CHAPTER 1

Introduction
1.1 Neurodegeneration

Neurodegeneration is defined as progressive loss of neuronal structure and function that ultimately leads to neuronal death. Neurodegeneration occurs in various diseases affecting the central nervous system. The loss of specific populations of neurons related to functional neuronal networks determines the clinical presentation of the neurodegenerative disease. For example, degeneration of neurons located in the frontal lobes and caudate nucleus/striatum of the basal ganglia are associated with Huntington’s disease, although the loss of neuronal function in the substantia nigra and striatum is related to Parkinson’s disease.

Because of the social and financial impact of these diseases to modern western societies, the development of affordable and effective therapies to prevent and protect against neurodegenerative diseases is of great interest. However, the prospects of adequate treatment of brain diseases are still very limited in spite of the impressive increase of neuroscience research during the last four decades. Due to the high level of complexity of brain function and dysfunction, the progress in development of new treatments and safe drugs is still impeded by insufficient knowledge of the causes and the mechanisms by which neurons die in neurodegenerative disorders.

Classification of neurodegenerative disorders has been a matter of serious dispute for a long time since many disorders overlap with one another in their clinical representation and their neuropathological characteristics. Traditionally, diseases of the brain were categorized based on the main clinical feature or the anatomical distribution of the predominant lesion. According to the anatomical regions, neurodegenerative disorders include roughly diseases of the cerebral cortex, the basal ganglia, the brainstem, the cerebellum or the spinal cord (Dickson, 2003). Within each group a further classification was made, based on particular clinical features. The cerebral cortex diseases were subdivided into dementing (e.g. Alzheimer’s disease (AD)) and non-dementing illnesses. However, dementia is not specific to AD only. It can accompany a diversity of conditions besides neurodegenerative disorders such as metabolic or infectious brain diseases. More recent classifications tend to be based not on anatomical dysfunctions but on common molecular defects. The major group of molecules implicated in neurodegenerative processes includes amyloid (Alzheimer’s disease (AD), some forms of Creutzfeldt-Jacob disease); tau (Alzheimer’s disease, Frontotemporal-17 dementia, Parkinson’s disease, Pick’s disease, Progressive Supranuclear Palsy), a-synuclein (Parkinson’s disease, Multiple System Atrophy dementia, Diffuse Lewy Body dementia and some forms of AD), trimucleotide-repeat sequence (Huntington disease, Spino-Cerebellar Atrophy, Myotonic Dystrophy) and prions (Creutzfeldt-Jacob disease, Fatal Familial Insomnia, Gerstmann-Sträussler-Scheinker, Kuru, Scrapie) (Dickson, 2003).

Given that regenerative capacities are rather limited in the adult central nervous system neuronal death represents a catastrophic event during neurodegenerative processes. Neuronal cell death can be roughly divided into necrotic and non-necrotic types. Necrotic cell death is a fast process characterized by cell swelling that requires...
no active contribution of the degenerative cell. In contrast, non-necrotic cell death is tightly regulated by autonomous processes, requires the engagements of active cellular processes and ultimately induces distinctive ultrastructural alterations. This cell death type can be further divided into apoptotic and autophagic type (Clarke, 1990). The hallmark features of apoptosis include chromatin condensation, nuclear fragmentation, margination and cytoplasmic blebbing (Clarke, 1999). Apoptosis is the most common and well-investigated form of programmed cell death. It is implicated in several neurodegenerative disorders and the main regulators include Bcl-2 and caspase families (Korsmeyer, 1999; Wellington and Hayden, 2000).

1.1.1 Alzheimer’s disease

AD represents the most common cause of dementia. Behavioral abnormalities followed by impairments in language proficiency, sensory perceptions and motor skills often occur during the progression of AD. The disease includes sporadic and familial forms with both “early-onset” (less than 65 years of age) and “late-onset” (over 65 years of age) forms. The diagnosis is rather difficult since the clinical features of AD overlap with the symptoms of various other neuropathological conditions. In addition, a definite confirmation of AD is achieved only by morphological and histological examination of the brain at autopsy (Jellinger, 1998). AD pathology is characterized by an accumulation of “senile plaques” and “neurofibrillary tangles” in brain regions involved in learning and memory processes and degeneration of basal forebrain cell groups. Senile plaques are extracellular deposits of fibrils and amorphous or diffuse aggregates of amyloid β-peptide (Aβ). Amyloid precursor protein (APP) is an integral membrane protein, highly expressed throughout the body. In AD, APP is abnormally cleaved by several secretases and this results in formation of the insoluble Aβ peptides (Fig. 1.1). Neurofibrillary tangles are intracellular accumulations of hyperphosphorylated microtubule-associated protein tau. As far as we currently know, tangle formation is for a large part the final result of amyloid-induced nerve cell degeneration. Another hallmark of AD is the degeneration of synapses and the death of specific groups of neurons. In particular cholinergic and glutamatergic neurons are affected but even those producing norepinephrine or serotonin have been observed to degenerate (Dickson, 2003).

1.1.2 Stroke/ischemia

Cerebrovascular accident (CVA) is a clinical definition used to describe the symptoms of a perturbation in the cerebral blood supply. Decreased or interrupted blood supply has several consequences. The major one is the strong reduction in glucose and oxygen availability in the territory of the affected vascular brain areas, a phenomenon designated as cerebral ischemia. Ischemia leads, within seconds or minutes, to a cellular energy crisis, initiation of anaerobic glycolysis, disruption in the activity of cellular pumps, increase in intracellular calcium and extracellular potassium,
**Figure 1.1:** Amyloid precursor protein (APP) is an integral membrane protein, highly expressed throughout the body. It can be cleaved by alpha, beta- and gamma-secretases. The beta- and gamma-secretase cleavage results in the splicing product amyloid beta-peptide (Aβ1-40 or Aβ1-42), which is able to aggregate due to abnormal peptide folding. Depending on the degree of aggregation, Aβ peptides are considered to be neurotoxic as in large quantities they induce amyloid plaque formation. Extracellular amyloid plaques produce loss of connections between neurons and decreased neuronal activity.

neurotransmitter release and in the end to neuronal death. The temporal profile of an ischemic injury can be described in a series of phases (ter Horst and Korf, 1997). The first phase includes the initiation of the expression of immediate early genes that transform the cells from a resting into an activated state. The second phase includes the activation of acute phase response proteins (such as heat shock proteins) with temporary repair functions. The third phase comprises the secretion of immediate early gene proteins from the first phase. The fourth phase is the most damaging since the proteinases activated in previous phases challenge the integrity of the cell. The fifth and last phase consists of activation of repair/remodeling molecular events to rescue the cell. Several mechanisms have been proposed to explain the pathophysiology of an ischemic injury, including increased excitotoxicity, calcium overload, free radical formation, immune response, and inhibition of protein synthesis. All these unbalanced events are ultimately causing neurodegeneration. Some of these aspects will be discussed in detail in the following sections.
1.2 Glutamate in neurodegeneration

The neurodegenerative molecular pathways are poorly understood largely due to the difficulty in distinguishing primary from secondary events. One important player in neurodegeneration is glutamate, the major excitatory neurotransmitter in particular in the forebrain regions. In many neurodegenerative disorders like cerebral ischemia and AD glutamate is locally released in high quantities and promotes an effect called excitotoxicity, which ultimately leads to programmed neuronal death (Smith-Swintosky and Mattson, 1994).

1.2.1 Glutamate-induced excitotoxicity

The neurotoxic action of the excitatory amino acid glutamate arises from its capacity to trigger a pathophysiological chain of reactions when it acts continuously on its receptors. L-Glutamate (L-Glu) is the major excitatory neurotransmitter in the brain being present at approximately two thirds of central synapses (Fonnum, 1984). Glutamate receptors are divided on the basis of their mode of action and pharmacological properties into two major subdivisions: ionotropic channel receptors (iGluR) and metabotropic G-protein coupled receptors (mGluR). iGluRs are characterized by their selective affinity for specific agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainic acid (KA). Upon binding of these ligands to the receptor, the channel opening occurs which allows the influx of mainly sodium and/or calcium ions into the nerve cell. A differential distribution and a specific pharmacological profile exists for each iGluR subtype (Monaghan et al., 1989).

The excitotoxicity theory asserts that the physiological excitatory transmission can be changed from a physiological into a pathological state leading to neuronal destruction (Whetsell and Shapira, 1993). The drastic increase in L-Glu in the synaptic cleft during brain injury could initiate two detrimental processes. These processes differ in time-dependency and ionic characteristics. The first process involves acute swelling of cell bodies and dendrites via the opening of membrane cation channels, causing depolarization. The Na\(^+\) influx and passive influx of Cl\(^-\) ions and H\(_2\)O precedes the cell volume expansion. Swelling occurs within minutes of L-Glu exposure and is critically dependent on the extracellular concentrations of Na\(^+\) and Cl\(^-\) ions. The second process is marked by delayed neuronal degeneration. In vitro observations suggest that the neuronal death is closely related with the increase in Ca\(^{2+}\) influx, mainly via NMDA receptors. NMDA receptors exhibit the highest permeability to Ca\(^{2+}\) compared to AMPA or KA receptors and posses a superior capacity for inducing intracellular Ca\(^{2+}\) influx and thus initiating neurodegenerative processes (Choi, 1992).
1.2.2 NMDA-receptors

NMDA receptors constitute a major class of Glu receptors in the mammalian central nervous system (MacDonald et al., 1989). They are localized at the postsynaptic membrane of excitatory synapses on almost all neurons, but are specifically enriched on pyramidal neurons in the neocortex and hippocampus. Pyramidal neurons are particularly susceptible to neurodegeneration and are e.g. massively lost in AD (Hynd et al., 2004). Normally speaking NMDA receptors are involved in a wide range of cellular processes, including neuronal differentiation, synaptic plasticity, and long-term potentiation (LTP) (Carroll and Zukin, 2002). In addition they mediate the neurotoxic effects of excitatory amino acids in the adult brain under pathological conditions of overstimulation (Choi, 1994).

NMDA receptors are activated by glutamate and glycine which makes NMDA receptors unique among other neurotransmitter receptors. NMDA receptor activity is modulated by several modulators such as divalent cations (Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$), redox substances, pH and polyamines.

NMDA receptors are ligand-gated channels and are composed of heteromultimeric subunits: NR1 and NR2 (NR2A-D) (McBain and Mayer, 1994). Although the structure and stoichiometry of the NMDA channel is unknown, in vivo receptors contain an obligatory NR1 and one of the NR2 subunits (Dingledine et al., 1999). The type of NR2 subunit determines agonist affinity, Mg$^{2+}$, Zn$^{2+}$ sensitivity, deactivation kinetics and channel conductance. In AD, NMDA receptors are significantly altered: NR1 and NR2B protein levels are significantly reduced, while the NR2A expression is increased in the regions involved in learning and memory processes (Mishizen-Eberz et al., 2004). Therefore, subunit composition defines the response of the receptor to glutamate activation, which subsequently affects neuronal function.

1.3 Neuroprotective signaling

1.3.1 TNF-α signaling

Cytokines are defined as small soluble proteins secreted by a cell, which can alter the behavior or properties of the cell itself or of another cell. Cytokines are involved in a variety of inflammatory and infectious conditions. They are not expressed constitutively but rather transiently after an inducing stimulus. The most potent signals for cytokine expression are other cytokines. In the end this led to the concept of a cytokine matrix in which they can stimulate or inhibit each other (Zhu and Emerson, 2002). This concept accounts for the complexity of the cytokine network found with any neurodegenerative disorder. Cytokine receptors are constitutively expressed and their activity is modulated by ligand interaction. Cytokine receptors are cleaved by metalloproteinase enzymes to produce soluble cytokine receptors (Williams et al., 1996), which are able to capture soluble cytokines, and hence act as inhibitors by competing with membrane-bound receptors.
1.3 Neuroprotective signaling

Tumor necrosis factor-alpha (TNF-α), one of the best-characterized cytokines was discovered in the 1970s by Old and colleagues (Carswell et al., 1975; Old, 1985). TNF-α is produced mainly by the monocyte/macrophage lineage, but T lymphocytes, neutrophils, mast cells, endothelial cells and neurons can express it also under particular circumstances. TNF-α is highly expressed under physical (UV, X-radiation, heat), chemical or immunological challenges. In vivo, TNF-α is considered to be the most rapidly secreted pro-inflammatory cytokine (Sorimachi et al., 1999) from preformed stores. It is produced by cleavage of the membrane TNF-α by TNF-α converting enzyme (TACE/ADAM17) (Cerretti et al., 1999). Membrane TNF-α is a 26-kDa cell surface transmembrane type II polypeptide. The result of the TACE cleavage consists of a 17-kDa soluble TNF-α form. TACE cleavage results in a decreased cell surface membrane-bound receptor density. Since clustering of TNF receptors is necessary for signaling, their overall activity is inhibited in this way. Most of the reported TNF-α-mediated biological effects are attributed to the soluble TNF-α form, whereas cell surface transmembrane TNF-α-mediated physiological effects are less known. The biological effects exerted by transmembrane TNF-α are mediated by direct cell-to-cell interaction (Probert et al., 1997).

In vivo TNF-α coordinates the cytokine response to injury. If its production is blocked, the expression of other cytokines, such as IL-1 and IL-6 or chemokines is down-regulated as well (Probert et al., 1996). Under pathophysiological conditions TNF-α acts as a switch-on molecule for the immune system. Following prolonged exposure to an excess of TNF-α its inflammatory properties are tailored towards immunosuppressive properties (Correale and Villa, 2004).

a) TNF-R1 and TNF-R2 signaling

TNF-α binds two distinct cell surface receptors: TNF-R1 and TNF-R2. TNF-R molecular pathways, either cooperatively or individually lead to cytotoxicity as well as differentiation and growth regulatory activities.

TNF-R1 activation can trigger fibroblast growth, and endothelial cell adhesion, while TNF-R2 signaling promotes proliferation of thymocytes and peripheral T cells and inhibition of early haematopoiesis (MacEwan, 2002). Because of its low affinity to soluble TNF, TNF-R2 was for a long time thought to have an “accessory function” by enhancing TNF-R1 signaling through a “ligand passing” process by which TNF-α binds to TNF-R2, dissociates and subsequently binds to TNF-R1.

Both TNF-Rs potentiate NF-κB complex activation. It was reported that neuroprotection is dependent on TNF-R1 expression in kainic acid-induced seizures model (Gary et al., 1998) or on TNF-R2 expression in a retinal-induced ischemic model (Fontaine et al., 2002). TNF-R2-induced neuroprotection was associated with the PKB/Akt pathway, since inhibition of PKB/Akt signaling abolished the neuroprotective effect (Fontaine et al., 2002). These in vivo studies were paralleled by studies in cultured cortical neurons where TNF-α induced neuroprotection by activation of TNF-R2 pathway. Moreover, it was suggested that activation of NF-κB by TNF-R1
and TNF-R2 displays differential temporal kinetics: while TNF-R1 signaling led to a transient NF-κB activity, TNF-R2 signaling resulted in a persistent NF-κB activation, which turned out to be crucial for neuroprotection (Marchetti et al., 2004). In addition, the TNF-R2 gene contains the consensus elements for transcription factors, such as nuclear factor-kappa B (NF-κB) in the 5′-flanking region suggesting receptor-self promotion (Rasmussen et al., 2001; Santee and Owen-Schaub, 1996).

b) TNF-α in pathology and therapy

Several reports showed the involvement of TNF-α in neurodegenerative illnesses (Probert et al., 1996; Ghezzi and Mennini, 2001; Sriram and O’Callaghan, 2007; Perry et al., 2001). It has been suggested that in CNS disorders, in which apoptosis is an underlying process for neuronal death such as AD, Parkinson’s disease, retinitis pigmentosa, cerebellar degeneration and ischemic injury, TNF-α is a major player.

TNF-α signaling in AD. A number of studies have shown that TNF-α is upregulated in AD (Perry et al., 2001). Furthermore, TNF promoter polymorphisms are associated with AD genes (Ma et al., 2004). It was reported that the chromosome 1p and chromosome 12p regions are involved in late-onset AD and these two regions harbor the TNF-R1 and TNF-R2 genes. However, only TNF-R2 exon 6 polymorphism was found to be linked to late-onset AD in families with no apolipoprotein E-epsilon 4 (ApoE-e4) genotype (Perry et al., 2001).

In addition, it has been demonstrated that overexpression of TNF-R1 promotes Aβ-induced neuronal death (Li et al., 2004). Cross-breeding transgenic APP23 mice, which are able to develop Aβ plaques, with TNF-R1−/− mice (APP23/TNF-R1−/−) resulted in a strong decrease of Aβ plaques compared with APP23 mice. APP23/TNF-R1−/− mice have lower expression of BACE1 and show increased learning abilities compared to APP23 mice. The authors suggested that TNF-R1 is connected to abnormal Aβ processing which could lead to Aβ plaque formation, neuronal damage and learning deficits (He et al., 2007). Thus, anti-TNF-R1-based therapies might be an efficient therapeutic target in treating AD (Rosenberg, 2005).

TNF signaling in ischemia. TNF-α expression in the cerebrospinal fluid (CSF) and in postmortem brain tissue correlates with the extent of ischemic injury in humans suffering from ischemic stroke (Zaremba et al., 2001). Similarly, TNF-α expression was increased both at the protein and mRNA level in an experimental focal ischemia model in rats (Botchkina et al., 1997). Additional evidence for the role of TNF-α signaling in models of focal cerebral ischemia was previously reported using mice double-deficient for both TNF receptors (Bruce et al., 1996). In these studies TNF-R1 ameliorated hippocampal damage (Gary et al., 1998). In contrast, the depletion of TNF-R2 signaling increased the cellular degeneration in retinal ischemia (Fontaine et al., 2002). These two differential effects of TNF-R1 suggest a distinct signaling with respect to cellular composition in the brain regions affected by the ischemic injury.
Furthermore, middle cerebral artery occlusion/focal cerebral ischemia induces a dramatic up-regulation of TNF receptors, with TNF-R1 appearing within 6 hr, followed by the appearance of TNF-R2 at 24 hr after the onset of ischemia (Botchkina et al., 1997). TNF-R1 can induce neuroprotection in some cell systems by increasing Fas-associated death domain-like interleukin-1-beta-converting enzyme-inhibitory protein (FLIP(L) (Taoufik et al., 2007) and NF-κB activation. TNF-R2 promotes neuronal survival by activation of PKB/Akt and NF-κB activation (Fontaine et al., 2002). The TNF-R2 neuroprotective effect persisted for 8 days following the retinal ischemic injury (Fontaine et al., 2002), which suggests that TNF-R2 may be a promising new approach in preventing irreversible neuronal loss by ischemic insults.

1.3.2 PKB/Akt signaling

Protein kinase B (PKB/Akt) belongs to the serine/threonine protein kinase family called AGC protein kinases (EC 2.7.11.1). PKB/Akt research started back in 1977, when Staal and co-workers identified a transforming leukemia virus in mice developing spontaneous lymphoma. This virus, termed Akt8, induced tumor formation confirming the oncogenic potential of the Akt gene (Staal et al., 1977). Together with this discovery, the PKB/Akt molecular homology was identified as significantly related to protein kinase A (PKA) and C (PKC) using a PCR screening approach (Coffer and Woodgett, 1991).

The first PKB/Akt transgenic mouse model was generated in 2000 (Jones et al., 2000). Overall, results from transgenic PKB/Akt mice demonstrate that PKB/Akt is an important modulator of cellular growth and cell survival and it controls the development and progression of various tumors.

PKB/Akt isoforms. Hitherto three isoforms of PKB/Akt have been identified and described in mice and humans (Brazil and Hemmings, 2001). Characterization and analyses of PKB/Akt isoform mutants provided new insight into the function of the three PKB/Akt proteins. PKBα/Akt1 is expressed in all organs and tissues and plays e.g. an essential role in the modulation of fetal growth. In the brain PKBα/Akt1 was shown to mediate neuroprotection in adult mice against ischemia-induced injury (Miao et al., 2005) by increasing endothelial nitric oxide synthase (eNOS) expression via phosphatidylinositol 3 (PI3)-kinase pathways (Hashiguchi et al., 2004). These data were confirmed in PKBα/Akt1 ko mice. These mice display diminished PKB/Akt phosphorylation and a reduction in eNOS (Yang et al., 2003), which could lead to enhanced neuronal degeneration. PKBβ/Akt2 is predominantly found in fat tissue, liver and skeletal muscle suggesting an involvement in glucose metabolism (Altomare et al., 1998). The role of PKBβ/Akt2 in the central nervous system is not known yet. PKBγ/Akt3 is found in the brain, testis, lung, mammary gland and fat (Yang et al., 2005). In contrast to PKBα/Akt1 or PKBβ/Akt2 mutant mice, PKBγ/Akt3 mutant mice display normal glucose metabolism and no growth retardation. However, their brain size is dramatically reduced by about 25% with a significant decrease in both
cell size and cell number (Tschopp et al., 2005). Characterization of the isoforms of PKB/Akt in double or triple knockout mice also provided more information on the specific function of the PKB/Akt isoforms. These studies concluded that the Akt1 gene is more important than Akt3 for embryo survival but that both are required for embryonic development (Yang et al., 2005; Tschopp et al., 2005).

**PKB/Akt activation.** PKB/Akt is activated by several cytokines, growth factors or neurotransmitters. PKB/Akt activation can be mediated via PI3-kinase (Datta et al., 1999). This pathway includes membrane phospholipids, especially phosphatidylinositol 3,4,5-triphosphate [PtIns(3,4,5)P3] that recruits PKB/Akt to the plasma membrane where it becomes phosphorylated. In the case of PKBα/Akt1, activation is reached when threonine 308 (Thr\(^{308}\)) and serine 473 (Ser\(^{473}\)) are phosphorylated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) and a still to be identified Ser\(^{473}\) kinase (Yang et al., 2003).

**PKB/Akt regulation.** So far several mechanisms of PKB/Akt regulation have been proposed. One hypothesis is that carboxyl-terminal modulator protein (CTMP) keeps PKB/Akt in an unphosphorylated and inactive state by physical protein-protein interactions (Maira et al., 2001). Another known negative regulator is protein phosphatase and tensin homologue deleted on chromosome 10 (PTEN) that inactivates PKB/Akt in a PI3-kinase dependent manner. PTEN knockout mice develop a broad range of tumors. Furthermore, they display atypical social interactions, exaggerated responses to stressful sensory stimuli in paradigms designed to assess anxiety and learning. Their brains are enlarged in the regions in which the PTEN gene was deleted and this effect is associated with hypertrophy of the cell bodies and with abnormal growth of neuronal processes (Kwon et al., 2006). Similar pathophysiological conditions were described for PKB/Akt overexpression in mice (Yang et al., 2003). In addition, heat shock proteins (Hsp) also bind and regulate PKB/Akt activity. Hsp are stress proteins that regulate protein stabilization and protect cells from several stress circumstances. It has been reported that overexpression of Hsp27 in neurons protects against excitotoxicity and may act as an inhibitor of neurodegeneration (Wagstaff et al., 1999). To date, Hsp27 and Hsp90 proteins have been reported to specifically bind and activate PKB/Akt leading to an inhibition of apoptosis (Rane et al., 2003).

PKB/Akt is interacting and regulated as well by scaffold proteins, such as scaffold proteins in the stress-mediated MAP-kinase (SAPK) signaling: JNK interacting protein 1 (JIP1) a scaffold protein for the c-jun amino-terminal kinase (JNK) pathway in neuronal cells and plenty of Src homology 3 (POSH), a scaffold protein for the mixed-lineage kinase (MLK)-JNK pathway (Kim et al., 2002; Figueroa et al., 2003). It has been suggested that PKB/Akt suppresses the JNK-dependent death mechanism not only upstream but also downstream of MLKs, thus leading to a decrease in the neuronal susceptibility to degeneration (Xu et al., 2001).
1.3 Neuroprotective signaling

**PKB/Akt substrates.** PKB/Akt isoforms are able to phosphorylate several substrates providing a variety of cellular responses. Glycogen synthase kinase-3 (GSK-3) was the first identified PKB/Akt substrate (Burgering and Coffer, 1995). Importantly, PKB/Akt phosphorylates the Bcl-2/Bcl-X antagonist (BAD). BAD binds the proteins Bcl-2 and Bcl-X at the mitochondrial membrane. Upon phosphorylation by PKB/Akt, BAD translocates from the mitochondrial membrane and Bcl-2 is released. Bcl-2 is then translocated to the nucleus where it promotes anti-apoptotic activities (Datta et al., 1999). Bcl-2 proteins exhibit neuroprotective functions against various excitotoxic challenges, such as glutamate or amyloid beta peptides. Another substrate which is activated and phosphorylated by PKB/Akt in a PI3-kinase-dependent manner (Fulton et al., 1999) is eNOS. eNOS activation has been shown to have neuroprotective function in various neurodegenerative conditions (Endres et al., 2004).

Other PKB/Akt phosphorylation targets essential for neuroprotection are several transcription factors, including cyclic AMP (cAMP)-response element binding protein (CREB) and NF-κB (Kane et al., 1999). Upon phosphorylation CREB possesses a higher affinity for its co-activator, resulting in transcriptional activation. The detailed mechanism of how CREB and PKB/Akt both lead to cellular survival still remains to be elucidated. Activation of NF-κB is dependent on the IκB kinase (IKK) complex. PKB/Akt is a direct regulator of IKK activity in a PI3-kinase-dependent manner. PKB/Akt-mediated NF-κB activation contributes to the suppression of apoptosis since NF-κB activity initiates the transcription of several anti-apoptotic proteins (Kane et al., 1999; Lawlor and Alessi, 2001).

1.3.3 NF-κB signaling

NF-κB is a transcription factor involved in the development and progression of several diseases such as autoimmune disease, cancer and neurodegenerative disorders. Depending on the cell type and its regulators NF-κB complex could induce the transcription of pro- and anti-apoptotic genes. It was first identified in B lymphocytes as an activator of immunoglobulin κ light chain transcription (Sen and Baltimore, 1987). The NF-κB complex consists of an inactive form of DNA-binding dimers and inhibitory subunits. In neurons, the most common subunits expressed are p50, p65 (RelA) and the inhibitory subunit IκB, which is composed of IκBα and IκBβ (Mattson and Meffert, 2006).

**NF-κB activation.** The NF-κB complex is activated in neurons by several molecules such as TNF-α, Fas ligands, glutamate, nerve growth factor (NGF), cell adhesion molecules and a secreted form of amyloid precursor protein (APP) or even by synaptic transmission between neurons (Mattson et al., 2000). These stimuli activate various cellular signaling pathways such as the protein kinase C (PKC), mitogen-activated protein (MAP) kinase kinase kinase-1 (MEKK1) and also the PKB/Akt signaling pathway, which all have the potential to phosphorylate IKK. This kinase consists of
two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ). IKK phosphorylates the inhibitory subunit IκB and induces the dissociation of IκB from the NF-κB complex. Upon dissociation of IκappaB from the NF-κB complex, the p50-p65 dimer of the NF-κB complex translocates from the cytosol to the nucleus where it binds to NF-κB responsive genes (Mattson and Meffert, 2006).

NF-κB substrates. The NF-κB complex induces the expression of genes involved in the regulation of several cellular processes, including cellular survival (Bcl-2, inhibitor of apoptosis proteins (IAP), TNF-α or TNF-R2 genes); immune response (TNF-α, interleukins (IL)-2, IL-6 genes); ion homeostasis (subunits of NMDA receptors and small conductance Ca²⁺ activated potassium (SK) channels). Furthermore, NF-κB can promote the transcription of the inhibitory subunit IκB and in this way regulates its own activity in a negative feedback loop (Mattson and Meffert, 2006).

The NF-κB complex is activated in neurons and glia cells in response to acute or chronic neurodegenerative processes. In several neurodegenerative models for traumatic brain injury, stroke, epilepsy or AD an increase in NF-κB activity was observed. These data were paralleled by in vitro studies which showed an activation of NF-κB in response to glutamate-induced excitotoxicity, metabolic insults or glucose deprivation. In neuronal cells sustained activation of NF-κB was shown to induce neuroprotection (Marchetti et al., 2004; Mattson et al., 2000). This beneficial outcome was in part the result of PI3-kinase-PKB/Akt pathway activation, increase in mitochondrial antioxidant Cu/Zn-SOD, Mn-SOD and the induction of anti-apoptotic proteins Bcl-2 and Bcl-x. Furthermore, NF-κB cellular survival mechanisms underlie cytokine-induced neuroprotective pathways, including transforming growth factor-beta1 (TGF-beta1) and TNF-α (Zhu et al., 2004).

NF-κB in relation to neurodegenerative conditions. Several reports showed that in AD NF-κB activity is increased in cells associated with neurodegenerative processes such as neurons and astrocytes located in close proximity to amyloid-β plaques (Lezoualc’h and Behl, 1998; Collister and Albensi, 2005). Furthermore, NF-κB activity is increased in cholinergic neurons in the basal forebrain and in the superior temporal lobe gyrus of AD patients (Lukiw and Bazan, 1998; Boissière et al., 1997). These brain regions are known to be susceptible to neurodegeneration. In cultured neurons amyloid-β and a secreted form of APP induce an up-regulation of NF-κB activity. This NF-κB activation was reported to be protective against amyloid-β toxicity in cultured neurons (Barger et al., 1995). Interestingly, in early-stage AD pathology NF-κB activity is strongly increased while in later stages NF-κB activity decreases tremendously.
1.4 Neuroprotective agents

1.4.1 Statins

Statins, 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors, are widely used as medication for lowering cholesterol levels. Today nine pharmaceutical compounds are available: lovastatin, mevastatin, simvastatin, pravastatin, fluvastatin, atorvastatin, rosuvastatin, pitavastatin and cerivastatin. They are classified in relation to their inhibitory properties for either the purified HMG-CoA reductase enzyme or for cellular cholesterol biosynthesis (McTaggart et al., 2001).

Statin signaling. Besides their acknowledged role on the regulation of cardiovascular functions, statins are able to exert neuroprotective effects, mainly attributed to anti-inflammatory effects (Leung et al., 2003), stimulation of eNOS (Harris et al., 2004; Hernández-Perera et al., 1998) and inhibition of inducible nitric oxide synthase (iNOS) (Vaughan and Delanty, 1999). In general, statins inhibit mevalonate synthesis and prevent the production of several isoprenoids, including farnesylpyrophosphate and geranylgeranylpyrophosphate that modulate small G proteins (GTPases). Thus, statins suppress the activation of GTPases, such as Rho, Ras and Rac by preventing their isoprenylation and thus their translocation from the inactive GDP-bound forms located in the cytoplasm to the active GTP-bound forms in the plasma membrane (Maltese, 1990; Seabra, 1998). Inhibition of Rho proteins by statins prevents down-regulation of eNOS expression and activity under hypoxia conditions, leading to the stabilization of eNOS mRNA (Laufs et al., 2000). Furthermore, Rho isoprenylation inhibits neuronal outgrowth in hippocampal neuronal cultures (Pooler et al., 2006).

Although our understanding of statin-mediated cellular pathways increased tremendously in the last decade, statin-induced neuroprotective mechanisms are still elusive. In vitro studies using primary neuronal cultures described merely three possible statin-mediated neuroprotective mechanisms. Although acute treatment with statins did not rescue neurons from cellular death and did not prevent the rise in $[\text{Ca}^{2+}]_{\text{c}}$ caused by NMDA treatment in their system, Zacco and colleagues managed to show the neuroprotective potential of various chronic statin treatments against NMDA excitotoxicity. In this case the neuroprotective effect of statins was not attributed to direct regulation of NMDA receptor-mediated events but rather to depletion of the cellular pool of cholesterol (Zacco et al., 2003). In an oxygen and glucose deprivation (OGD)/reoxygenation-evoked neuronal death model simvastatin treatment was reported to induce neuroprotection (Lim et al., 2006). Simvastatin mediated this beneficial effect by reducing the production and toxicity of 4-hydroxy-2E-nonenal (HNE), a major cytotoxic end product of lipid peroxidation. The statin-induced neuroprotective mechanism was not ascribed to NMDA or AMPA receptor-mediated events but rather to modulation of NF-κB activity, since HNE directly inhibits NF-κB basal activity (Lim et al., 2006). It was concluded that simvastatin treatment suppresses HNE cytotoxicity by restoring the NF-κB activity in the OGD model leading to increased
neuronal survival. Johnson-Anuna and colleagues reported simvastatin-mediated neuroprotective effects against Aβ toxicity via increased Bcl-2 mRNA and protein levels in primary cortical neurons (Johnson-Anuna et al., 2007).

Several in vivo studies have shown statin-mediated neuroprotective effects against various neurodegeneration-like events. This beneficial neuroprotective effect was mainly attributed to increased eNOS expression (Kurosaki et al., 2004), Hsp cooperation, Bcl2 up-regulation, NF-κB and PKB/Akt pathways (Lu et al., 2007). In a model for brain injury, statins (simvastatin and atorvastatin) were shown to increase neurogenesis in neurogenic brain areas such as the hippocampal dentate gyrus and to reduce neuronal loss in the non-neurogenic brain areas such as the cornu ammonis 3 (CA3) (Lu et al., 2007). In a different study simvastatin enhanced neuronal survival in axotomized retinal ganglion cells after optic nerve injury via the overexpression of Hsp27 and activation of PKB/Akt pathways (Kretz et al., 2006). Furthermore, statin treatment increases the expression level of 26 genes (as observed in a microarray study) in particular genes related to apoptosis (c-myc, Bcl2) (Johnson-Anuna et al., 2005) and PKB/Akt phosphorylation (Li et al., 2006). These microarray studies were paralleled by in vivo chronic simvastatin administration to investigate whether statins would provide neuroprotection in brain cells after a challenge with the Bcl-2 inhibitor HA 14-1 or the NO donor sodium nitroprusside (SNP). Bcl-2 levels were significantly increased in brains of simvastatin-treated and these brains were less vulnerable to mitochondrial dysfunction or caspase activation (Franke et al., 2007).

1.4.2 SK channels

SK channels are voltage insensitive channels and belong to the KCNN family (potassium intermediate/small conductance calcium-activated channel, subfamily N). In the mammalian brain, SK channels are the product of three paralogous genes, namely KCNN1/KCa2.1/SKCa1 (SK1), KCNN2/KCa2.2/SKCa2 (SK2) and KCNN3/KCa2.3/SKCa3/hKCa3 (SK3). Recently, a new KCNN4/KCa3.1/SK4/IK1 channel was discovered being highly expressed in rat microglia (reviewed by Stocker (2004)).

SK1 and SK2 channel subunits show extensive colocalization. They are mainly found in the entorhinal cortex, the subiculum, in pyramidal cortical neurons, the CA1 - CA3 region from the hippocampus and in the thalamus. SK3 channel subunits have a complementary distribution, and are mainly located in the brain stem and in mononinergic neurons (Sailer et al., 2002).

Functional SK channels are composed of heteromeric subunits with constitutively bound calmodulin (CaM). The channels are activated in response to low (< 1μM) intracellular Ca$^{2+}$ that binds to CaM. It has been shown that SK channels contribute to basal synaptic integration at least in four different ways. In cortical and hippocampal pyramidal central neurons, SK channel activity dampens membrane excitability, contributes to dendritic response and regulates the synaptic integration. In certain neuronal cell types, SK channels are localized to dendritic spines where they interact with NMDA receptors. There SK channel activity suppresses the amplitude of
evoked synaptic potentials by inhibiting NMDA receptor-dependent activation (Ngo-Anh et al., 2005; Faber et al., 2005). In tonically firing cells, including midbrain dopamine neurons SK channels affect pacemaking by decreasing interspike intervals during a burst of action potentials as well as the length of the burst. In high firing-frequency cells, including cerebellar Purkinje neurons, SK channel activity is required for enduring basal rapid firing and SK channels act as modulators for firing pattern. In auditory hair cells SK channels are able to convert excitatory neurotransmitter signal into inhibitory signals (reviewed by Bond et al. (2005)). Overall, it can be concluded that SK channels are important regulators of synaptic integration and neuronal excitability in such a way that an increase in SK channel activity normally leads to decreased excitability of the cells (reviewed by Stocker (2004)).

Hitherto, there is only limited information on the role of SK channels in aging and neurodegenerative disorders. Changes in neuronal excitability are currently thought to underlie learning and memory processes. Aging is often characterized by a decrease in neuronal excitability and cognitive deficits. Since SK channel activity leads to a reduction of neuronal excitability it was hypothesized that increased SK channel expression and activity can in part account for the cognitive impairments found in aged individuals. In agreement with this hypothesis SK3 channel expression was found to be up-regulated in the hippocampus of aged mice. This elevation in SK3 channel expression was shown to result in a strong decrease in neuronal excitability and was paralleled by impaired memory and learning abilities (Blank et al., 2003). Since SK channels can regulate neuronal excitability a role in neurodegeneration and neuroprotection may be expected. Indeed overexpression of SK2 channels in in vitro cultured hippocampal neurons induced neuroprotection against kainite and glutamate excitotoxicity. Furthermore, in vivo overexpression of SK2 channels in dentate gyrus diminished a kainate-induced CA3 lesion (Lee et al., 2003). Thus, SK channels may prove to be important drug targets in the development of new effective therapeutic strategies against degenerative events.

1.5 Outline of this thesis

Neurodegenerative processes lead to neuronal loss which may ultimately result in functional impairments or even death. Thus, the understanding of the molecular basis of neuroprotective mechanisms for developing effective therapies to prevent and treat neurodegenerative diseases is of paramount importance. Hitherto, a number of studies showed that TNF-α is able to promote protection against glutamate-induced excitotoxicity in cortical neurons. Until now, the molecular mechanisms underlying these beneficial effects of TNF-α are largely unknown. An initial study showed that TNF-α increases neuronal survival by mobilizing the TNF-R2 pathway. TNF-R2-associated neuroprotective effects are mediated by the activation of PKB/Akt and subsequent sustained NF-κB activation.

In the first part of this thesis (Chapters 2, 3 and 4) the TNF-α-mediated pro-
Tective mechanisms in cortical neurons are investigated in more detail. In Chapter 2 we studied the kinetics of the neuroprotective effect of TNF-α treatment. Since PTEN is a counter-regulator of PKB/Akt signaling we investigated the time course of PKB/Akt and PTEN expression during TNF-α-treatment in wild-type, TNF-R1-/- and TNF-R2-/- neurons. In particular, we checked whether PTEN activation precedes PKB/Akt activation during long-term TNF-α treatment. Finally, we identified which PKB/Akt isoform is important in TNF-α-induced neuroprotective pathways.

To gain further insight into the regulation of PKB/Akt we studied the phosphorylation of PKB/Akt by two cAMP effector systems, PKA and Epac in cortical neurons in Chapter 3. PKA activation was shown to dephosphorylate PKB/Akt whereas Epac activation lead to increased PKB/Akt phosphorylation. Since both PKA and Epac were found to be complexed to the A-kinase anchoring protein AKAP150, we hypothesized that AKAP150 acts as a coordinator of PKA/Epac signaling to modulate PKB/Akt phosphorylation.

To date, only limited data is available on potential neuroprotective downstream targets of TNF-α induced NF-κB activation. SK channels are important regulators of neuronal excitability and were recently identified as a downstream target for NF-κB-mediated promoter regulation. Therefore, we investigated whether increased SK channel expression is part of the mechanism of TNF-α-induced cellular survival (Chapter 4).

Chapter 5 describes the neuroprotective effect of lovastatin pretreatment on glutamate-induced neuronal death in neurons. Interestingly, in primary human vascular endothelial cells (HUVEC) lovastatin treatment was shown to increase the expression of TNF-R2 proteins, without affecting TNF-R1 expression levels. We investigated whether lovastatin can also induce an increase in TNF-R2 expression levels in cortical neurons and whether activation of TNF-R2 signaling might be the mechanism that underlies the neuroprotective effect exerted by lovastatin. Moreover, we investigated the effect of lovastatin on PKB/Akt and NF-κB phosphorylation.

In Chapter 6 we wanted to confirm the observed in vitro neuroprotective effect of lovastatin in an in vivo mouse model. The damage of cholinergic neurons and their cortical target areas from the magnocellular nucleus basalis (MNB) that occurs during aging or AD could be mimicked using an NMDA-induced lesions into MNB model. In this in vivo model we found that lovastatin has a neuroprotective effect on NMDA-induced excitotoxic lesions. In addition, we investigated whether this neuroprotective effect of lovastatin administration was dependent on PKB/Akt activation. Since the degeneration of cortical cholinergic fibers is associated with a cognitive decline in AD we studied the effects of lovastatin treatment on learning and memory abilities using different behavior paradigms.

It is known that glutamatergic and cholinergic systems interact functionally at the level of the cholinergic basal forebrain. The N-methyl-D-aspartate receptor (NMDA-R) is a multiprotein complex composed of NR1, NR2 and/or NR3 subunits. The subunit composition of NMDA-R of cholinergic cells in the nucleus basalis has not yet
been investigated. By means of choline acetyl-transferase and NR2B or NR2C double staining, we investigated whether mice express both the NR2C and NR2B subunits in nucleus basalis cholinergic cells (Chapter 7). Since we found increased acetyl choline (ACh) in the frontal cortex and amygdala, we assessed behavioral habituation to novel environments and objects as well as object recognition in NR2C-2B subunit exchange mice.

Overall this thesis provides more insight into TNF-α mediated neuroprotective signaling. We investigated both upstream and downstream targets of TNF-α pathways. In addition, we provide more information about PKB/Akt regulation, lovastatin-induced neuroprotective mechanisms.

References


References


