CHAPTER 1
NEURODEGENERATION: APOPTOSIS AND MONOAMINE OXIDASE B

1 Neurodegenerative diseases

Neuronal loss plays an important role in the normal development of a functional integrated nervous system (Oppenheim, 1991). Initially there is an excess of neuronal cells in the nervous system, which take part in a competitive survival process, with only those neurons that are functionally, temporally, and spatially correct surviving (Cowan *et al.*, 1984). Under normal conditions most of the surviving neurons stay viable and functional throughout the lifetime of an individual (Mattson, 2000), whereas the neurons that do not survive the competition die through apoptosis, an intrinsic cell suicide program (Holbrook *et al.*, 1996).

This intrinsic cell suicide program involves the induction of specific proteins, such as p53, and activation of degrading enzymes, such as endonucleases. What follows is a characteristic pattern that involves rapid nuclear collapse and DNA destruction. Although apoptosis during development is a beneficial process, it's occurrence in the mature brain is harmful, and leads to a decrease in the number of functional neurons, which can not be replenished by cell division (Holbrook *et al.*, 1996).

The inappropriate activation of apoptosis in neuronal cells can be a result of their high metabolic rates and free radicals that are produced as a normal part of cell metabolism. As the processes of aging or disease impair mitochondrial activity, modify DNA and induce protein modifications, the equilibrium between survival and apoptosis shifts toward apoptosis. Neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease, may mimic an accelerated aging process by increasing the levels of free radicals or disruption of calcium homeostasis (Holbrook *et al.*, 1996), and thereby lead to neuronal cell death through apoptosis.

Death of neurons in different anatomic parts of the brain give rise to the particular symptoms associated with these neurodegenerative diseases, with hippocampal and cortical neurons dying in Alzheimer's disease (AD) and midbrain dopaminergic neurons dying in Parkinson's disease (PD). The prevalence of neurodegenerative disorders is rapidly increasing as average lifespan increases (Mattson, 2000).

1.1 Parkinson's disease (PD)

1.1.1 Definition

Parkinson's disease (PD) is an idiopathic, slowly progressive, degenerative central nervous system (CNS) disorder, characterised by slow and decreased movement, muscular rigidity, resting tremor, and postural instability. These symptoms are a result of pigmented neuronal loss of the substantia nigra, locus ceruleus, and other brain stem dopaminergic cell groups (Beers *et al.*, 2006), which give rise to dopamine level depletion in the caudate nucleus and putamen, and results in reduction of cortical activation (Wells *et al.*, 2003). Contributing to the tremor of PD, there is also a relative elevation of striatal cholinergic interneuron activity (Wells *et al.*, 2003), which makes it clear that there is an imbalance of dopamine and acetylcholine neurotransmitter levels in the corpus striatum (Tierney *et al.*, 2006). Apart from dopaminergic neuronal loss in PD brains, development of neurofibrillary structures, termed Lewy bodies, also occur (Holbrook *et al.*, 1996).

1.1.2 Epidemiology

PD is the second most common neurodegenerative disease, with an overall prevalence of 0.1% in the total population (Bahr, 2004). It affects about 1% of people over 65 years of age and 0.4% of those above 40 years. PD rarely begins in childhood or adolescence (juvenile Parkinsonism), with a mean age of onset being 57 years (Beers *et al.*, 2006).

1.1.3 Aetiology

Until recently, primary Parkinsonism was described as an idiopathic disease, and although the cause of the disease still remains largely unidentified, emerging evidence suggests that multiple factors, both genetic and acquired (exposure to environmental factors) contribute to the neurodegeneration of the dopaminergic cells (Bahr, 2004). Evidence that dopaminergic neurons die by an apoptotic process in PD is accumulating, with analysis of post mortem brain tissue from PD patients revealing evidence for neuronal apoptosis. This evidence includes nuclear condensation, chromatin fragmentation and formation of apoptotic bodies (Mochizuki *et al.*, 1996; Tompkins *et al.*, 1997; Tatton *et al.*, 1998). The causes of the neuronal death are likely to involve age-related increased oxidative stress and mitochondrial dysfunction in dopaminergic neurons, as a result of sensitisation by environmental and genetic factors (Jenner & Olanow, 1998; Polymeropoulos, 1998).

Secondary Parkinsonism results from loss of or interference with dopamine's action in the basal ganglia due to other degenerative disorders, drugs or exogenous toxins (Beers *et al.*, 2006).

1.1.4 Pathogenesis

The pathological hallmarks of Parkinson's disease (PD) are a loss of dopaminergic neurons in the mesencephalon and the presence of Lewy bodies in altered neurons.

Genetic factors playing a role in the pathogenesis include Par-4 and α-synuclein. Par-4 levels are selectively increased in the substantia nigra dopaminergic neurons prior to their death, with suppression of Par-4 expression protecting dopaminergic neurons against death (Duan *et al.*, 1999a). The protein, α-synuclein, is a major component of the PD brain lesions called Lewy bodies, and mutations in α-synuclein are responsible for a small percentage of PD cases, with the expression of mutant α-synuclein in cultured cells promoting apoptosis (el-Agnaf *et al.*, 1998).

Biochemical abnormalities in PD substantia nigra include:

(a) Abnormal iron accumulation, and alteration in the concentration of iron-binding proteins (Mattson, 2001);

Iron is capable of functioning as a catalysing agent in oxidative reactions that may generate hydrogen peroxide as well as hydroxyl ions. Thus, if iron is available in a free and reactive form, it has the potential for exacerbating oxidative stress and damage (Mattson, 2001). There is a 35% increase in substantia nigra iron levels in PD (Dexter *et al.*, 1987; Sofic *et al.*, 1991), which is a reflection of neuronal cell loss rather than any specific pathogenetic factor (Mattson, 2001). Abnormally high concentrations of iron can also be found in macrophages, astrocytes, and reactive microglia in the PD substantia nigra (Jellinger *et al.*, 1990).

(b) Increased oxidative stress and oxidative damage (Mattson, 2001);

Based on recent evidence it appears that there are increased levels of oxidative stress and oxidative damage to bio-molecules in PD substantia nigra. These results suggests:

- Enhanced free-radical generation in the PD nigra;
- Increased super oxide generation in PD nigral neurons, with the super oxide dismutase (SOD) levels being the indicative factor of super oxide generation. In PD there appears to be increased levels of copper/zinc and manganese SOD (Marttila *et al.*, 1988a; Saggu *et al.*, 1989);
- Increased levels of poly-unsaturated fatty acids (Dexter *et al.*, 1986), malondialdehyde and hydro-peroxides (Jenner, 1991) in PD substantia nigra, which are the products of free-radical damage to lipid membranes (Mattson, 2001).

(c) Mitochondrial complex I deficiency (Mattson, 2001);

Through oxidative phosphorylation (OXPHOS) the mitochondria is responsible for producing adenosine triphosphate (ATP). OXPHOS also produces 95% of the cell's super oxide ions during aerobic metabolism (Mattson, 2001). In the PD nigra there is approximately a 35% deficiency in complex I (Schapira *et al.*, 1990). Defects in complex I activity may generate increased super oxide ions that may enhance oxidative stress and oxidative damage in the presence of elevated concentrations of iron (Mattson, 2001).

(d) Increased nitric oxide (NO) formation (Mattson, 2001) and

The free radical gas NO•, which is generated by the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS), is present in many tissues, including the CNS. It acts as an atypical molecular messenger, which may have a toxic role, and has been implicated in the neurodegeneration that occurs in PD. As a free radical, NO• could potentially contribute to dopaminergic neuronal death by mechanisms such as increased lipid per-oxidation, release of iron (II), and damage to DNA. It is also an inhibitor of numerous enzymes such as cytochrome c oxidase and SOD (Itzhak & Ali, 1996). In non-human primates and humans, NOS activity is at its highest in the nigrostriatal system (Kuiper *et al.*, 1994; Molina *et al.*, 1994, 1996; Qureshi *et al.*, 1995).

(e) The generation of nitro tyrosine residues within PD substantia nigra (Mattson, 2001).

The PD brains that become available for analysis are inevitably at the end stage of the disease process when the majority of the nigral neurons have already disappeared and gliosis is often widespread. For this reason it is important to distinguish between biochemical abnormalities and those that might be a consequence of post-mortem changes (Mattson, 2001).

1.2 Alzheimer's disease (AD)

1.2.1 Definition

Alzheimer's disease is a progressive dementia affecting both cognition and behaviour with no known cause or cure (Wells *et al.*, 2003), and is characterised by senile plaques, β-amyloid deposits, and neurofibrillary tangles in the cerebral cortex and sub cortical grey matter (Beers *et al.*, 2006).

1.2.2 Epidemiology

AD is the most common cause of dementia, and accounts for more than 65% of dementias in the elderly. The disease is twice as common amongst women as amongst men, partly because women have a longer life expectancy. It affects about 4% of people aged 65 to 74 and 30% of those older than 85. Prevalence in industrialised countries is expected to increase as the proportion of the elderly increases (Beers *et al.*, 2006).

1.2.3 Aetiology

Most cases of AD are sporadic, with late onset (over 60 years) and unclear aetiology. About 5-15% of AD cases are familial, with half of these having an early onset (younger than 60 years), and is typically related to specific genetic mutations (Beers *et al.*, 2006).

Dementia associated with the more advanced stages of AD is believed to be caused by neuronal degeneration in cognition-related brain regions. In the AD brain, large-scale cell death of mature neurons is a pathologic process that remains unsolved. Apoptosis and cell cycle re-entry are amongst the new types of cell death processes that have been proposed for the neuronal loss, wherein β-amyloid (Aβ) can be a driving force. The amyloid protein induces apoptosis through oxidative stress while also driving cell division and cell death in cultured neurons (Copani *et al.*, 1999). Evidence for DNA fragmentation, expression of apoptosis-related genes, and caspase activation support an apoptotic mechanism in AD neurodegeneration (Olariu *et al.*, 2005).

In PD there is a subset of patients who develop a dementia, termed sub-cortical dementia, which shows cortical pathology in the form of neuritic plaques (Holbrook *et al.*, 1996).

1.2.4 Pathogenesis

In AD the neurons in the cortex and limbic structures of the brain responsible for higher learning, memory, reasoning, behaviour and emotional control are degenerated (Wells *et al.*, 2003). Synapses and neurons in brain regions that serve learning and memory functions include the hippocampus, entorhinal cortex, basal forebrain and neocortical association corticles (DeKosky *et al.*, 1996). Cholinergic pathways, especially a large system of neurons located at the base of the forebrain in the nucleus basalis of Mynert as well as serotonergic neurons of the raphe nuclei and noradrenergic cells of the locus ceruleus are profoundly damaged and monoamine oxidase type B (MAO-B) activity is increased (Wells *et al.*, 2003).

The extra cellular β-amyloid deposits, intracellular neurofibrillary tangles, and extra cellular senile plaques/neuritic plaques that develop in AD, lead to neuronal loss (Beers *et al.*, 2006), and their presence is necessary for AD to occur (Wells *et al.*, 2003).

The progressive impairment of cognition and emotional disturbances that occur in AD result from degeneration of synapses and death of neurons in limbic structures such as the hippocampus and amygdale, and associated regions of the cerebral cortex. The damaged neurons exhibit aggregates of hyperphosphorylated tau protein and evidence of excessive Ca^{2+}-mediated proteolysis and oxidative stress (Yankner, 1996; Mattson, 1997). In AD there is abnormally increased levels of the tau protein (a component of neurofibrillary tangles and β-amyloid) in the brain and cerebrospinal fluid (CSF), and reduced levels of choline acetyltransferase. The latter plays an important role in the synthesis of acetylcholine (Beers *et al.*, 2006).

The mitochondrial function of brain cells in AD patients is compromised (Mattson, 2001) with increased levels of cellular oxidative stress in vulnerable regions of the AD brain (Bruce *et al.*, 1997; Moccoci *et al.*, 1994; Smith *et al.*, 1991). There is increased protein oxidation, protein nitration, and lipid per-oxidation in neurofibrillary tangles (NFT's) and neuritic plaques (NP's) (Good *et al.*, 1996; Smith *et al.*, 1997). Other alterations include membrane depolarisation, increased levels of mitochondrial oxyradicals, and membrane permeability transition that are commonly present in cells undergoing apoptosis (Mattson, 2001).

Expression of brain-derived neurotrophic factor (BDNF) and its high-affinity receptor, tyrosine kinase receptor B (trkB), are selectively decreased in the frontal cortex and hippocampus of AD patients (Ferrer *et al.*, 1999), and could possibly contribute to the neurodegenerative process in AD (Mattson, 2001).

Exposure of synaptosomes or intact synaptically connected neurons to Aβ and related oxidative insults, result in caspase activation (which is increased in degenerating neurons and neuritis (Chan *et al.*, 1999)), loss of plasma membrane phospholipid asymmetry, increased Par-4 levels, mitochondrial calcium uptake, and release of factors capable of inducing nuclear chromatin condensation and fragmentation into the cytosol (Mattson *et al.*, 1998b, 1998c; Duan *et al.*, 1999b). Intraneuronal accumulation of Aβ occurs in normal aging without deposition of Aβ in amyloid plaques (Naslund *et al.*, 1994), whereas it is deposited extra cellular in the AD brain (Olariu *et al.*, 2005). Increased Aβ levels lead to deposition and fibrillary aggregation thereof (Beers *et al.*, 2006). It can induce apoptosis directly (Loo *et al.*, 1993; Mark *et al.*, 1995) and can greatly increase neuronal vulnerability to death (Mattson, 1997) and excitotoxicity (Mattson *et al.*, 1992). It may lead to neuronal death and formation

of the neurofibrillary tangles and senile plaques, which consist of degenerated axonal or dendritic processes, astrocytes, and glial cells around an amyloid core (Beers *et al.*, 2006). Disordered glial immunity also plays a key role in the pathogenesis of Alzheimer's disease (AD) (McGeer & McGeer, 1999), with Aβ being able to activate super oxide forming nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase in mononuclear phagocytes (MP), resulting in H_2O_2 production (Bianca *et al.*, 1999). sAPPα and Aβ synergistically activate MP, in the absence of pro-inflammatory cytokines, with glutamate secretion and reactive oxygen species (ROS) production as a result (Ikezu *et al.*, 2003).

Experiments on isolated neurons have provided evidence that alterations in proteolytic processing of amyloid precursor protein (APP) may play a major role in the increased levels of oxidative stress in neurons in AD (Mattson, 1997). APP mutations may cause AD by altering proteolytic processing of APP, such that levels of Aβ are increased and levels of the secreted form of APP (sAPPα) are decreased (Mattson, 1997; Lannfelt *et al.*, 1995; Furukawa *et al.*, 1996b). Due to APP mutations in AD, soluble/secreted Amyloid Precursor Protein alpha (sAPPα) levels are decreased with the neuroprotective actions thereof not being reduced. sAPPα increases the resistance of neurons to oxidative injury induced by Fe^{2+} and Aβ (Furukawa *et al.*, 1996b). It also induces an increase in the basal level of glucose transport, and attenuates oxidative impairment of glucose transport in cortical synaptosomes (Mattson *et al.*, 1999a). A signal transduction pathway that mediates the neuroprotective effects of sAPPα has been elucidated and involves cyclic guanosine-5-monophosphate (GMP) production, activation of potassium channels (Furukawa *et al.*, 1996a), and activation of the transcription factor NF-κB (Barger & Mattson, 1996), which has been shown to protect neurons against apoptosis and excitotoxicity in several different cell culture and *in vivo* models (Barger *et al.*, 1995; Mattson *et al.*, 1997; Yu *et al.*, 1999).

In addition to APP, two proteins called presenilin-1 and presenilin-2, can harbour mutations that cause early onset AD (Hardy, 1997; Mattson *et al.*, 1998a), by altering Ca^{2+} homeostasis in such a way that Aβ production is increased and neurons are made vulnerable to apoptosis and excitotoxicity (Mattson, 2001). Some of these mutations lead to increased production of β-amyloid peptides, which contribute to the pathology of AD (Olariu *et al.*, 2005). Other genetic determinants which influence β-amyloid deposition, cytoskeletal integrity, and efficiency of neuronal repair include the apo-lipoprotein (apo) E alleles (ε) (Beers *et al.*, 2006).

Protein kinase C (PKC) is a key signal transduction system that plays an important role in the production of Aβ and generally declines with aging. Not all aged individuals develop AD

although aging seems to be a prerequisite of AD. It can be hypothesised that in aging in the presence of high risk AD factors, PKC deficiency would imbalance the APP α-processing towards a β- and/or γ-processing with generation of soluble Aβ. Gradual elevation of soluble Aβ will initially activate PKC and related downstream pathways, while constant high levels of Aβ, as in the late stage of AD, will down regulate PKC and dampen the PKC-related intracellular pathways (Olariu *et al.*, 2005). In AD brain tissue, there also exists increased mitogen-activated protein kinase (MAPK) activity, an intracellular enzyme located downstream to PKC that could be partly responsible for the generation of neurofibrillary tangles (NFT) (Swatton *et al.*, 2004).

2 Apoptosis in neurodegenerative diseases

2.1 Overview of apoptosis

Apoptosis, otherwise known as type I cell death (Schweichel & Merker, 1973), is a genetically encoded, ubiquitous pathway enabling cells to undergo highly regulated death in response to pro-death signalling (Wyllie *et al.*, 1980). It describes the morphology of cells disappearing in a non-inflammatory manner (Kerr *et al.*, 1972), and is a form of programmed cell death that involves a stereotyped sequence of biochemical and morphological changes (Mattson, 2001). Death by apoptosis often occurs as part of normal development of homeostasis (Holbrook *et al.*, 1996).

Apoptosis is characterised by a number of unique distinguishing features, including cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation, intra-nucleosomal DNA fragmentation, phosphatidylserine exposure and, finally, fragmentation into membrane-enclosed apoptotic bodies sequestered by macrophages or other engulfing cells (Wyllie *et al.*, 1980). These cellular remains that are removed by phagocytosis do not invoke an inflammatory response (Holbrook *et al.*, 1996).

It is triggered by a variety of stimuli that cause susceptible cells to execute the apoptotic program. Such triggers include:

(a) Neurotrophic factor deprivation:

Neurotrophic factor support is an intensively studied neuronal death signal, and lack thereof may trigger apoptosis during development of the nervous system and in neurodegenerative disorders (Mattson & Lindvall, 1997).

(b) Excitotoxicity:

The second most prominent trigger of neuronal apoptosis is the activation of glutamate receptors, of which glutamate is an excitatory amino acid neurotransmitter. Calcium influx through ionotropic glutamate receptor channels and voltage-dependent calcium channels mediates glutamate-induced neuronal apoptosis and necrosis (Ankarcrona *et al.*, 1995; Glazner *et al.*, 2000). Such "excitotoxicity" may occur in AD, PD, HD and ALS (Wong *et al.*, 1998; Mattson *et al.*, 1999b). Over activation of glutamate receptors under conditions of reduced energy availability or increased oxidative stress, results in Ca^{2+} influx into postsynaptic regions of dendrites. Ca^{2+} entering the cytoplasm through plasma membrane channels and endoplasmic reticulum (ER) channels induces apoptotic cascades that involve Par-4, pro-apoptotic Bcl-2 family members (Bax and Bad), and/or p53. These factors act on mitochondria to induce Ca^{2+} influx, oxidative stress, opening of permeability transition pores (PTP), and release of cytochrome c. This results in caspase activation and execution of the cell-death process (Mattson, 2001).

(c) Oxidative stress:

Oxidative stress (in which free radicals such as super oxide anion radicals and hydroxyl radicals damage cellular lipids, proteins, and nucleic acids by attacking chemical bonds in those molecules) is a very important trigger of neuronal death in neurodegenerative disorders (Mattson, 1998; Mattson, 2001; Sastry & Rao, 2000). Because the signals for apoptosis involve the same molecules that are produced during oxidative stress, it is increasingly evident that oxidative stress is a common inducer of apoptosis. Many of the agents that can induce apoptosis are oxidants or stimulate the production of free radicals through cellular metabolism (Holbrook *et al.*, 1996). Calcium and free radicals are able to induce proteins that are involved in apoptotic pathways, such as p53 (Holbrook *et al.*, 1996). Free radical-induced apoptosis especially, is dependent upon expression of functional p53 protein (Holbrook *et al.*, 1996). Even though p53 functions primarily as a trigger for apoptosis (e.g. Yonish-Rouach *et al.*, 1991), it does not participate in the execution phase of apoptosis, with apoptosis sometimes occurring in the absence of p53 (Holbrook *et al.*, 1996).

(d) Reduced energy availability & DNA damage:

Reduced energy availability to neurons (Beal, 1995; Bruce-Keller *et al.*, 1999; Duan *et al.*, 1999c), as well as DNA damage (Holbrook *et al.*, 1996) may also initiate neuronal apoptosis.

After being triggered, the process of apoptosis is mediated by specific biochemical cascades involving mitochondrial changes and activation of proteases called caspases. It provides a mechanism for cells to die without adversely affecting their neighbours (Wyllie, 1997). The process of apoptosis is very complex and involves several pathways, of which only two have been identified: A pathway that is directly activated by death receptors and a pathway that involves the mitochondria (Lei *et al.*, 2003). In the current study the focus will be on the mitochondrial pathway.

The different triggers explained give rise to certain events, which eventually lead to mitochondrial changes, which are central to the apoptotic process and ultimately leads to cell death, with the mitochondrion being the final controller of the cell death decision (Kroemer *et al.*, 1998). These changes that occur in mitochondria of cells undergoing apoptosis include increased oxyradical production, opening of pores in their membranes, and release of cytochrome c (Keller *et al.*, 1998; Matsumoto *et al.*, 1999). The events that occur upstream of the mitochondrial changes are complex and involve interaction of several types of proteins, such as Bcl-2, Par-4, caspases and telomerase. The signalling pathways that initiate or prevent apoptosis are highly concentrated in synaptic terminals, which are major sites of intercellular communication between neurons (Mattson, 2001).

The neuroprotective activity of the compounds studied, may contribute to inhibition of the mitochondrial pathway leading to apoptosis. For this purpose it is necessary to have a basic understanding of the different components, which form part of this pathway. These components will be explained in the following sections.

2.1.1 Growth factors

Neurotrophins (also called "neurotrophic factors") are a family of protein growth factors that control the survival of neurons. They are secreted proteins, usually found in the blood stream, that signal particular cells to survive, or differentiate, or grow. Neurotrophic factors are secreted by target tissue, and act by prohibiting the neurons from initiating apoptosis - thus signalling the neurons to survive. Neurotrophins also induce differentiation of progenitor cells, which act as precursor cells, to form neurons (Hempstead, 2006; Reichardt, 2006; Allen & Dawbarn, 2006).

There are two classes of growth factor receptors (GFR) namely nerve growth factor receptor/low-affinity neurotrophin receptor (p75) and the tyrosine kinase receptor ("Trk") family. p75 is a low affinity neurotrophin receptor, to which all neurotrophins bind, whereas the Trk family include TrkA, TrkB, and TrkC, and will only bind with specific neurotrophins,

but with a much higher affinity. The Trks mediate the functional signals of the neurotrophins (Arevalo & Wu, 2006).

Brain-derived neurotrophic factor (BDNF) is a neurotrophic factor found in the brain, but also in the periphery. More specifically, it is a protein that has activity on certain neurons of the central nervous system and the peripheral nervous system - it helps to support the survival of existing neurons, and encourage the growth and differentiation of new neurons and synapses through axonal and dendritic sprouting (Acheson *et al.,* 1995; Huang & Reichardt, 2001). In the brain, it is active in the hippocampus, cortex, cerebellum, and basal forebrain areas vital to learning, memory, and higher thinking (Yamada & Nabeshima, 2003), which makes it a neurotrophin that plays a vital role in the pathogenesis of AD. BDNF stimulates production of antioxidant enzymes, which may account, in part, for its ability to protect neurons against oxidative and metabolic insults relevant to the pathogenesis of AD and PD (Cheng & Mattson, 1994; Frim *et al.*, 1994). In addition to suppressing oxidative stress, it can enhance neuronal calcium homeostasis by modulating the expression and/or function of glutamate receptors, ion-motive ATPases, and calcium-binding proteins. BDNF may also induce production of anti-apoptotic proteins such as Bcl-2 (Allsopp *et al.*, 1995; Furukawa *et al.*, 1997), and binds to the TrkB receptor, with TrkB mediating the multiple effects, which includes neuronal differentiation and survival (Huang & Reichardt, 2001; Patapoutian & Reichardt, 2001).

Glial cell-line derived neurotrophic factor (GDNF) is a small protein that potently promotes the survival of many types of neurons. The most prominent feature of GDNF is its ability to support the survival of dopaminergic and motor neurons, which are of therapeutic importance in Parkinson's disease (Carnicella *et al.,* 2008; Arevalo & Wu, 2006). GDNF also signals through the tyrosine kinase receptor, with GFRα1 being the GDNF-family-receptor (Arevalo & Wu, 2006).

The pathway, by which GDNF and BDNF act, is explained in figure 1.1. The specific neurotrophic factor (NF), binds to a growth factor receptor (GFR), which transmits activating signals to the Raf/MEK/ERK cascade through Ras. After binding to the GFR, the Growth factor receptor-bound (GRB) complex activates the Ras-activation guanine nucleotide exchange factor, Son of Sevenless (SOS), which activates Ras. This gives way to a cascade of phosphorylation and activation reactions, ultimately leading to the raf/MEK/ERK pathway and finally the functional neuroprotective effects of the neurotrophic factors (fig. 1.1). In section 2.1.7 a detailed explanation of the mitogen-activated protein kinase (MAPK) – pathway is given.

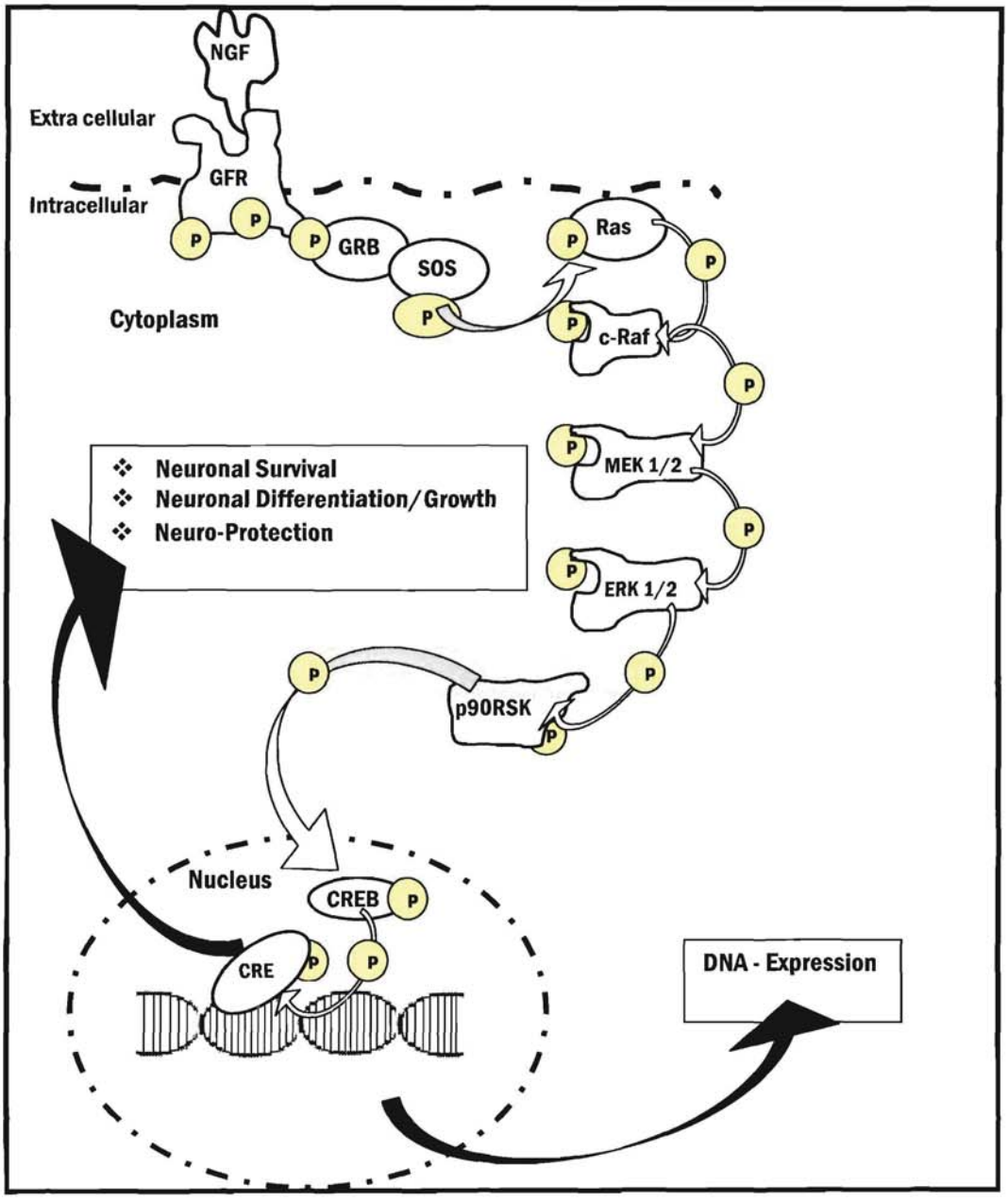

Figure 1.1: Pathway by which neurotrophic growth factors contribute to cell survival

Activation of nuclear factor kappa B (NF-κB) can protect cultured neurons against death induced by trophic factor withdrawal and exposure to excitotoxic, oxidative, and metabolic insults (Yu *et al.*, 2000). Gene targets that mediate the survival-promoting action of NF-κB may include manganese super oxide dismutase, Bcl-2, and apoptosis inhibitor proteins (Mattson, 2001). Increased Ca^{2+} levels or activation of membrane receptors (such as the receptor for secreted amyloid precursor protein α (sAPPα)) can stimulate cyclic guanosine 5'-monophosphate (GMP) production via a nitric oxide (NO)-mediated pathway, and cyclic

GMP can induce activation of K^+ channels and the transcription factor NF-κB and thereby increase resistance of neurons to excitotoxic apoptosis (Furukawa *et al.*, 1996a) (fig. 1.2).

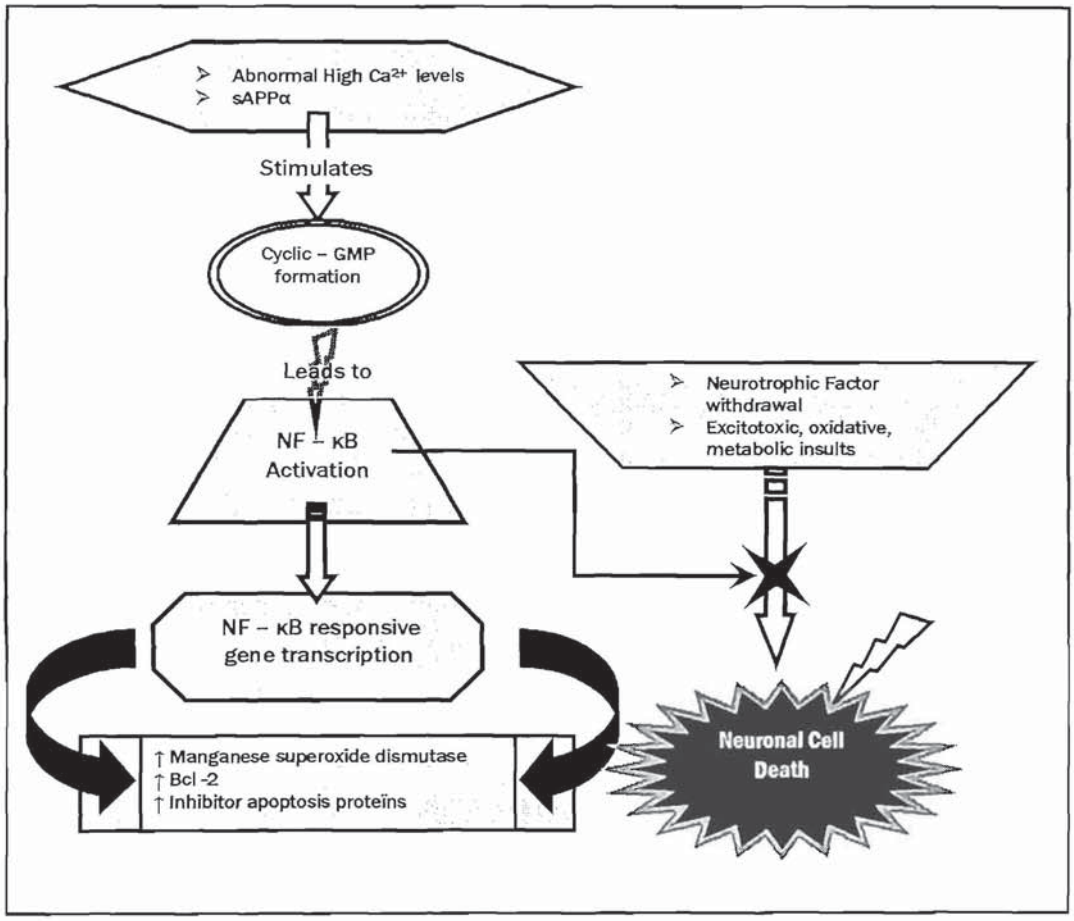

Figure 1.2: Neuroprotection by NF-κB

2.1.2 p53 dependent apoptosis

p53 is a sequence-specific DNA-binding protein (e.g., El Deiry *et al.*, 1992) that functions as a transcription factor (Farmer *et al.*, 1992; Kern *et al.*, 1992; Seto *et al.*, 1992) and interacts directly with proteins involved in both DNA replication (Dutta *et al.*, 1993) and DNA repair (Wang *et al.*, 1994). It can induce programmed cell death, suppress normal cell growth, or facilitate DNA repair (Oren, 1994). Endogenous p53 has been implicated in the cellular response to DNA damage (Kastan *et al.*, 1992), although p53-dependent apoptosis has previously occurred, in several instances, under circumstances not known to damage DNA (Holbrook *et al.*, 1996).

p53 presumably acts on an early event in the apoptotic pathway (Baffy *et al.*, 1993; Kane *et al.*, 1993), and can be induced by several factors. One of these factors include Aβ, which induces oxidative injury (Shearman *et al.*, 1994; Hensley *et al.*, 1994; Behl *et al.*, 1994) that

is an established inducer of p53 (Tishler *et al.*, 1993) (fig. 1.3). It activates the p53 dependent apoptotic pathway, with extra cellular deposition of Aβ occurring secondarily to the neuronal cell death. For Aβ to be able to activate the p53 dependent apoptotic pathway, it needs to accumulate to a threshold level (LaFerla *et al.*, 1996).

The downstream effectors of p53 in the apoptotic program are presently unknown. In principle, p53 may interact with the apoptotic machinery directly, transcriptionally regulating apoptotic genes, or indirectly by producing a cellular environment that facilitates apoptosis. Intriguing candidates for regulation by p53 during apoptosis are the products of the bcl-2 gene family (Holbrook *et al.*, 1996). Decreases in bcl-2 and increases in bax mRNA levels are associated with apoptosis induced by p53 over expression, suggesting that p53 regulates apoptosis by influencing the Bcl-2/Bax ratio (Miyashita *et al.*, 1994a, 1994b; Selvakumaran *et al.*, 1994). p53 also participates in a cell cycle checkpoint that arrests cell growth in response to DNA damage (Kastan *et al.*, 1992) (fig. 1.3).

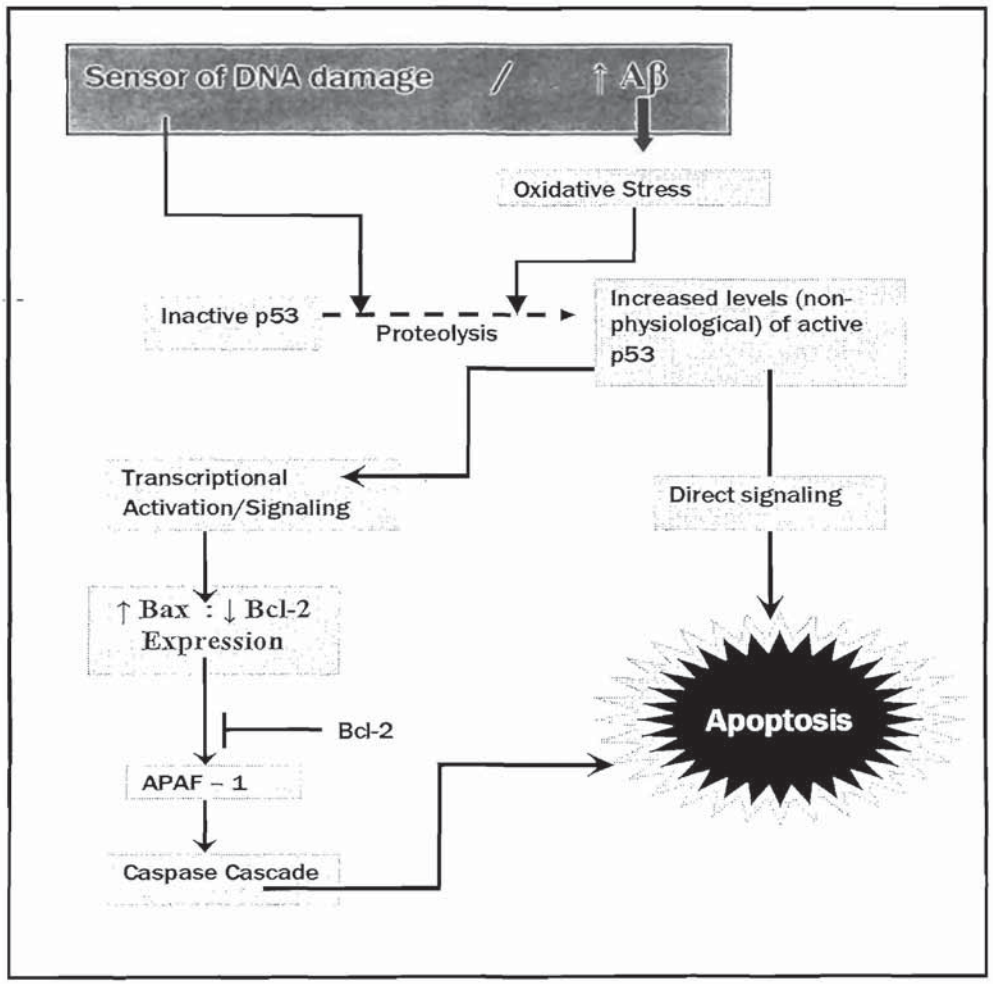

Figure 1.3: Apoptosis through the p53-pathway

2.1.3 Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a central glycolytic protein with a pivotal role in energy production, acting as a cellular kinase (Kawamoto & Caswell, 1986). It has a role in DNA replication and repair and possibly functions as a molecular chaperone. It acts as a pro-apoptotic enzyme downstream of Bcl-2 in the cell signal transduction cascade of apoptosis (Schulze *et al.*, 1993). In cells undergoing apoptosis, GAPDH is localised in the nuclear compartment, whereas this accumulation of GAPDH is normally suppressed in cells not undergoing apoptosis (Ishitani *et al.*, 1998; Saunders *et al.*, 1999; Sawa *et al.*, 1997). Its nuclear translocation in combination with other factors may be a key event during apoptosis and could have an influence in several neurodegenerative diseases (Schulze *et al.*, 1993). It is also important to note that the sub-cellular localisation and expression levels of endogenous GAPDH are different between neuronal and non-neuronal cell lines (Dastoor & Dreyer, 2001).

GAPDH exerts a role in neurodegenerative diseases that are characterised by the expansion of cytosine adenine guanine (CAG) repeats, in the genes that cause them. Some of these gene products include huntingtin and atrophin (Burke *et al.*, 1996), ataxin (Koshy *et al.*, 1996), androgen receptor and the β-amyloid precursor protein (Schulze *et al.*, 1993). They result in protein-protein interaction of GAPDH with the mentioned gene products of the different neurodegenerative diseases. When binding to GAPDH, these extended CAG repeats lead to inhibition of GAPDH activity, rendering it inactive, so that it can not play a role in DNA replication and repair (Burke *et al.*, 1996). GAPDH possibly serves as a carrier to mediate the translocation of these gene products into the nucleus (*Dastoor & Dreyer*, 2001).

Despite increasing evidence that GAPDH is involved in apoptosis, its role is unclear. Even though most cells that display nuclear GAPDH translocation do not have fragmented nuclei or show membrane blebbing (*Dastoor & Dreyer*, 2001), it is still possible that there is a correlation between its translocation into the nucleus and programmed cell death, since increased GAPDH expression, observed in apoptotic cells, is accompanied with nuclear fragmentation (Schulze *et al.*, 1993). Thus it can be concluded that the presence of GAPDH *in the nucleus* does not induce apoptosis *per se*, although nuclear localisation thereof might be an early indication of apoptotic cells or might even be responsible for apoptosis (Epner *et al.*, 1999; Ishitani *et al.*, 1998; Saunders *et al.*, 1997). GAPDH sometimes being present in the nucleus, without inducing or enhancing apoptosis, suggests that the nuclear localisation thereof is not responsible for the apoptotic action (Schulze *et al.*, 1993), but can be seen as an early event in the apoptotic cascade, most probably before the point of no return, where the cell death program can no longer be stopped (*Dastoor & Dreyer*, 2001). It is rather an

event coupled to the import of GAPDH into the nucleus that is responsible for the apoptotic action that follows (Schulze *et al.*, 1993).

2.1.4 Caspase-3 and poly(ADP-ribose)polymerase (PARP)

The presence of many different caspase substrates in synapses (Chan & Mattson, 1999), suggests that caspase-mediated cleavage of synaptic proteins may control the process of neuronal apoptosis (Mattson, 2001).

The caspases are a large family of cysteine proteases, which are responsible for the execution of apoptosis (Cryns & Yuan, 1998). They can act during the pre-mitochondrial phase (e.g., caspases 2 and 8) or post-mitochondrial phase (e.g., caspases 3 & 9) of apoptosis (Mattson, 2001).

Initially caspases are synthesised in inactive forms, called caspase zymogens (Degterev *et al.*, 2003). They can be activated in a reversible manner after trophic factor withdrawal or activation of glutamate receptors (Glazner *et al.*, 2000; Mattson, 2001). The apoptotic signalling pathways that lead to caspase zymogen processing can be subdivided into two major categories: cell surface sensor-mediated and intracellular sensor-mediated pathways. The cell surface sensor-mediated pathway is activated in response to extra cellular signals, indicating that the cell's existence is no longer needed for the well-being of the organism. These cell surface sensor-directed apoptotic signals are initiated by ligands binding to cell surface death-mediating receptors, and are exemplified by the signalling of the death receptor family. The other major category of apoptotic signalling, the intracellular sensor-mediated pathway, is activated by stimuli such as DNA damage and catatonic drugs, which act inside the cell. Cells possess multiple means of targeting mitochondria, which play an important role in these pathways, but most of these signals integrate at the level of the Bax/Bak gateway (Degterev *et al.*, 2003).

The caspases can be classified into two groups according to their function. These two groups are initiator (upstream) and execution (downstream) caspases. The initiator caspases (caspase-1, -2, -4, -5, -8, -9, -10, -11 and -12) are activated upon apoptotic signals. When activated this group of caspases activate the execution caspases (caspase-3, -6, -7), which perform the downstream execution steps of apoptosis by cleaving multiple cellular substrates (Degterev *et al.*, 2003).

In the present study the focus will be on caspase-3 and caspase-9, since they are of significance in the mechanism of action of the compounds studied.

The release of cytochrome c, induced by a variety of death stimuli, results in the activation of a complex, which consists of apoptosis-activating factor 1 (Apaf-1) and caspase-9. This complex leads to the cytochrome c dependent processing of pro-caspase-3 to active caspase-3, and ultimately to apoptosis (Kluck *et al.*, 1997; Reed, 1997; Zou *et al.*, 1997). Apart from caspase activation, cytochrome c release causes slow irreversible loss of mitochondrial function and respiration, leading to the death of the cell. Therefore, the mitochondrial apoptotic pathway appears to result in a bipartite 'point-of-no-return' event, consisting of fast caspase activation and slow caspase-independent death through mitochondrial dysfunction (Degterev *et al.*, 2003).

Downstream from the mitochondria, caspase-9 is an important intracellular amplifier of caspase signalling (Degterev *et al.*, 2003), and acts as a critical upstream activator of caspase-3 (Porter & Janicke, 1999). It forms part of the apoptosome complex, and is activated through an apoptosome-induced conformational change. This forms active caspase-9, which processes the executioner caspase-3 to enable the initiation of the execution phase of apoptosis (Li *et al.*, 1997; Slee *et al.*, 1999). It is thus a critical upstream activator of caspase-3 (Porter & Janicke, 1999) (fig. 1.4).

Caspase-3 is a frequently activated death protease (Porter *& Janicke*, 1999), which plays a major role in the executive phase of apoptosis (Nicholson *et al.*, 1995; Tewari *et al.*, 1995), mediating nuclear apoptosis (Hirata *et al.*, 1998). Caspase-3 is important for cell death in a remarkable tissue-, cell type- or death stimulus-specific manner, and is essential for some of the characteristic changes in cell morphology to take place as well as certain biochemical events associated with the execution and completion of apoptosis (Porter & Janicke, 1999).

Caspase-3 activation in neurons possibly marks the beginning of the effector (downstream) phase of apoptosis (Hartmann *et al.*, 2000). Once it has been activated, downstream death substrates (Nicholson & Thornberry, 1997; Cryns & Yuan, 1998; Reed, 1997), such as Poly(ADP-ribose) polymerase (PARP), a protein that repairs damaged DNA and regulates chromatin structure, are cleaved and inactivated, irrespective of the involvement of cytochrome c (Nicholson & Thornberry, 1997; Cryns & Yuan, 1998; Reed, 1997). This proteolytic cleavage and inactivation of PARP by caspases is an early indicator of apoptosis, and is a process which consumes large amounts of NADP, thereby indirectly depleting the cellular ATP store (Kaufmann *et al.*, 1993). As a result of the inactivation of PARP, DNA fragmentation takes place.

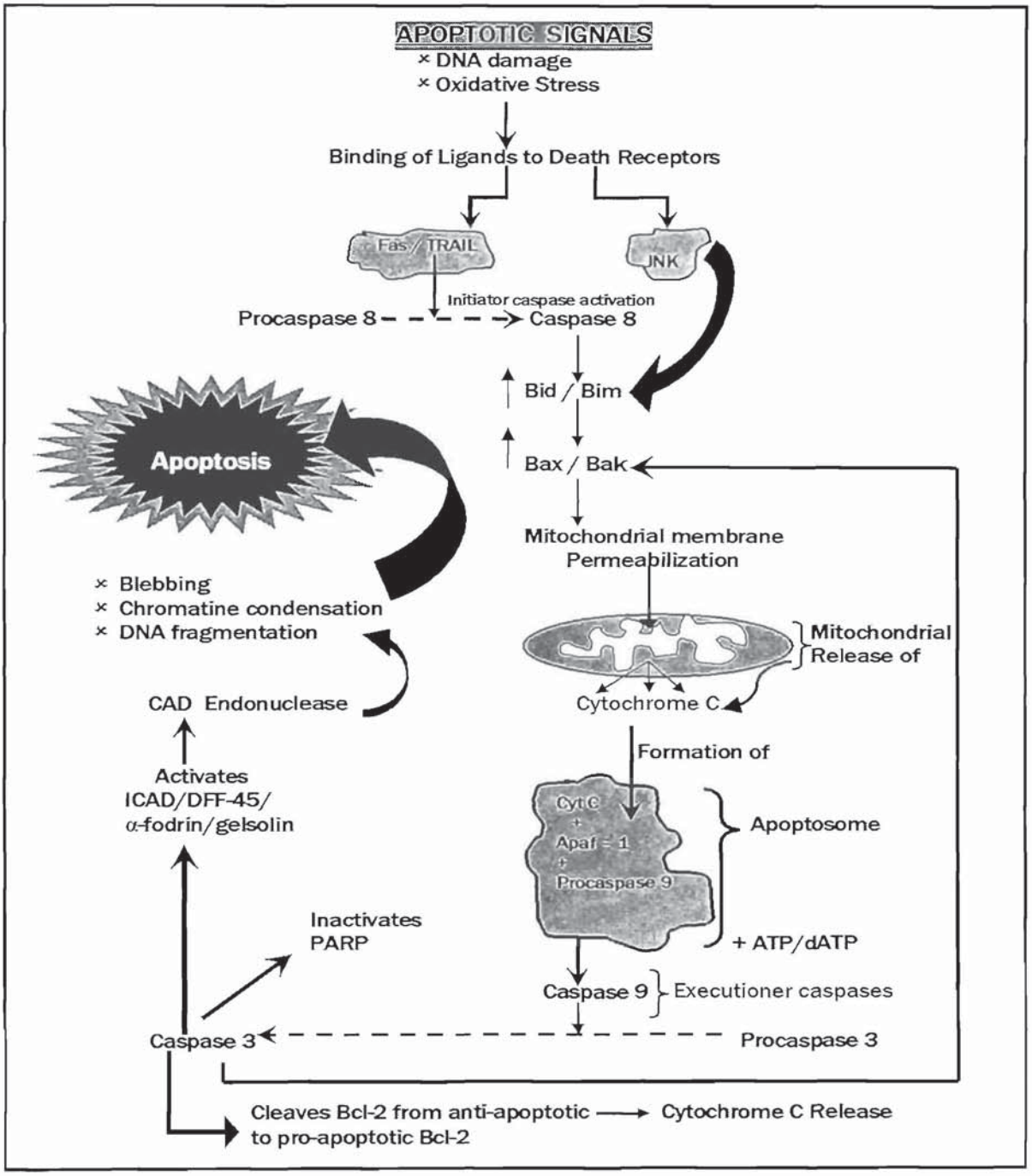

Figure 1.4: Bcl-2 regulated mitochondrial apoptosis

Caspase-3 might also amplify the upstream death cascade, including cytochrome c release from mitochondria, by cleaving Bcl-2 and converting it from an anti-apoptotic to a pro-apoptotic protein (Cheng *et al.*, 1997). Caspase-3 can exert multiple effects of which some are critical for cell death to occur. Other effects of caspase-3 may ensure the efficient completion of the apoptotic process once the cell has been committed to die (Porter & Janicke, 1999) (fig 1.4). Once the program has been completed, activated caspase-3 can no longer be detected, possibly because of degradation of the protease itself (*Hartmann et al.*,

2000). The cleavage of inhibitor of caspase activated DNAse/DNA fragmentation factor-45 (ICAD/DFF-45), that results in the induction of the caspase activated DNAse (CAD) endonuclease, is a caspase-3-dependent step in a major pathway to DNA fragmentation, by inter-nucleosomal degradation (Porter & Janicke, 1999). The DNA fragmentation mentioned forms part of the final steps of apoptosis.

The activation of caspase-3 has been associated with neuronal death in several experimental models of acute and chronic neurodegenerative disorders (Bergeron & Yuan, 1998; Pettmann & Henderson, 1998; Schulz *et al.*, 1999). Neurotoxins commonly used to induce experimental parkinsonian syndromes, e.g., 1-methyl-4-phenylpyridinium (MPP^+) and 6-hydroxydopamine (6-OHDA), have been shown to exert their pro-apoptotic actions via activation of caspase-3-like proteases in neuronal *in vitro* models (Dodel *et al.*, 1998; Dodel *et al.*, 1999; Lotharius *et al.*, 1999). The increased expression of caspase-3 in Lewy body-containing neurons is in line with the hypothesis that caspase-3 is a probable effector of apoptotic cell demise (Hartmann *et al.*, 2000). Furthermore, it has been shown that the oligomerisation of Aβ 1-42 and Aβ 1-40 peptides, which arise from the β and γ-secretase cleavage of the trans-membrane portion of the amyloid precursor protein (APP), is critical to the progressive dysfunction and loss of cholinergic neurons in Alzheimer's disease (Selkoe, 2000, 2001). Caspase-3 has also been found to cleave the cytosolic tail of APP, stimulating the subsequent γ–secretase cleavage step approximately five-fold (Gervais *et al.*, 1999). The released cytosolic portion of APP has been suggested to play a separate role in the disease pathophysiology (Nishimura *et al.*, 2002).

2.1.5 Bcl-2 family proteins

The Bcl-2 family proteins are key regulators of apoptosis (Eisenmann *et al.*, 2003). They are integral membrane-bound proteins (Tsujimoto *et al.*, 1984) that are concentrated at the mitochondrial, nuclear, and endoplasmic reticular membranes, which are all sites of free radical production (Krajewski *et al.*, 1993).

Normal cellular homeostasis requires the suppression of pro-apoptotic players by various mechanisms, including phosphorylation, intracellular localisation, and heterodimerisation with pro-survival Bcl-2 family proteins. Disruption of the balance between pro- and anti-apoptotic Bcl-2 family members is suggested to be fundamental to the development of many diseases (Eisenmann *et al.*, 2003). These proteins may control the cell-death process by interacting with mitochondrial membranes in a manner that either promotes or prevents ion movements across mitochondrial membranes (Green & Reed, 1998).

Three sub-families have been identified: (a) the pro-survival Bcl-2 (e.g., Bcl-2 and Bcl-XL) proteins, which are anti-apoptotic; (b) the pro-apoptotic Bax (e.g., Bax and Bak) proteins and (c) the BH3 domain-only (e.g., Bad, Bim, and Bid) proteins, with Bad being pro-apoptotic (Adams & Cory, 1998; Cory & Adams, 2002; Gross *et al.*, 1999). Structurally, the BH3-only proteins are divergent from other Bcl-2 family members (Eisenmann *et al.*, 2003) (fig. 1.5).

Bcl-2 and Bax form homodimeric complexes or can heterodimerise with each other, and the ratio of Bcl-2 to Bax expression may ultimately determine cell survival following an apoptotic stimulus. Bcl-2 acts downstream of p53 to inhibit apoptosis (Oltvai *et al.*, 1993) by increasing resistance of neurons to death induced by excitotoxic, metabolic, and oxidative insults relevant to AD, stroke, and other disorders (Martinou *et al.*, 1994; Guo *et al.*, 1998). It also protects neurons against hydrogen peroxide and other agents that cause free radical damage, such as radiation. Bcl-2 prevents apoptosis by blocking the redistribution of calcium (Lam *et al.*, 1994; Holbrook *et al.*, 1996). Bax is structurally related to Bcl-2, but functionally promotes apoptosis (Oltvai *et al.*, 1993). In addition to direct p53 regulation (Miyashita *et al.*, 1994b), Bcl-2 appears to be regulated at the protein level by Bax (Oltvai *et al.*, 1993), which is a p53 immediate early response gene. Bcl-2 and Bax proteins appear to compete with one another to control the relative susceptibility of cells to p53-mediated apoptosis (Selvakumaran *et al.*, 1994). Another mechanism, by which Bcl-2 possibly protects cells from apoptosis, is its efficient prevention of translocation of endogenous GAPDH into the nucleus (*Dastoor & Dreyer*, 2001) (see section 2.1.3).

Bad is regulated through its phosphorylation and cytosolic sequestration. Dephosphorylated Bad promotes apoptosis, by binding to either Bcl-2 or Bcl-XL, and titrating them away from pro-apoptotic Bax/Bak proteins. Unbound Bax oligomerises which disrupts mitochondrial integrity, causing cytochrome c release and initiating the caspase cascade (Cory & Adams, 2002; Downward, 1999). Phosphorylated Bad is however bound and sequestered in the cytosol by the chaperone protein 14-3-3. Phosphorylation of one of at least three serine residues inactivates Bad by regulating interactions with either 14-3-3 or Bcl-2 family members. Several survival kinases are implicated in the direct phosphorylation of Ser^{75}, Ser^{99} and Ser^{118}, including RSKs, Akt or p70S6K and PKA, indicating that Bad functions as an important convergence point in signal transduction pathways affecting cell survival (Bonni *et al.*, 1999; Shimamura *et al.*, 2000; Fang *et al.*, 1999) (fig. 1.5).

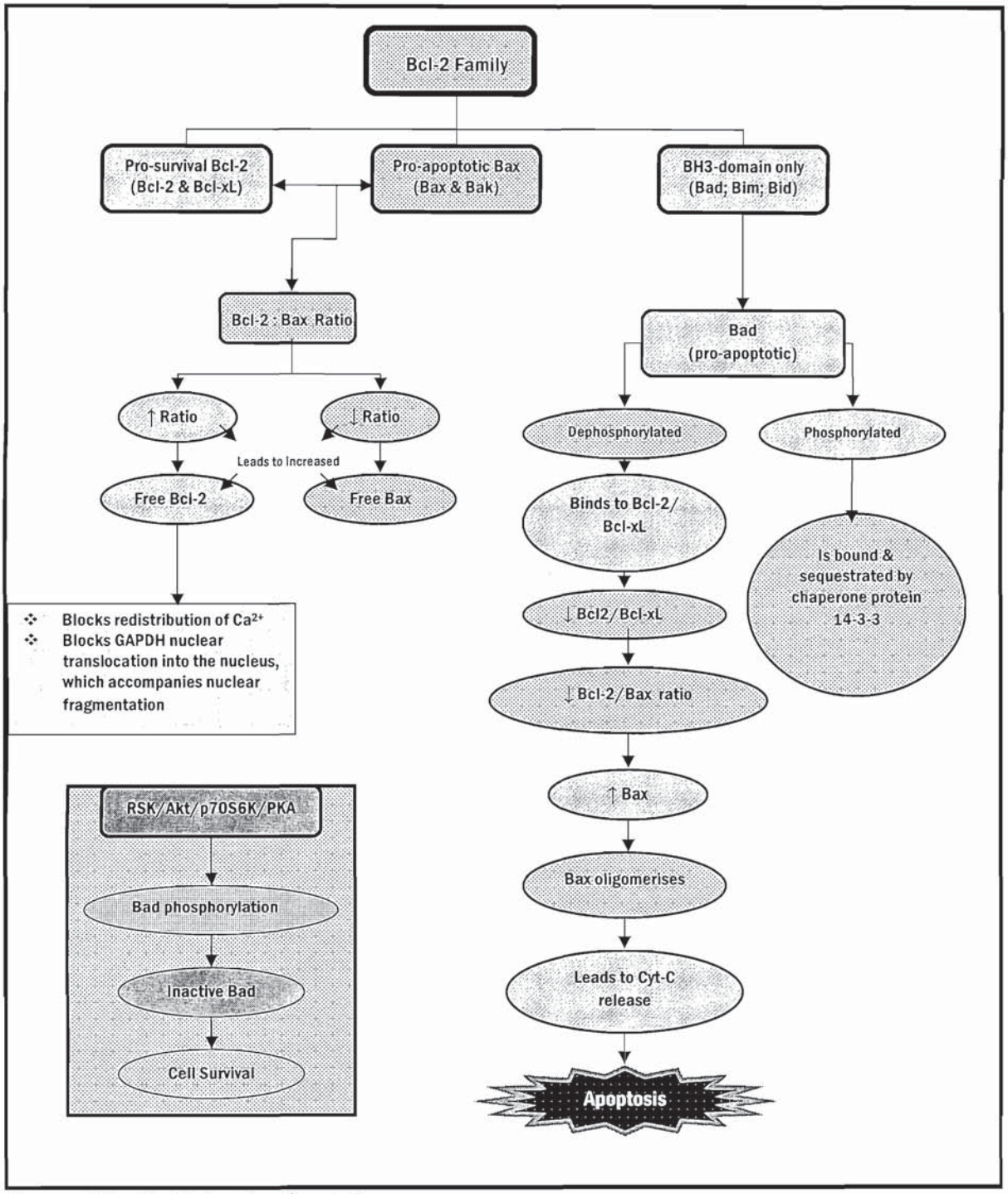

Figure 1.5: Bcl-2 family of proteins

2.1.6 Protein kinase C (PKC)-pathway/amyloid precursor protein (APP)

The β-amyloid protein and amyloid precursor protein (APP), are critical components of Alzheimer's' disease pathology and are greatly controlled and regulated by the PKC pathway. PKC is a phospholipid-dependent protein kinase that plays a crucial role in various cellular functions in neuronal and non-neuronal cells. In neurons it is a key enzyme in

neurotransmission, synaptic plasticity, learning and memory. Certain PKC isoforms are intimately involved in cell survival by suppressing apoptosis induced by Aβ (Weinreb *et al.*, 2004).

With regard to AD, PKC is linked to amyloid precursor protein (APP) processing (Fluhrer *et al.*, 2004). Non-amyloidogenic sAPPα is released by PKC- and MAPK-dependent pathways, with the activation of MAPK being dependent on PKC signalling pathway activity (Yogev-Falach *et al.*, 2003).

Proteolytic processing of the amyloid precursor protein (APP) can proceed via two opposing paths, with vastly different outcomes (Wilquet *et al.*, 2004): The amyloidogenic path, which produces β-amyloid (Aβ) fragments, the etiological agents of AD pathology, and the non-amyloidogenic path, which produces neuroprotective sAPPα fragments (Mattson, 1994; Meziane *et al.*, 1998).

The amyloidogenic path involves sequential cleavages of APP, by β-secretase/beta-site APP-Cleaving Enzyme (BACE) and γ-secretases, with the generation of β-amyloid (Aβ) fragments, which are the etiological agents of AD pathology (Allinson *et al.*, 2003). When Aβ is in an aggregated form, it can induce membrane lipid peroxidation in hippocampal and cortical neurons (Mark *et al.*, 1995; 1997a) as well as in cortical synaptosomes (Keller *et al.*, 1997). Lipid peroxidation promotes neuronal death in part by impairing the function of membrane ion-motive ATPases (Na^+/K^+-ATPase and Ca^{2+}-ATPase) and glucose transporters (Mark *et al.*, 1995, 1997b), decreasing ATP levels, which promotes membrane depolarisation, energy depletion, and disruption of cellular Ca^{2+} homeostasis. Membrane lipid per-oxidation also impairs glutamate transport in astrocytes and synaptosomes (Keller *et al.*, 1997; Blanc *et al.*, 1998), which would be expected to further promote excitotoxic injury (Mattson, 2001). The oxidative stress induced by Aβ can also render neurons vulnerable to excitotoxicity and apoptosis (Mattson *et al.*, 1992; Mark *et al.*, 1995, 1997a; Kruman *et al.*, 1997). Aβ also induces time- and dose-dependent decreases in catalase activity and increases in Cu/Zn- and Mn-superoxide dismutase (SOD) activities (Bruce *et al.*, 1997). Besides the mentioned toxic effects of β-amyloid, it also appears to stimulate calcium release directly and hydrogen peroxide/super oxide production, which again, could lead to apoptosis (Holbrook *et al.*, 1996). Although Aβ is secreted throughout life, it begins to accumulate in old age (Olariu *et al.*, 2005). It is now understood that neurons are the main source of Aβ in AD and it has been hypothesised that Aβ gradually accumulates in the extra cellular space due to excess secretion and/or deficient clearance (Chaney *et al.*, 2003).

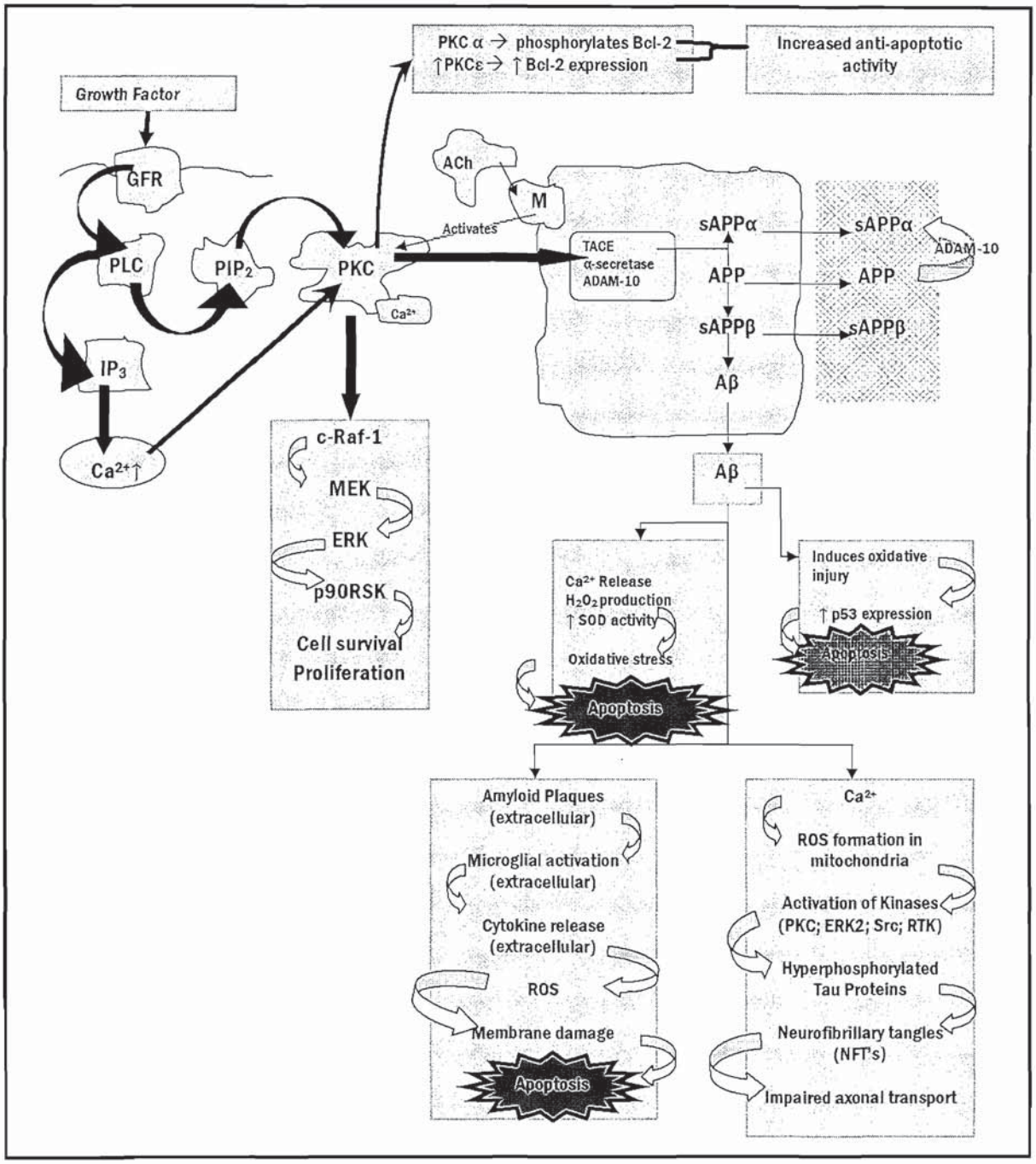

Figure 1.6: β-amyloid peptide and PKC in apoptosis

The non-amyloidogenic path, controlled by PKC (Takahashi *et al.*, 2002), involves APP cleavage by α-secretases (Allinson *et al.*, 2003), at a site which will preclude BACE cleavage, and release a neuroprotective sAPPα fragment (Mattson, 1994; Meziane *et al.*, 1998) (fig. 1.6). PKCα and -β are key regulators of α-secretory APP processing (Rossner *et al.*, 2001), with PKCα being specifically involved in sAPPα release. PKCϵ is involved in coupling cholinergic receptors with APP metabolism (Lanni *et al.*, 2004), with stimulation of muscarinic receptors, increasing cleavage of APP through the α-secretase pathway, and

inhibiting Aβ production (Muller *et al.*, 1997). Increasing the α-secretase processing pathway could be beneficial for the treatment of AD, by shifting the balance of APP processing toward a presumably non-pathogenic pathway (Yogev-Falach *et al.*, 2003). Even though it does not necessarily affect Aβ generation or slow amyloid plaque formation (Olariu *et al.*, 2005) (fig. 1.6), the non-amyloidogenic sAPPα has potent neurotrophic and neuroprotective activities against excitotoxic and oxidative insults in various cellular models and can serve as a neuroprotective agent against the toxic activity of Aβ (Yogev-Falach *et al.*, 2003).

2.1.7 Mitogen-activated protein kinase (MAPK)-pathway

Cells recognise and respond to extracellular stimuli by engaging specific intracellular programs, such as the signalling cascade that leads to activation of the mitogen-activated protein kinases (MAPKs) (Roux & Blenis, 2004). These intracellular signalling pathways strictly control cell function and fate, i.e. differentiation, death and survival. The Ras/Raf/MEK/ERK pathway can regulate cell cycle progression and apoptosis, and plays a fundamental role in Bad inactivation (Chang *et al.*, 2003).

Each family of MAPKs is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) (Dan *et al.*, 2001; Kolch, 2000).

Five distinct groups of MAPKs have been characterised in mammals: Extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK1/2), c-Jun amino-terminal kinases (JNKs) 1, 2, and 3, p38 isoforms α, β, and δ, ERK's 3 and 4 and ERK5. ERK1/2 is preferentially activated in response to growth factors and phorbol esters, while the JNK and p38 kinases are more responsive to stress stimuli (Roux & Blenis, 2004). A decrease in neurotrophic growth factors is shown to be part of the pathology of numerous neurodegenerative disorders (Mattson & Lindvall, 1997). For this reason the focus will be on the ERK1/2 pathway in this study. This mammalian ERK1/2 module, is also known as the classical mitogen kinase cascade, consisting of the MAPKKKs, A-Raf, B-Raf and Raf-1, the MAPKKs, MEK1/2, and the MAPKs, ERK1/2 (Roux & Blenis, 2004). B-Raf and Raf-1 presumably participates in neuronal apoptosis, with A-Raf mainly presiding in urogenital tissues (Wellbrock *et al.,* 2004). Apart from activating MEK1/2, B-Raf and Raf-1 also inactivates Bad indirectly, by increasing the activity of PKCθ (Hindley & Kolch, 2007).

Cell surface receptors such as tyrosine kinases (RTK) and G protein-coupled receptors transmit activating signals to the Raf/MEK/ERK cascade through Ras (Campbell *et al.,* 1998; Wood *et al.*, 1992). Activation of Ras is achieved through recruitment of Son of Sevenless (SOS), a Ras-activation guanine nucleotide exchange factor. SOS stimulates Ras to change

GDP to GTP, allowing it to interact with and activate Raf (Geyer & Wittinghoffer, 1997), a small GTP-binding protein of the Ras/Rho family. The activated Raf gives way to the activation of a MAPKK, e.g. MEK1/2, through phosphorylation in response to extra cellular stimuli (Dan *et al.*, 2001; Kolch, 2000). MAPKKK activation leads to the phosphorylation and activation of a MAPK, e.g. ERK1/2 (Hallberg *et al.*, 1994), which then stimulates MAPK activity through dual phosphorylation (Roux & Blenis, 2004), and acts as a key regulator of cell proliferation (Kohno & Pouyssegur, 2003; Roux & Blenis, 2004).

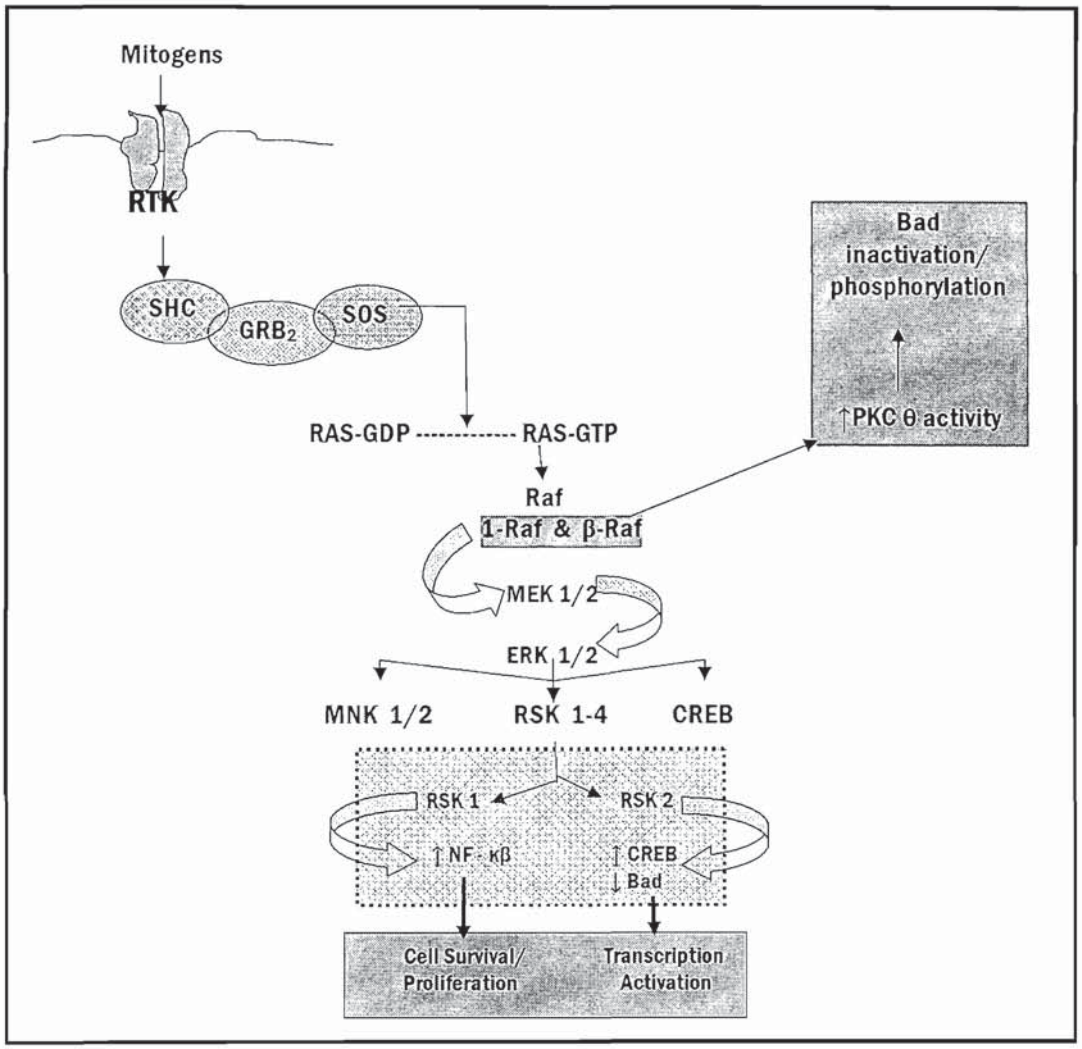

Figure 1.7: Ras/Raf/MEK/ERK pathway to cell survival

Once activated, MAPKs phosphorylate target substrates such as MAPK-activated protein kinases (MKs), of which the ribosomal S6 kinases (RSKs) are relevant. These RSKs form part of the MK (MAPK-activated protein kinases) family (Roux & Blenis, 2004), and are exclusively activated by the ERK's (Frodin & Gammeltoft, 1999; Roux & Blenis, 2004). Activated RSK phosphorylates multiple transcription factors (Thomson *et al.*, 1999). It has

been shown that the neurotrophic factor-stimulated RSK2 isoform promotes cortical neuron survival by phosphorylating Bad and CREB (Bonni *et al.*, 1999; Ginty, *et al.*, 1994; Xing *et al.*, 1996), whereas RSK1 can promote survival by activating NF-κB (Ghoda *et al.*, 1997; Schouten *et al.*, 1997; Roux & Blenis, 2004). RSK2 phosphorylation of Bad facilitates Bad's inactivation through binding to 14-3-3 and sequestration from heterodimerising with mitochondria-bound Bcl-2 or Bcl-XL (Tan *et al.*, 1999; Lzcano *et al.*, 2000; Harada *et al.*, 2001; *Datta et al.*, 1997). Bad inactivation occurs through hyper-activation of the MEK/ERK/RSK signalling module, with B-Raf being an upstream activator of the MEK/ERK signalling pathway (Brose *et al.*, 2002; Davies *et al.*, 2002; Satyamoorthy *et al.*, 2003; Pollock *et al.*, 2003). Activation of the MEK/ERK pathway inhibits apoptotic cell death (Eisenmann *et al.*, 2003) (fig. 1.7).

3 Monoamine oxidase B (MAO-B) in neurodegenerative diseases

Monoamine oxidases are enzymes that catalyse the oxidation of monoamines. They are bound to the outer membrane of mitochondria in most cell types in the body, and belong to a protein family of flavin containing amine oxidoreductases (Kearney *et al.*, 1971).

In mammals, MAO is present as two isoforms (MAO-A and MAO-B), which are separate gene products and separate enzymes, that exhibit over 70% sequence identity and distinct but overlapping substrate specificities in the catabolism of neurotransmitters such as dopamine and serotonin (Weyler *et al.*, 1990; Shih *et al.*, 1999). They are both implicated in a large number of neurological disorders and are targets for drugs against Parkinson's disease and depression (Cesura & Pletscher, 1992). Mammalian MAOs are bound to the outer mitochondrial membrane and have a FAD molecule as the co-factor, which is covalently bound to the protein (Kearney *et al.*, 1971). The FAD site is the site at which irreversible inhibitors of MAO are covalently linked (Youdim *et al*, 2005). The enzymes are expressed in both a tissue-dependent and an age-dependent manner (Binda *et al.*, 2002) and have different patterns of tissue distribution and different substrate and inhibitor specificity (Johnston, 1968).

These enzymes catalyse the oxidative deamination of monoamine neurotransmitters and neuromodulators such as dopamine, noradrenalin, adrenaline, serotonin (5-hydroxytryptamine) and β-phenylethylamine (PEA), as well as some exogenous bioactive monoamines (Johnston, 1968). Serotonin, noradrenalin and adrenalin are mainly deaminated by MAO-A, whereas PEA is preferentially deaminated by MAO-B. Dopamine on the other hand is metabolised by both isoforms, MAO-A and MAO-B (Riederer *et al.*, 2004).

MAO-B constitutes about 80% of the total MAO activity in the human brain (Riederer *et al.*, 1978; Sonsalla & Golbe, 1988) and is the predominant form of the enzyme in the striatum (Riederer *et al.*, 1989, 2004). For this reason the focus will be mainly, but not exclusively, on the development of MAO-B specific inhibitors.

3.1 Mechanism of action of monoamine oxidase B (MAO-B)

Monoamine oxidase oxidises primary aliphatic amines as well as some secondary and tertiary amines according to equation 1 (Tipton *et al.*, 2004):

$$RCH_2NR'R'' + O_2 + H_2O \longrightarrow RCHO + NHR'R'' + H_2O_2 \qquad \text{(eq. 1)}$$

Kinetic studies have shown the reaction to involve the binding of the amine substrate to the enzyme before oxygen. The reaction proceeds in two steps: In the first of these, reduction of the enzyme-bound FAD results in the formation of the aldehyde product and ammonia, whereas the second step entails the re-oxidation of the enzyme-bound FAD by O_2 with the formation of hydrogen peroxide (Tipton *et al.*, 2004) (eq. 2 and eq. 3):

$$RCH_2NH_2 + FAD \xrightarrow{+H^+} RCHO + NH_4^+ + FADH_2 \qquad \text{(eq. 2)}$$

$$FADH_2 + O_2 \xrightarrow{+H_2O} FAD + H_2O_2 \qquad \text{(eq. 3)}$$

With primary amines the first partial reaction is believed to proceed *via* an imine intermediate, which is then hydrolysed by water to the final product (Tipton *et al.*, 2004) (eq. 4):

$$RCH_2NH_2 \xrightarrow{-H^+} RCH{=}N^+H_2 \xrightarrow{+H_2O} RCHO + NH_4^+ \qquad \text{(eq. 4)}$$

The hydrolysis of this type of intermediate does not occur in the case of some irreversible inhibitors or with the neurotoxin MPP^+ (Tipton *et al.*, 2004).

3.2 Protective strategies

Ontogenetic studies have demonstrated that MAO-B activity stays unchanged until about the 60th year of life, after which it increases non-linearly (Delumeaeu *et al.*, 1994; Strolin & Dostert, 1989), with the expression levels increasing ~4-fold with age (Fowler *et al.*, 2003). Because MAO-B is predominantly located in glial cells, the increase of this enzyme with age may be attributed to glial cell proliferation associated with neuronal loss (Shih *et al.*, 1999; Mellick *et al.*, 1999). This age-related increase of brain MAO B activity is believed to cause an augmentation in oxidative stress (Barnham *et al.*, 2004), by the production of increased levels of hydrogen peroxide, and an increased level of dopamine metabolism. This is

thought to play a role in the etiology of neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Kumar *et al.*, 2003), where there is indeed an increased level of MAO activity (Mandel *et al.*, 2005). In conjunction with this, studies have demonstrated that elevated MAO-B levels induce apoptosis in neuronal cells (Boulton *et al.*, 1998).

The development of specific, reversible MAO-B inhibitors can lead to clinically useful neuroprotective agents (Hubalek *et al.*, 2005). The hydrogen peroxide (H_2O_2) produced during the mentioned amine oxidation can accumulate in PD patients, making it available for the Fenton reaction, wherein a highly active free radical, the hydroxyl radical, is formed and can damage nucleic acids, proteins, and membrane lipids, leading to neuronal degeneration (Nicotra *et al.*, 2004). MAO-B inhibitors, which decrease the rate of MAO-B catalysed oxidative deamination and consequently, the production of reactive oxygen species (ROS), might thus beneficially contribute to the treatment of Alzheimer's and Parkinson's diseases through neuroprotection (Youdim *et al.*, 2004). Moreover, in the context of Parkinson's disease, MAO-B inhibitors present a second therapeutic application, due to MAO being one of dopamine's major metabolising enzymes. As MAO-B is present in excess in the tissue in which metabolism occurs, the inhibition of the iso-enzyme B blocks the metabolism of dopamine, enhancing both the endogenous dopamine level and dopamine produced from exogenously administered precursor levodopa (L-DOPA) (Foley *et al.*, 2000; Yamada & Yasuhara, 2004). The inhibition of dopamine degradation by MAO-B inhibitors combined with supplementation of dopamine by L-DOPA has been shown to be successful in the treatment of PD patients (Palhagen *et al.*, 2006).

It is important to note that selective inhibition of MAO-A or -B, will not change the levels of dopamine drastically in the human striatum (Riederer & Youdim, 1986). This is in contrast with those monoamines that are substrates for only one isoform. Although selective inhibition of MAO-A or -B do not affect the steady state level of dopamine in the brain, such inhibition did affect its release, which would explain the anti-symptomatic effects observed in PD patients, with some of these drugs (Youdim & Bakhle, 2006). Non-selective MAO inhibitors, such as ladostigil, increase levels of all three monoamines, noradrenaline, 5-HT and dopamine in the hippo-campus and striatum of rats and mice. It also shows anti-depressant activity in animal models (Sagi *et al.*, 2005), which makes it a useful and beneficial side effect in PD patients, as a significant proportion (40-60%) of patients exhibit signs of depression (Youdim & Bakhle, 2006). Inhibitors of MAO-A have been proved to be effective anti-depressants, while MAO-B blockers have been emphasised in the treatment of Parkinson's disease (Riederer *et al.*, 2004).

4 Concluding remarks

In focusing on the neurodegenerative disorders, Alzheimer's disease and Parkinson's disease, it is clear that an intervention is necessary to slow down the progression of the neuronal breakdown process, which takes place as part of the pathophysiology of these diseases. The neurodegeneration in PD and AD takes place by an intrinsic cell suicide program known as apoptosis, which consists of several pathways and cascades, which ultimately lead to the death of neuronal cells in certain areas of the brain, depending on the disorder. Monoamine oxidase B also has a role in this cell death process, but also contributes to the signs and symptoms presented by PD patients.