and chick retinal cell cultures exposed to an AChE inhibitor showed axonal changes and cell size reduction, respectively. Recent evidence demonstrates that AChE supports mammalian neurite extension and interneuronal interactions through non-catalytic activities, independently of its enzymatic capacity to hydrolyze acetylcholine (Grifman et al., 1998). In embryos, this property explains the

well-known transient expression of AChE in numerous non-cholinergic neurons. In adults, such activities may shed new light on the puzzling association between changes in AChE activities and various neurodegenerative diseases. Within the vertebrate retina, non-catalytic neurogenic roles have been attributed to AChE based on the spatiotemporal pattern of its expression during development and in retinal explant cultures (Robitzki et al., 1997). On the other hand, cultures of embryonic chick sympathetic neurons grown on an AChE-enriched medium presented a stimulated neurite outgrowth. The cells of neuronal clusters are AChE positive staining, while glial cells on the surface have lower or no AChE activity (Keller et al., 2001). It was suggested that AChE have a novel role in neuronal differentiation and use to be a neuronal differentiation marker (Hoshi et al., 1997; Hayashi et al., 2000).

Therefore, P19 cells have been used as an in vitro model of the CNS cells after differentiation by retinoic acid. Further advantage in using these cellular systems will be applicable: (1) for studying commitment and neuronal specification in vitro, (2) as pharmacological assays for drug screening.

Detection of Aβ-induced neurotoxicity

Many laboratories have investigated significant resources in establishing and validating cell culture models of Aβ toxicity. In some circumstances the word "toxicity" has applied to any compromise of cellular function, although this may not necessary result in cell death. Typical assay endpoints employed to assess cell viability following Aβ treatment include changes in neuronal morphology (neurite sprouting or retraction, reflactile properties of the cell soma, membrane blebbling), redox-dye reduction, cell proliferation, trypan blue or fluorescent-dye exclusion, and release of cellular lactate dehydrogenase activity. The neurotoxic properties of Aβ have been described by inhibiting tetrazolium dye reduction. The reduction of tetrazolium is an index of the cell's metabolic integrity. Such compromising of cellular events appears to be an early indicator of impending of cell loss. Effects on tetrazolium reduction, observed for Aβ, suggest an indication of metabolism leading to cell death. The correlation between the impairment of the tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl -

2,5-diphenyltetrazolium bromide (MTT), reducing activity and the magnitude of cell death in the experimental condition was examined. In cortical cultures, the impairment of the MTT reducing activity is correlative to cell death. In PC12 cells, $A\beta_{25-35}$ exerts the impairment of the MTT reducing activity without cell death. Thus, we consider that the impairment of the tetrazolium dye reducing activity is a early indicator of $A\beta$ toxicity (Shearman et al., 1994; Shearman et al., 1995). One parameter used as the basis for colorimetric assays is the metabolic activity of viable cells. The mitochondrial dehydrogenase activity that reduced tetrazolium dye was used to determine cellular redox activity, an initial indicator of cell death, in a quantitative colorimetric assay. Because tetrazolium salts are reduced to a colored formazan only by metabolically active cells, these assays detect viable cells exclusively. Tetrazolium salts, one is XTT. XTT is cleaved to formazan by the "succinate-tetrazolium reductase" system (EC 1.3.99.1) which belongs to the mitochondrial respiratory chain and is active only in viable cells. When XTT entered to cells along with a secondary electron transfer reagent such as menadione or phenazine methosulfate (PMS), results in the formation of an aqueous, soluble formazan product. This eliminated the need for a solubilization step in the procedure. XTT forms an orange-colored water soluble product that can be directly measured by absorption spectrophotometry. However, the XTT tetrazolium compound has the disadvantages of limited solubility and stability in solution, requiring preparation of fresh solutions prior to each assay.

The second cytotoxic assay is the release of lactate dehydrogenase (LDH) from cells, a measurement routinely used to quantitative cytotoxicity in cultured neurons (Behl et al., 1994). Membrane integrity can be evaluated by measuring LDH activity. Because LDH is a cytoplasmic enzyme, when LDH is found within the media on the cells, there are two possible causes: cellular death and leak within the cell membrane. While XTT measures primarily early redox changes within the cell reflecting the integrity of the electron transport chain, the release of LDH is thought to be cell lysis. The activity of LDH, is assessed by measuring the pyruvate-dependent oxidation of nicotinamide adenine dinucleotide (NADH), which resulting in the conversion of a tetrazolium salt into a red formazan product. The amount of color formed is proportional to the number of

lysed cells once the appropriate controls have been subtracted. Visible wavelength absorbance (OD 490nm) data are collected using an ordinary 96-well plate reader. Two factors in tissue culture medium can contribute to background absorbance using this assay: phenol red and LDH from fetal calf serum. Background absorbance from both factors is corrected for by including a culture medium control. The absorbance value determined from this control is used to normalize the absorbance values obtained from other samples.

The third assay is visual counting in conjunction with trypan blue exclusion. Trypan blue exclusion assay is routinely performed by counting cells that internalize the dye to define non-viable cells. Exclusion of the dye corresponds to cell survival.

Different methods were used on the same experiment to ensure that consistency and reliability of the results were not interfered by artifact. The quantification of neuronal death in these experimental systems is achieved using a variety of in vitro assay with different techniques. In this study, three assays were used to assess effect of VPA, VPU, and $A\beta_{1-42}$ on neuron-like cells viability.