On the Role of Reactive Oxygen Species in Signal Transduction, Neuronal Degeneration and Protection

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1 Introduction

1.1 Epidemiology of neurodegenerative diseases and stroke

Despite enormous developmental progress in medicine within the past decades, diseases of the central nervous system still represent a severe problem concerning therapeutic availability. Increasing incidence of neurodegenerative diseases such as Alzheimer´s disease (AD) and Parkinson´s disease (PD) or of stroke derives from a self-created burden of industrialized countries: the prolonged life-span.

Concerning AD, which is the most common neurodegenerative disease, the incidence proportionally correlates with age. Currently, there are more than 12 million AD patients world-wide and already in Germany the prevalence of AD increased to 800000 patients. Furthermore, this number is expected to double within the next 25 years. Besides therapeutical aspects, such development also causes profound economical problems. In Germany, the annual expenses for the therapy and care of AD or stroke patients exceeds DM 50 billion. Such problems reveal the urgent necessity to establish effective therapeutic treatments which reduce neuronal damage reliably or improve the neurologic outcome after stroke. Using symptomatic pharmacological interventions that solely substitute the loss of neurotransmitters such as dopamine (PD) or acetylcholine (AD), only temporary success could be achieved because the neurodegenerative processes still continue. Thus, it is important to apply therapies that interfere directly with neuronal damage.

Many substances with neuroprotective properties such as calcium antagonists, NMDA-antagonists, antioxidants or growth factors have been investigated in clinical trials but the results were mostly disappointing. Already the application of potential candidate drugs caused severe problems because many of them, especially proteins such as growth factors or cytokines, were blood-brain barrier impermeable. Invasive treatment like intraventricular and intrahippocampal administration of growth factors (Fisher et al. 1995; Shigeno et al. 1991), implantation of genetically engineered cells constantly secreting neuroprotective proteins (Pechan et al. 1995) or structural alterations for vehicle transport (Charles et al. 1996; Friden et al. 1993) have been investigated to improve the clinical use of these substances. But as such complex, invasive applications represent an obstacle and as the therapeutic success of such interventions has been rather low, there is still no commonly accepted effective treatment of neurodegenerative diseases or stroke. Therefore, an important step is to understand the exact

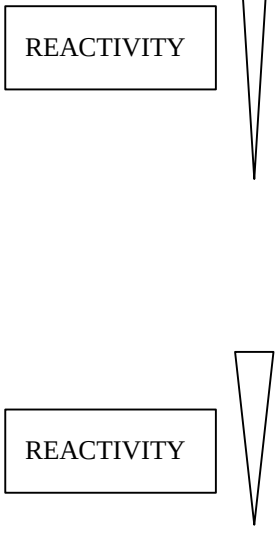
pathological mechanisms of neurodegeneration which would be a basis to block or to promote certain pathways of signal transduction that are initiated in neurons under pathological conditions. Especially the support of endogenous neuroprotective pathways is a hopeful strategy for the treatment of neurodegenerative diseases and stroke.

Among such endogenous signaling mediators, reactive oxygen species (ROS) are very interesting target molecules which are subject of investigation in the current thesis.

1.2 Role of reactive oxygen species in neurodegenerative diseases and stroke

1.2.1 General principles about radical function in the brain

One of the most important pathological events promoting deleterious consequences after stroke or in neurodegenerative diseases is the formation of reactive oxygen species (ROS).



Free radicals as ROS	
Hydroxyl radical	$OH^{-\cdot}$
Nitric oxide radical	$NO^{\cdot}$
Singulet oxygen	$½\ O_2^{\cdot}$
Alkoxyl radical	$RO^{\cdot}$
Peroxyl radical	$ROO^{\cdot}$
Superoxide anion radical	$O_2^{-\cdot}$
other ROS	
Peroxynitrite	$ONOO^-$
Hydrogen-peroxide	H_2O_2
Molecular oxygen	O_2

Fig 1. Classification, chemical structure and reactivity of reactive oxygen species (ROS)

Within the brain, different types of reactive oxygen species are known. ROS can be sub-classified into free radicals such as superoxide anion radicals ($O_2^{-\cdot}$) or hydroxyl radicals (OH^{-}) and into oxygen-containing molecules with oxidative properties such as hydrogen peroxide (H_2O_2) or peroxynitrite ($ONOO^-$) (Fig. 1). Free radicals contain an unpaired electron in the outer orbital which makes them unstable having only a half life of milliseconds. This instability causes a high reactivity with cellular target structures such as proteins, DNA and membranes-lipids, and explains the toxicity of ROS (Fig 2). Such radical effects are normally controlled by intracellular antioxidative mechanisms which prevent an excess release of ROS including the antioxidative enzymes superoxide dismutase (SOD), catalase or glutathione peroxidase (Fig. 2). In addition to such controlled enzymatic antioxidative defense mechanisms, there are also some endogenous molecules that can chemically detoxify ROS such as vitamin E, uric acid, glutathione, ascorbic acid and thioredoxin.

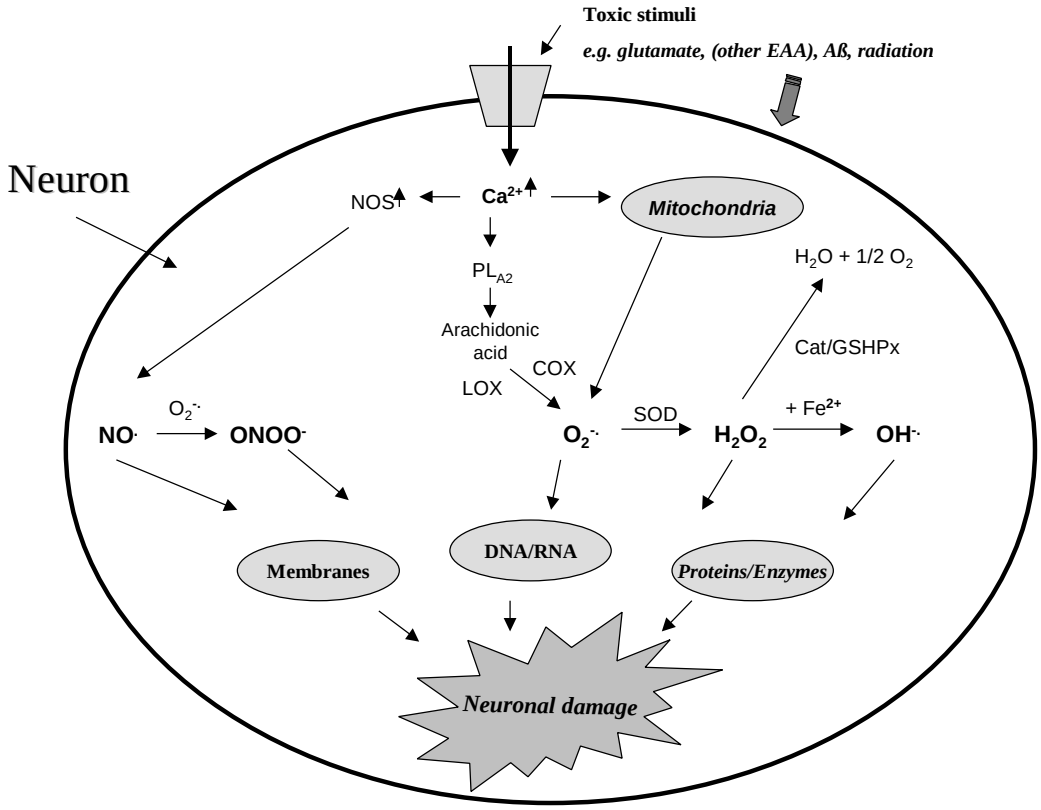


Fig 2. Mechanisms of ROS-induced damage and endogenous antioxidant activity

NOS = nitric oxide synthase, PL_{A2}=phospholipase A2; Cat = catalase; SOD = superoxide dismutase; GSHPx = glutathione peroxidase, EAA = excitatory amino acids, Aß = amyloid ß protein

In neurodegenerative diseases either the amount of ROS-generation exceeds the capacity of endogenous defense mechanisms or the antioxidative enzymes exhibit structural alterations as found in patients with amyotrophic lateral sclerosis (ALS). Numerous studies have shown that the excess release or generation of ROS entails deleterious events, and no doubt such over-stimulation leads to neuronal cell death. Recent data revealed that ROS also are involved in the process of aging. Increased levels of oxidative stress have been shown to occur during aging of many different organ systems including the brain (Stadtman 1992). Although the molecular basis of the increased oxidative stress is uncertain, experimental data point to accumulation of ROS generated in mitochondria. Strong evidence for this scenario comes from studies showing that rodents placed on calorie restricted diets have extended life-spans and reduced incidence of age-related diseases (Sohal and Weindruch 1996). Levels of age-related oxidative stress in calorie restricted rodents were significantly reduced in all tissues so far examined, including brain (Sohal et al. 1994). Similar data from non-human primates strongly suggest that ROS contributes to the process of aging (Lane et al. 1996). Therefore, the role of reactive ROS in the age-related neurodegenerative diseases AD, PD and ALS is described in more detail in the following part of the thesis.

On the other hand, increasing evidence also points to an important signaling function of ROS under physiological conditions or upon moderate ROS-stimulation. Recently, such possible functional role of ROS has been discussed to be of pivotal relevance for cellular function, integrity and survival (Mattson 1997). Thus, ROS might act with “janus-face-character” depending on the radical stimulus, the amount of ROS, and the duration of exposure. The current studies investigate the functional role of ROS and try to enlighten this discrepancy of radical action.

1.2.2 Reactive oxygen species and Alzheimer`s disease

Several authors described a pivotal role for ROS in the pathology of AD which is the most important neurodegenerative disease in the westernized countries. Studies of post-mortem brain tissue from AD patients and age-matched neurologically normal controls have shown that a variety of markers of oxidative stress are increased in AD brain, with clear correlation to Aß deposition and neurofibrillary degeneration. Levels of lipid peroxidation, determined by the thiobarbituric acid reactive substances assay were significantly increased in several regions of AD brain (Subbarao et al. 1990; Lovell et al. 1995). Further, protein carbonyl

levels, indicating protein oxidation, were markedly increased in vulnerable brain regions and in tangle-bearing neurons (Smith et al. 1991a; Smith et al. 1996). Some studies also provided evidence for oxidative damage to DNA in the AD brain. Activity or protein levels of several antioxidant enzymes such as Cu/Zn-SOD, Mn-SOD and catalase were reported to be altered in vulnerable regions of AD brain, as well as in individual tangle-bearing neurons consistent with ongoing oxidative stress (Pappolla et al. 1992; Smith et al. 1994a). Thus, disturbed endogenous antioxidative function seems to represent a key event in neurodegenerative diseases.

Several laboratories recently showed that amyloid plaques and neurofibrillary tangles contain high levels of glycated proteins (Vitek et al. 1994; Smith et al. 1994b; Yan et al. 1996). Glycation (cross-linking of sugars to proteins) is a modification which indicates ongoing oxidative stress. Both Aß and tau-protein (tau) which is the major component of straight and paired-helical filaments in neurofibrillary tangles have been shown to undergo glycation in AD. Interestingly, oxidation can induce the formation of Aß fibrils and tau filaments that appear indistinguishable from the protein fibrils present in senile plaques and neurofibrillary tangles, respectively (Dyrks et al. 1992; Troncoso et al. 1993). Because levels of oxidative stress increase with normal aging, it is reasonable to consider that such an oxidizing environment could initiate a cascade of events in which oxidation promotes Aß and tau fibril formation, which then promotes further oxidative stress in brain cells. Indeed, previous studies showed that insults inducing oxidative stress such as glutamate, glucose deprivation, or Aß entailed changes in tau similar to those seen in the neurofibrillary tangles of AD (Cheng and Mattson 1992; Stein-Behrens et al. 1994).

The chemical process appears to involve the generation of ROS which could conceivably propagate to cell membranes and induce lipid peroxidation (Butterfield et al. 1994). Alternatively, a cell surface receptor for Aß could mediate induction of oxidative stress. Recently, Yan et al. (Yan et al. 1996) provided evidence that a cell surface receptor for advanced glycation end products (RAGE), binds Aß fibrils and induces oxidative stress in microglia. Some studies have reported that Aß can form ion-conducting pores in membranes (Arispe et al. 1993; Shigeno et al. 1991), and the resulting elevation of intracellular calcium levels could induce accumulation of various reactive oxygen species including H_2O_2 and $O_2^{-\cdot}$. (Lafon-Cazal et al. 1993a).

The induction of oxidative stress by Aß was found in different experimental systems including cultured neurons, synaptosomes and endothelial cells (Thomas et al. 1996; Behl et al. 1994). Electron paramagnetic resonance spectroscopy studies of rodent cortical

synaptosomes, using nitroxyl stearate spin labels that intercalate into cell membranes, showed that Aß induced lipid peroxidation (Butterfield et al. 1994). Several studies have shown that various antioxidants such as vitamin E, propyl gallate, tert-phenyl butyl nitrone, nordihydroguaiaretic acid or the synthetic EUK-8 could protect cultured neurons against Aß toxicity (Behl et al. 1992; Goodman et al. 1994). On the other hand, some investigators were not able to demonstrate that antioxidants protect neurons against Aß toxicity (Lockhart et al. 1994). These different results could mean that the success of radical scavenging is dependent on the cell type or experimental conditions. Especially, the duration of exposure could be relevant for the effect of antioxidants. It was demonstrated that in the case of some lipophilic antioxidants, vitamin E being a prime example, long-term (i.e., 12-24 h) pretreatment with the antioxidant prior to Aß exposure is required to observe neuroprotection (Goodman and Mattson 1994).

Antioxidants further prevented the disruptive effect of Aß on muscarinic cholinergic signal transduction demonstrating that ROS were mechanistically involved in this action of Aß. Pedersen et al. (Pedersen et al. 1996) reported that Aß suppressed acetylcholine production in a cholinergic cell line. Such actions of Aß mediated ROS elevation were supposed to contribute to the well-known deficit in cholinergic signaling pathways in AD.
However, the most important mechanisms of ROS-induced damage in AD include direct radical attack on proteins (Smith et al. 1991b); alterations of the lipid microenvironment of proteins (Dinis et al. 1993), changes in membrane fluidity (Pettegrew 1989), liberation of toxic compounds or precursors of toxic compounds such as arachidonic acid (Barbour et al. 1989).

As ROS have been described to be involved in multiple pathways associated with the progression of AD they are obviously important molecules that trigger the observed neuronal loss. Thus, effective radical scavenging could be an appropriate solution to interfere with a final executioner of AD, overcoming the problems of insufficient monocausal therapy.

1.2.3 Role of reactive oxygen species in Parkinson`s disease and amyotrophic lateral sclerosis

The second important neurodegenerative disease with an incidence of about 0.1% among people in industrialized countries is Parkinson`s disease (PD) (Checkoway and Nelson 1999). Neurodegeneration in PD predominantly affects dopaminergic neurons in the substantia nigra

that project to the striatum. The resulting striatal dopamine deficit as well as the associated imbalance of the neurotransmitters dopamine, acetylcholine and glutamate entails the well known symptoms of PD (Ebadi et al. 1996). Oxidative stress and mitochondrial dysfunction have been suggested to play important roles in the pathogenesis of PD (Owen et al. 1996). In PD, the major mitochondrial defect appears to be associated with complex I of the respiratory chain. Implication of oxidative stress in the pathogenesis and progression of PD is supported by the decrease in glutathione content, increase in levels of lipid peroxidation products, enhanced iron content in the substantia nigra and elevated production of ROS (Simonian and Coyle 1996; Jellinger 1999). ROS can also be produced during the oxidative deamination of catecholamines. Dopamine within nigral neurons undergoes spontaneous autoxidation to neuromelanin. This process generates ROS and neuromelanin itself may contain toxic quinones and hydroxyquinones (Graham 1978; Graham 1984). Also, H_2O_2 which is produced in the synthesis of dopamine by tyrosine hydroxylase and in the oxidative deamination of dopamine by monoamine oxidase may be involved in the progress of Parkinson's disease concerning the loss of nigral neurons (Olanow and Arendash 1994; Linert et al. 1996).

Amyotrophic lateral sclerosis (ALS) is considered to be an appropriate example for the direct involvement of ROS in neurodegenerative diseases as the pathological basis of ALS often implies a deficit of cytosolic Cu,Zn superoxide dismutase (SOD-1). SOD-1 has been identified as a factor that prevents the deleterious cascade of radical reactions by dismutiation of superoxide anion radical to oxygen and hydrogen peroxide which can be transferred to oxygen and water in the presence of catalase or glutathione peroxidase. In patients with familial amyotrophic lateral sclerosis (FALS), mutations in SOD have been demonstrated (FALS) (Deng et al. 1993; Rosen 1993). The mechanisms by which FALS-SOD mutants exert their toxic properties in the pathogenesis of this disease is still very controversial. One hypothesis is that FALS-SOD mutations cause the appearance of a pro-oxidant, pro-apoptotic function in a typically antioxidant enzyme (Yim et al. 1996).

However, the complete pathological mechanisms of neurodegenerative diseases such as PD or ALS are still not fully elucidated and future work is required to find the exact biopathological basis where therapeutic interventions can appropriately interfere with.

1.2.4 Involvement of reactive oxygen species in stroke

Stroke is the most common life-threatening neurological event. In westernized countries, stroke is the third leading cause of death after heart disease and cancer, and in the elderly it is

a major source of disability leading to institutionalization (Carr and Kenney 1992). Although pharmacological therapy to reduce ischemic damage is being pursued, prevention and rehabilitation are still the only strategies to reduce disability and lethality of stroke patients. Several processes have been described to be involved in deleterious events after ischemia including the release of excitatory amino acids, elevated Ca^{2+} with succeeding activation of enzymes, release of intracellular Fe^{2+}, energy deficit with disturbed mitochondrial function and anaerobic glycolysis which leads to lactate-acidosis (Choi 1988; Lafon-Cazal et al. 1993b; Siesjo et al. 1995). In nearly all processes that follow such ischemic damage, an aberrant amount of ROS is generated which triggers the progression of neuronal damage (Kuroda and Siesjo 1997) (Fig 3).

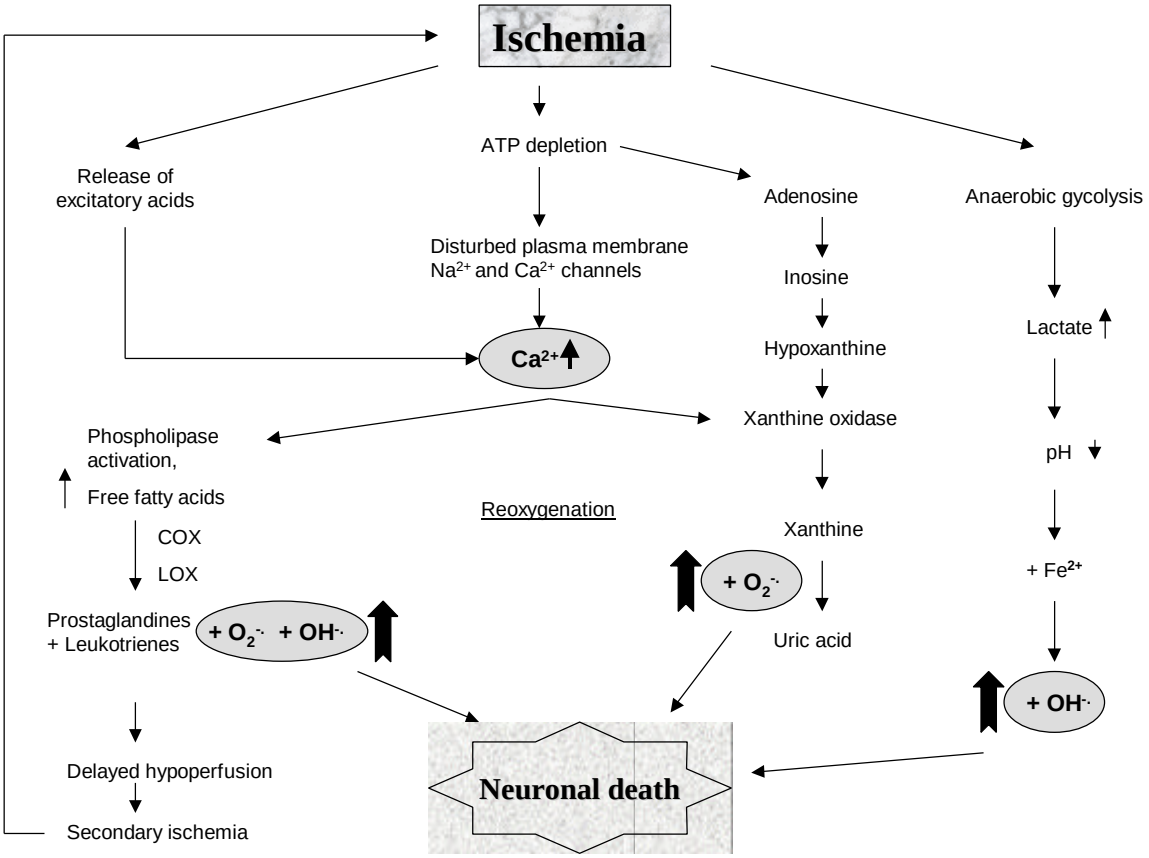


Fig 3 Proposed pathway for ROS-mediated events linking cerebral ischemia to neuronal cell death (modified from Phillis 1994))

These ROS and related reactive chemical species mediate much of damage that occurs after transient brain ischemia, and in the penumbral region of infarcts caused by permanent ischemia. One of those candidates is nitric oxide, a water- and lipid-soluble free radical which

is generated by the action of nitric oxide synthases. After ischemia, an immediate increase in nitric oxide synthase 1 (NOS 1; n-NOS)-activity in neurons and in NOS 3 (e-NOS)-activity in vascular endothelium has been described (Bolanos and Almeida 1999). Afterwards, also an increase in NOS 2 (i-NOS) activity in infiltrating neutrophils and macrophages, activated microglia and astrocytes was found in the same study. The effects of ischemia on the activity of NOS 1 are thought to be secondary to impairment of glutamate reuptake at synapses, activation of NMDA receptors, and resulting elevation of intracellular Ca^{2+} (Love 1999). In the context of cerebral ischemia, the activity of NOS 1 and NOS 2 was broadly deleterious, and their inhibition or inactivation was neuroprotective (Dawson and Dawson 1996; Iadecola 1997).

However, the production of nitric oxide in blood vessels by NOS 3, causes vasodilatation and improves blood flow in the penumbral region of brain infarcts (Szabo and Billiar 1999; Szabo and Billiar 1999). In addition to causing the synthesis of nitric oxide, brain ischemia leads to the generation of superoxide anion radicals, through the action of nitric oxide synthases, xanthine oxidase, and leakage from the mitochondrial electron transport chain. Nitric oxide and superoxide are themselves reactive but can also combine to form a highly toxic anion, peroxynitrite (Beckman 1991; Darley-Usmar and Halliwell 1996; Darley-Usmar and Halliwell 1996). The toxicity of the free radicals and peroxynitrite results from their modification of macromolecules, especially DNA, and from the resulting induction of apoptotic and necrotic pathways (Samdani et al. 1997; Dawson and Dawson 1996). The mode of cell death that prevails possibly depends on the severity and precise nature of the ischemic injury (Nicotera and Lipton 1999). Recent studies have emphasized the role of peroxynitrite in causing single-strand breaks in DNA, which activate the DNA repair protein poly(ADP-ribose) polymerase (PARP) (Pieper et al. 1999; Lipton 1999). This catalyses the cleavage and thereby the consumption of NAD+, the source of energy for many vital cellular processes. Over-activation of PARP, with resulting depletion of NAD+, has been shown to contribute to brain damage after transient focal ischemia in experimental animals. Neuronal accumulation of poly(ADP-ribose), the end-product of PARP activity has been demonstrated after brain ischemia in man (Endres et al. 1997).

In conclusion, brain ischemia initiates a complex cascade of metabolic events, several of which involve the generation of ROS. This knowledge will probably lead to a range of further pharmacological strategies that interfere with ROS to limit brain injury in stroke patients.

1.2.5 Neurodegeneration by reactive oxygen species: apoptosis or necrosis?

During the last years the discussed participation of apoptosis in stroke and neurodegenerative diseases has risen an increasing interest in the mechanisms of neuronal apoptosis. This was largely stimulated by the identification of several families of pro- and anti-apoptotic genes that are linked with death of mammalian cells (Bredesen 1996)). In the vast majority of cases examined, oxidative stress and disruption of calcium homeostasis have been mechanistically related to apoptotic or necrotic cell death. This is the case for excitotoxicity and metabolic insults such as occur in ischemic brain injury, traumatic brain injury, PD ALS or AD (Siesjo et al. 1995 Hall and Braughler 1993; Cotman 1998). Even in systems where initial studies failed to provide evidence for free radical and calcium involvement, subsequent data strongly support roles for oxidative stress in the cell death process (Greenlund et al. 1995; Martin et al. 1992).

In studies of cultured CNS neurons, it was shown that neuronal cell death induced by Aß can manifest as apoptosis (Loo et al. 1993). Although morphological and biochemical criteria have been established that allow investigators to categorize cell death as either apoptosis or necrosis, it is increasingly recognized that many of the mechanistic pathways and characteristics of apoptosis and necrosis are similar. A study investigating the influence of glutamate-induced oxidative stress described a form of neuronal cell death with criteria of both apoptosis and necrosis (Tan et al. 1998). They found membrane-blebbing, positive tunel-staining and cell shrinkage. Further, protein synthesis inhibitors blocked the oxidative toxicity pointing to an active form of cell death. On the other hand, no DNA-laddering, no chromatin condensation and no nuclear fragmentation was found after ROS stimulation.

Recent studies have shown that the same insult can kill the same population of neurons either by apoptosis or necrosis depending on the severity and/or duration of the insult. For instance, Ankarcrona et al. (Ankarcrona et al. 1995) reported that low concentrations of glutamate damaged neurons slowly by apoptosis, whereas neurons exposed to higher concentrations of glutamate underwent a rapid necrotic death. Apoptosis induced by Aß is correlated with an increased cellular accumulation of ROS, and is prevented by expression of Bcl-2, strongly implicating free radicals in the apoptotic process (Saille et al. 1999). Luo et al. (Luo et al. 1999) reported that ROS induce expression of a set of immediate early genes that were also induced in neurons subjected to well-established apoptotic paradigms. Levels of mRNA for c-fos and c-jun were increased relatively rapidly with a time course that preceded

cell death. Recent data from studies of post-mortem brain tissue from AD patients suggest that neuronal apoptosis probably occurs. It was demonstrated that neurons in vulnerable regions of AD brain exhibit DNA strand breaks, a feature of both oxidative stress and apoptosis (Su et al. 1994). Other authors even went further and described oxidative stress by excitotoxicity as apoptosis-necrosis-continuum (Portera-Cailliau et al. 1997).

However, the question if ROS rather induced necrosis or apoptosis remains to be further clarified. In the current study, the broadly used apoptosis inducer staurosporine as well as the excitotoxin glutamate strongly promoted the elevation of ROS suggesting that ROS are at least involved in both necrotic and apoptotic processes.

Several therapeutic strategies have been used to prevent oxidative damage and its consequences like apoptotic or necrotic neurodegeneration. Although some of the antioxidant drugs used in early studies were ineffective or had unacceptable side effects, other trials with radical scavenging properties have proven highly encouraging. Neuroprotection by antioxidants is therefore still a promising therapeutic strategy for the treatment of chronic neurodegenerative diseases and stroke (Hall 1993; Wolz and Krieglstein 1996).

1.3 Investigated neuroprotective drugs with potential antioxidative properties

1.3.1 Angiotensine converting enzyme (ACE)-inhibitors as potential neuroprotectants – an application beyond clinical use.

ACE inhibitors are established drugs for the treatment of hypertension and cardiac insufficiency (Govantes and Marin 1996). Because of their world-wide use, reliable efficacy of treatment and minimal side-effects, ACE-inhibitors have been proven as safe, convenient and effective drugs. Apart from hypertension and chronic heart failure, they also seemed to have additional beneficial effects. It has been shown that ACE-inhibitors were able to protect ischemic rat hearts against reperfusion injury (Ferrari et al. 1992; Liu et al. 1992), to block the progression of renal damage (Kohara et al. 1993) and to reduce angiotensin II-induced myocyte and coronary vascular necrosis (Kabour et al. 1995). Furthermore, captopril and quinapril attenuated cardiomyocyte apoptosis in spontaneously hypertensive rats (Diez et al. 1997). Recent data suggested that ACE-inhibitors could have a beneficial influence on cellular apoptosis which is thought to play a role in the pathology of the mentioned

neurodegenerative diseases ALS, multiple sclerosis, PD and AD as well as in stroke (Bredesen 1995).

Since the existence of an intracerebral brain renin angiotensin system has been revealed (Unger et al. 1988), various studies on the effect of ACE-inhibitors on brain function were performed. Recent data described that ACE-inhibitors ameliorated ischemic brain metabolism in spontaneously hypertensive rats by preventing the ischemia-induced increase in tissue lactate concentration and by stabilizing ATP-levels (Sadoshima et al. 1993). Moreover, ACE-inhibitors have been shown to reduce mortality in spontaneously hypertensive rats (Fujii et al. 1992; Vacher et al. 1993; Lee et al. 1996), and captopril improved neurologic outcome from incomplete cerebral ischemia in rats (Werner et al. 1991). Nevertheless, it is still unclear whether ACE-inhibitors are able to reduce infarct volume after cerebral ischemia in normotensive animals. Although many investigations on the protective capacities of ACE-inhibitors were performed, the mechanism of action remained poorly understood. However, there is growing evidence that the protective effects of ACE-inhibitors are independent of blood pressure reduction (Gohlke et al. 1996) or inhibition of angiotensin II formation (Takeda et al. 1997).

As ROS obviously play an important role in necrotic (Mattson et al. 1995), as well as in apoptotic cell damage (Ratan et al. 1994), radical scavengers such as 21-aminosteroids, thiols or tocopherol as well as overexpression of antioxidative enzymes could protect against several forms of neuronal damage (Lin and Chang 1997; Stoyanovsky et al. 1998). Investigators who so far determined radical scavenging properties of ACE-inhibitors observed different results. Some authors found that only the ACE-inhibitors containing a sulfhydryl (SH)-group such as captopril or zofenopril were capable of scavenging reactive oxygen species (Chopra et al. 1990;Mak et al. 1990; Noda et al. 1997). Others reported that free radical scavenging was independent of the SH-group and that the ACE-inhibitors with carboxylat or phosphonic acid structure such as enalapril, lisinopril, ramipril or fosinopril were equally potent antioxidants indicating that also these ACE-inhibitors might influence oxidative injury (Mira et al. 1993;Suzuki et al. 1993; Fernandes et al. 1996).

One aim of the current work concerning ACE-inhibitors was to determine whether the ACE-inhibitors enalapril and moexipril can protect cultured neurons from glutamate-, $Fe^{2+/3+}$- or staurosporine-induced neuronal damage and whether suppression of ROS-generation is involved in the mechanism of neuroprotection.

1.3.2 Role of estrogens as potential antioxidants

Estrogens are well known endogenous hormones that regulate the function of several endocrine-gated pathways and functions that are indispensable for the female organism but also appeared to be important for males. Since decades, estrogens are established in the therapy of several functional disturbances such as postmenopausal hormone deficit, dysmenorrhoea, prostata cancer as well as for contraception. Some recently discussed side-effects of estrogen treatment seemed to restrict the wide-spread use of estrogens (for review see Persson 1985). Primarily the incidence of breast cancer and endometrium carcinoma was suspected to correlate with the intake of estrogens. The long-term use of hormonal contraceptives that contained estrogens was also suspected to promote the risk of thrombosis. Nowadays, one has to be cautious with such findings because other studies proved the opposite (for review see Lupulescu 1993; Lupulescu 1995). Especially in the presence of simultaneous gestagen-treatment the cancer incidence was reduced and the progress of thrombotic complications rather seemed to depend on secondary risk factors such as smoking. An increasing number of investigations revealed cardio- or neuroprotective properties of estrogens which were also called “beneficial side-effects” (Schwartz et al. 1995; Hurn and Macrae 2000).

Some prior studies of mechanisms of estrogens demonstrated that they could have an inherent antioxidant activity (Keaney, Jr. et al. 1994; Liehr and Roy 1998; Liehr and Roy 1998). Estrogens can prevent oxidative processes because they contain a phenolic structural moiety in the A ring of these steroids (Behl et al. 1997; Subbiah et al. 1993). Recent data suggest that estrogens may protect against AD by an antioxidant mechanism. Postmenopausal women receiving estrogen replacement therapy were reported to have a significantly reduced risk of developing AD (Henderson et al. 1994; Simpkins et al. 1994). Exogenous application of the physiological 17ß-estradiol protected cultured neuroblastoma cells (Behl et al. 1995) and primary hippocampal neurons (Goodman et al. 1996) against Aß toxicity. Other estrogenic steroids that were investigated in these studies were similarly effective in protecting hippocampal neurons against Aß toxicity, whereas non-estrogen-steroids such as androgens, gestagens or glucocorticoids were either ineffective or exacerbated Aß toxicity. Assays of lipid peroxidation in isolated cortical membranes exposed to $FeSO_4$ demonstrated that estrogens suppress lipid peroxidation (Goodman et al. 1996). Also, the enhanced synthesis of neurotrophic factors (Singh et al. 1995) and inhibition of N-methyl-d-aspartate (NMDA) receptors (Weaver, Jr. et al. 1997) are suggested to contribute to the neuroprotective

mechanism of estrogens. Collectively, these data are consistent with the possibility that estrogens might protect neurons against oxidative injury and thereby avoid the development of AD and other neurodegenerative diseases.

In most of the in vitro studies micromolar concentrations of estrogens were used to demonstrate their neuroprotective effect against oxidative stress. Thus, it remained to be clarified whether estrogen derivatives can also protect neurons against oxidative damage at physiological nanomolar concentrations in vitro. Furthermore it was still unclear if the neuroprotection is mediated through estrogen receptor stimulation or rather due to structure-related antioxidative properties. In the current work, the effects of the 3-OH steroids 17ß-estradiol and 2-OH-estradiol on the intracellular ROS level and the oxidative damage in primary chick neurons after incubation with $FeSO_4$ were investigated. Moreover, the effect of ER estrogen receptor blockade was studied using the unselective estrogen receptor antagonist tamoxifen.

1.4 Role of ROS in neuroprotection and signal transduction

As already mentioned , the exogenous application of drugs that are directed against single pathological mechanisms is mostly insufficient. A promising strategy seems to be the exogenous stimulation of endogenous neuroprotective signal transduction. Optimal conditions for such processes would be mediated by a stimulus that initiates several endogenous pathways in parallel. Short and moderate episodes of sub-lethal stimuli like short ischemia, hypoxia and exposure to toxic agents have been described to mediate protection against subsequent severe damage.

This phenomenon of preconditioning, which was reported first by Murry et al. (1986), was further investigated by several authors in different animal species and organs. Most of these investigations were performed in heart tissue and myocardial cell cultures which provided insight into the underlying mechanisms. Cardiac preconditioning was shown to be mediated by adenosine through adenosine A_1 receptor stimulation which initiated an intracellular cascade including G-protein-linked protein kinase C (PKC) activation and opening of ATP-dependent potassium (KATP) channels as well as of calcium channels (Speechly-Dick et al. 1995; Zhou et al. 1996). Also, phospholipase C, MAPK, AMPA-receptor activation, nitric oxide, eNOS and reactive oxygen species ROS appeared to be involved in the mechanism of preconditioning (Qiu et al. 1997; Gidday et al. 1999; Leon et al. 1998; Mullane 1992). Particularly the question whether ROS participate in preconditioning has been discussed controversially. Some authors demonstrated that the preconditioning effect

implicated ROS that were generated by mitochondria, and radical scavengers at least partially abolished preconditioning-mediated myocardial protection (Vanden et al. 1998; Tritto et al. 1997; Baines et al. 1997; Kaeffer et al. 1997). Others suggested that neither ROS nor an up-regulation of antioxidative enzymes were involved in preconditioning (Iwamoto et al. 1991; Omar et al. 1991; Richard et al. 1993).

As compared to the heart, preconditioning of the brain, firstly described by Kitagawa et al. (1990), or of neuronal cell cultures is still poorly understood. Despite some similarities, the mechanisms of preconditioning seem to be different as far as Tauskela et al. (1999) reported that in contrast to the heart, factors like PKC, MAPK, AMPA receptors and Ca^{2+} were probably not involved in brain. Brain preconditioning which could be induced in vivo by short episodes of ischemia (mostly MCA occlusion or CCA occlusion) or in vitro by stimuli like hypoxia, oxygen-glucose deprivation, nitropropionic acid, iodoacetate, glutamate or MPP^+ seems to employ distinct mechanisms (Prass et al. 1998a). An upregulation of heat shock proteins (HSP70 and HSP27) (Sakurai et al. 1998; Xi et al. 1999), bcl-2 (Shimazaki et al. 1994), bFGF (Sakaki et al. 1995), opening of ATP sensitive potassium channels (Heurteaux et al. 1995) or HIF-1 suppression (Ruscher et al. 1998) have been considered to be responsible for the observed protection against subsequent insults. As ROS can be generated by most of the known preconditioning stimuli (Ohtsuki et al. 1992; Peters et al. 1998a; Ravati et al. 1999) they are suggested to be important mediators. Like in the heart, conflicting data exist concerning the relevance of ROS in brain preconditioning, and the effect of exogenously produced ROS as a preconditioning stimulus on pure neuronal cultures has hardly been investigated.

Further, the neuroprotective signal transduction pathways that are initiated by ROS remain to be clarified. One important candidate that could mediate such neuroprotective signaling is the transcription factor nuclear factor kappa B (NF-kB). NF-kB is a transcription factor with inducible activity that regulates the transcription of several genes upon activation. Within the brain, NF-kB, which was recently found to be present in all cells of the central nervous system, predominantly consists of the heterodimeric subunits p50 and p65 (Kaltschmidt et al. 1999a; Grilli and Memo 1999a). In the cytosol of neurons those DNA-binding subunits are either complexed with the inhibitory protein I-kB-α or constitutively active (O'Neill and Kaltschmidt 1997). Upon activation, the intracellular pathway involves the phosphorylation of I-kB kinase (IKK). The phosphorylation of I-kB which can take place at different amino acids depending on the activating stimuli (mostly serine 32 and serine 36) is the marking for subsequent ubiquitination and degradation of I-kB by the proteasome. The

unmasked homo- or heterodimeric complex of NF-kB is then translocated to the nucleus and initiates transcription by binding to a decameric consensus sequence. The pathway of NF-kB activation is illustrated in figure 4.

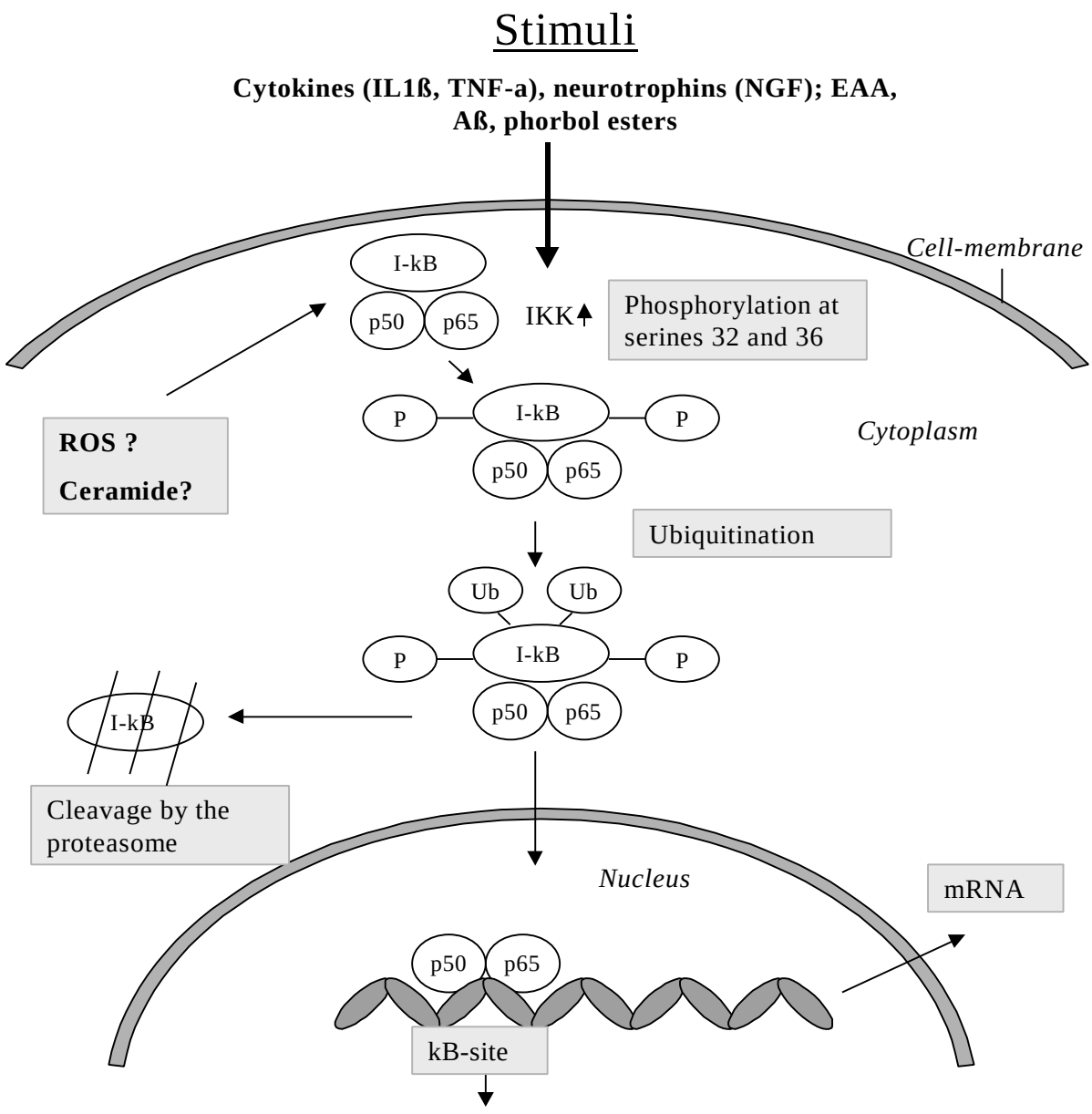


Fig. 4. Proposed mechanism of NF-kB activation

Such activation of NF-kB mediates the transcription of NF-kB-consensus sequence containing genes which are either indispensable to maintain neuronal growth and integrity or which account for the translation of deleterious proteins (Kaltschmidt et al. 1994). Therefore, the functional role of NF-kB has been discussed controversially. For instance, activation of NF-kB was demonstrated to mediate excitotoxin-induced apoptosis in rat striatum and to promote Aß neurotoxicity (Qin et al. 1998; Bales et al. 1998). Further, the infarct volume after transient focal ischemia was found to be reduced in p50 knock-out mice indicating that activated NF-kB contributes to ischemic cell death (Schneider et al. 1999). In contrast, NF-kB suppressed apoptosis by inhibition of caspase-8 activation or modulation of bcl-2 and mediated the antiapoptotic properties of growth factor NGF in neurons (Wang et al. 1998), (de Moissac et al. 1998; Herrmann et al. 1997; Maggirwar et al. 1998). In the same line, inhibition of NF-kB induced apoptosis in PC 12 cells and potentiated amyloid-ß-mediated apoptotic damage in primary neurons (Taglialatela et al. 1997; Kaltschmidt et al. 1999b). Further, a lack of the p50 subunit increased the vulnerability of hippocampal neurons to excitotoxic injury (Yu et al. 1999).

Also, the phenomenon of preconditioning which can be induced by short and moderate episodes of sub-lethal stimuli requires transcriptional activity to mediate protection against subsequent severe damage (Currie et al. 2000). As ROS can be potentially generated by most of the known preconditioning stimuli (Peters et al. 1998b) and are assumed to be a common mediator of NF-kB activation, a link between preconditioning and NF-kB activation was proposed (Prass et al. 1998b). Recently, it was reported that moderate pretreatment with Aß in an NF-kB activating concentration protects neurons against subsequent severe Aß toxicity (Kaltschmidt et al. 1999b).

As illustrated in figure 4, NF-kB regulates the transcription of various proteins associated with either neuroprotection or neurodegeneration. Among the beneficial proteins, bcl-2 and superoxide dismutase (SOD) seem to play a pivotal role for neuronal survival. Especially SOD provides an important defence against ROS. SOD catalyses the dismutation of superoxide to O_2 and H_2O_2, with the latter being metabolized to water in the presence of catalase or glutathione peroxidase (Fridovich 1989). Three isoforms of SOD have been identified in mammalian cells: the cytosolic Cu/Zn-SOD (SOD-1), encoded by the sod1 gene; the mitochondrial Mn-SOD (SOD-2), encoded by the sod2 gene; and the extracellular Cu,Zn SOD, encoded by the sod3 gene. SOD overexpression has been shown to be protective in several types of oxidant injury (Huang et al. 1992; Park et al. 1998). Transgenic mice that overexpress SOD exhibit reduced brain injury in different stroke models (Yang et al. 1994;

Murakami et al. 1997). Conversely, ischemic cerebral injury is exacerbated in mice deficient in SOD (Kondo et al. 1997). These results suggest that SOD can play a crucial role in limiting ROS damage associated with cerebral ischemia. Besides direct detoxification of ROS, preserving the function of glutamate transporters that play an important role in preventing excitotoxicity by rapid clearance of synaptic glutamate from oxidative impairment, could be another way through which SOD protects against neuronal damage. Concerning the functional correlation of NF-kB and ROS, especially SOD-2 seems to be important role for the balance of intracellular ROS levels (Pang et al. 1992).

To identify the executioners of the NF-kB-mediated rescue pathway the current study also focused on the effect of moderate ROS-stimulation on the regulation of SOD-1 and SOD-2.

1.5 Aims of the current thesis

An aberrant elevation of ROS that occurs in many neurodegenerative disorders or in stroke obviously initiates or executes several deleterious pathways leading to neuronal cell death. On the other hand, an important functional relevance of ROS for the survival and integrity of neurons has been proposed under certain conditions. Thus, ROS seem to possess a dual role for cellular function. The current study investigates both sides of radical action: the possibility to inhibit the damaging ROS elevation using promising neuroprotective drugs with antioxidative properties and the principle to promote ROS-gated intracellular pathways signaling for neuronal survival.

The main purposes of the current study are indicated as follows:

1. ROS kinetics were investigated in neuronal cell death induced by the damaging agents glutamate, iron and staurosporine. Morphological studies also focused on the appearance of ROS-gated apoptotic or necrotic cell damage.
2. The role of ACE-inhibitors for neuronal survival and their corresponding effect on the abundant formation of ROS was studied in cultured neurons.
3. The antioxidative properties of estrogen steroid hormones were examined concerning the mechanism of estrogen-mediated protection and the receptor-dependency of the observed effect.
4. In cultured neurons, a model for moderate stimulation with ROS by xanthine/xanthine oxidase [(X/XO) pre] or by $FeSO_4$ was established and characterized for ROS-formation kinetics.
5. The current thesis investigated whether moderate ROS generation could precondition cultured neurons against different forms of subsequent neuronal damage including

apoptosis and necrosis and whether ROS-scavengers were able to abolish such neuroprotection. Furthermore, the influence of ROS-preconditioning on the elevation of oxygen radicals by the damaging agents within single neurons was studied.

6. To evaluate the mechanism of ROS-mediated neuroprotection against staurosporine-induced apoptosis the role of the inducible transcription factor NF-kB and its ROS-dependent activation were examined.
7. Finally, the cellular expression of the antioxidative enzymes SOD-1 and SOD-2 that could potentially execute the neuroprotection of ROS-mediated NF-kB activation were investigated

2 Materials and Methods

2.1 Materials

2.1.1 Animals

For primary hippocampal cultures neonatal day 1 Fischer-344-rats from domestic breeding were used. The animals were kept under controlled light and environmental conditions (12 h dark/light circle, 23 ± 1^0C, 55 ± 5% relative humidity) and had free access to food (Altromin, Germany) and water.

2.1.2 Drugs

Xanthine	Sigma, Deisenhofen
Xanthine oxidase	Fluka, Buchs
Ferrous sulfate (Fe_2SO_4)	Sigma, Deisenhofen
17-ß-estradiol	Sigma, Deisenhofen
2-OH-estradiol	Sigma, Deisenhofen
Enalapril	Sigma, Deisenhofen
Moexipril	Schwarz, Monheim
Cycloheximide	Sigma, Deisenhofen
Lactacystine	Sigma, Deisenhofen
Pyrrolidindithiocarbamate (PDTC)	Sigma, Deisenhofen
Sodium Cyanide (NaCN)	Merck, Darmstadt
MK 801	Sigma, Deisenhofen

2.1.3 Materials for cell culture

Dulbeccos modified eagle medium (DMEM)	Gibco, Life Technologies, Eggenstein
Fetal bovine serum	Gibco, Life Technologies, Eggenstein
Antibiotic-Antimycotic mixture (100x)	Gibco, Life Technologies, Eggenstein
Containing: penicilline G sodium 10000	

U/ml, streptomycine 10000 µG/ml, amphotercine B 25 µg/ml	
Neurobasal Medium	Gibco, Life Technologies, Eggenstein
Leibovitz L15 medium	Gibco, Life Technologies, Eggenstein
B27 supplement	Gibco, Life Technologies, Eggenstein
Glutamin	Gibco, Life Technologies, Eggenstein
Papain	Gibco, Life Technologies, Eggenstein
Poly-L-lysine-HBr (30000-70000)	Sigma, Deisenhofen
Poly-L-Lysine-HBr (70000-150000)	Sigma, Deisenhofen
Petri dishes Easy Grip, 35x10 mm	Becton Dickinson, Heidelberg
Falcon	
Culture flasks Easy access, 25 cm^2, 50 ml,	Becton Dickinson, Heidelberg
Falcon	
Culture flasks Heraeus Petriperm	Bachhofer, Reutlingen
Multiwells (24), Nuclon Delta	Nunc, Wiesbaden

2.1.4 Materials for investigation of neuronal viability and apoptosis

Trypan blue solution 0.4%	Merck, Darmstadt
Hoechst 33258 (bisbezimide)	Sigma, Deisenhofen
Methanol 99.8% (gradient grade)	Merck, Darmstadt
Phosphate buffered saline (PBS)	Sigma, Deisenhofen
Lactate Dehydrogenase (LDH)-KIT	Sigma, Deisenhofen
with LDH reagent A, containing phosphate-buffer pH 7.5 and NADH;	
LDH reagent B, containing Pyruvate	
UV-meter	Pharmacia Biotech, Cambridge, U.K.
Microscope (trypan blue-staining)	Leica, Benzheim
Axiovert 120 Microscope	Zeiss, Jena
Camera	Olympus OM-4Ti, Japan

2.1.5 Materials for measurement of reactive oxygen species and mitochondrial membrane potential

Dihydrorhodamine 123	Molecular Probes, Göttingen
CCD-camera	Hamamatsu, Herrsching
argus 50 software	Hamamatsu, Herrsching
Axiovert 100 microscope	Zeiss, Jena
Rhodamine 123	Molecular Probes, Göttingen

2.1.6 Materials for immunocytochemistry

Methanol	Boehringer Mannheim, Mannheim
Bovine serum albumin	Sigma, Deisenhofen
Laser Scanning microscope LSM 510	Zeiss, Jena
Primary neurofilament (NF) polyclonal antibody	Sigma, Deisenhofen
Primary p65 monoclonal NF-kB antibody	Boehringer Mannheim, Mannheim
Secondary FITC-coupled anti-mouse antibody	Boehringer Mannheim, Mannheim
Secondary rhodamine-coupled anti-mouse antibody	Boehringer Mannheim, Mannheim
Slides	IDL, Nidderan

2.1.7 Materials for western blotting

Enhanced chemilumenescent (ECL) detection KIT	Pierce, Rockford, USA
Acrylamide	Sigma, Deisenhofen
Amino-n-caproic acid	Sigma, Deisenhofen
Aprotinine	Sigma, Deisenhofen
Blotter	Biometra, Göttingen
Bovine serum albumin	Sigma, Deisenhofen
Bromphenole-blue	Promega, Heidelberg
Calpain inhibitor	Sigma, Deisenhofen

Calpain-inhibitor	Sigma, Deisenhofen
Developer	Sigma, Deisenhofen
Electrophoresis supplier	Pharmacia, Sweden
Films	Kodak, Stuttgart
Fixative	Agfa Gevaert, Leverkusen
Glycerol	Sigma, Deisenhofen
Glycin	Sigma, Deisenhofen
Horse-radish-peroxidase-conjugated sheep anti-rabbit IgG	Sigma, Deisenhofen
I-kB_α polyclonal rabbit antibody	Santa Cruz, Heidelberg
Lauryl sulphate sodium	Sigma, Deisenhofen
Mercaptoethanol	Sigma, Deisenhofen
Methanol 99.8%	Merck, Darmstadt
Milk powder	Heirler GmbH, Radolfzell
Nitrocellulose-membranes, Protran BA 83	Amersham, Braunschweig
p65 monoclonal mouse antibody	Boehringer, Mannheim
Phenylmethylsulfonyl fluoride (PMSF)	Sigma, Deisenhofen
Ponceau S	Sigma, Deisenhofen
SOD-1 and SOD-2 polyclonal rabbit antibody	RDI, Flanders, USA
TEMED	Applichem, Gatersleben
Tris-HCl	Sigma, Deisenhofen
Tween 20	Sigma, Deisenhofen
Whatman paper	Schleicher und Schüll, Dassel

2.1.8 Materials for nuclear extraction and electrophoretic mobility shift assay (EMSA)

Ammoniumperoxy sulfate (APS)	Sigma, Deisenhofen
Boric acid	Sigma, Deisenhofen
Dithiothreitol	Sigma, Deisenhofen
EDTA	Nunc, Wiesbaden
EGTA	Merck, Darmstadt

Ficoll 400	Sigma, Deisenhofen
Films	Kodak
glycerol	Sigma, Deisenhofen
HEPES	Boehringer Mannheim, Mannheim
KCl	Merck, Darmstadt
NF-kB oligonucleotide	Sigma, Deisenhofen
Nonidet P40	Boehringer Mannheim, Mannheim
Phosphor^{32}-labeled ATP	Sigma, Deisenhofen
PMSF	Sigma, Deisenhofen
Poly(dl-dC)	Amersham, Braunschweig
Polynucleotide kinase	Serva, Heidelberg
TEMED	Applichem, Gatersleben
Tris-HCl	Sigma, Deisenhofen
Whatman paper	Whatman, England

2.2 Methods

2.2.1 General principles

Coating of culture dishes, preparation of primary cultures, medium exchanges, treatment with drug solutions and with all other solutions that had contact with the cultured cells were conducted in sterile laminar air flows with vertical or horizontal ventilation (Lamin Air ELB 2448, Heraeus, Hanau; Envirco C424H, Ceag Schirp, Borken und Prettl Typ H-1 06.12, Reinraumtechnik GmbH, Pfullingen). All glass materials and cannula of metal were sterilized at 180 ºC for 2 h (TV 40 UT, Memmert, Emmndingen). Aqueous solutions for cultivation of the cells and for drug treatment and buffers were distilled twice (Milli-Q, Millipore, Neu-Isenburg). All other tools that had to be sterilized were autoclaved at 120ºC and 2 bar for 25 minutes. Solutions that were expected to be instable at high temperatures were filtered through membrane filters with a pore size of 200 nm. Instruments for surgery were incubated in ethanol (70%) for 30 min before preparation of the animals. Media used in the experiments were obtained sterile and heated to 37 ºC before contacting the cells. All demonstrated experiments were reproduced in duplicate if not stated otherwise.

2.2.2 Cell culture models

2.2.2.1 Primary neuronal cultures from chick embryo telecephalons

Primary neuronal cultures from 7 d old chick embryo telencephalons were prepared as described previously (Pettmann et al. 1979a). For preparation of primary neuronal cultures from chick embryos fertilized eggs of the breed “Weiße Leghorn” were derived (“Geflügelfarm, Ch. Thome, Biedenkopf-Wallau”). After preliminary storage at 18 ºC for maximally 4 days, the eggs were brooded for 7 days in an incubator with 60-65% relative humidity at 37 ºC

After the eggs were cleaned with ethanol (70 %) the embryos were taken out with a pincette and placed in a petri dish containing DMEM. The cerebral telencephalic hemispheres were isolated and mechanically dissociated through nylon meshes of 48 µm mesh width. The homogenized cell suspension was seeded onto poly-L-lysine coated Petri dishes containing 15-mm glass coverslips or into culture flasks (25 mm^2) with a density of $4x10^4$ cells/cm^2. The cells were cultured in DMEM supplemented with penicillin-streptomycin solution and 20%

fetal bovine serum at 37°C, 5% CO_2 and 95% relative humidity. Culture medium was replaced every 2 d and the neuronal cells were used for experiments on day 5 after seeding. The percentage of neurons at this time point was > 98% because gliogenesis was known to occur later than 8 d during the development of the chick embryo and the few glial cells in the initial suspension were inhibited by the polylysine substrate. Neuronal cells were identified by an immunohistochemical demonstration of tetanus toxin binding sites (Pettmann et al. 1979b) and by immunocytochemistry which revealed that nearly all cells reacted with neurofilament antibodies.

Poly-L-Lysin-buffer pH 8.5	Poly-L-Lysin hydrobromide 70000-150000	5 mg
	Boric acid	3.09 g
	Distilled water	ad 500 ml
	pH adjusted with NaOH (1M)	
Cell culture medium	DMEM	500 ml
	fetal calf serum	100 ml
	penicillin-streptomycin solution	5 ml

2.2.2.2 Primary mixed hippocampal cultures from postnatal rats

Cultured hippocampal cells were prepared from postnatal (p 1) Fischer 344 rats as described previously (Sengpiel et al. 1998). The pups were washed with ethanol (70%) and decapitated. After the skull was opened, the brains were removed and placed onto a petri dish containing solution 1. The hippocampi were carefully isolated, purified and dissected. Then the hippocampi were incubated at 37°C for 20 min in solution 2, and gently triturated with a fire-polished Pasteur pipette. Thereafter, the cell suspension was layered onto solution 3, centrifuged at 200 x g for 10 min and the pellet was re-suspended. For image analysis/immunocytochemistry, cells were plated onto poly-L-lysine-coated glass coverslips that were placed into Petri dishes. For cytotoxicity assay, western blotting analysis, and EMSA cells were seeded at a density of 2 x 10^4 cells/cm² into poly-L-lysine-coated Petri dishes. Cells were cultured in a humidified atmosphere of 5% CO_2 and 95 % air at 37°C in

neurobasal medium with 0.5 mM glutamine, B27 supplement and antibiotics for 10 days before experiments. Medium was exchanged after 3 days in culture. The mixed cultures contained 61.2% neurons and 38.8% astrocytes as evaluated by an immunocytochemical staining with anti-neurofilament antibodies (1: 2000) and anti-GFAP antibodies (1: 10) (Fig. 5). Animal care followed official governmental guidelines.

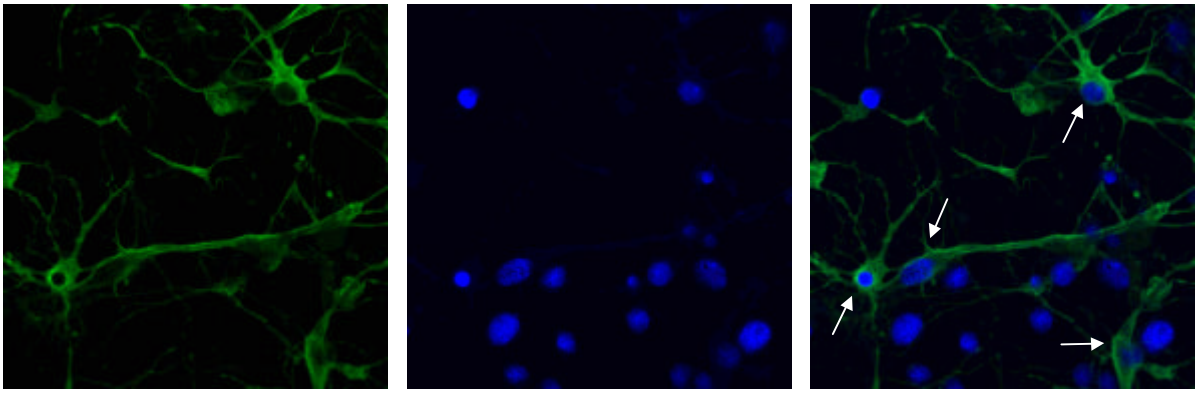


Fig. 5. Immunocytochemical characterization of mixed hippocampal cultures

The illustration shows double-staining with FITC-conjugated anti-GFAP antibodies (A) and with the nuclei-sensitive dye Hoechst (B) In C an overlay of both staining is demonstrated. Arrows indicate the localization of astrocytes. Nuclei which are not co-localized with GFAP-staining are considered to be of neuronal origin.

Poly-L-Lysin-buffer	Poly-L-lysin hydrobromide 30000-70000	1 mg
	Boric acid solution (1.25%)	5 ml
	Borax solution (1.91%)	5 ml
Cell culture medium	Neurobasal medium	100 ml
	B27 supplement	2 ml
	L-Glutamine (50 mM)	1 ml
	Penicillin-streptomycin solution	5 ml
Solutions for preparation		
Solution 1	Bovine serum albumin	30 mg
	DMEM	150 ml

Solution 2	Papaine	30 mg
	Bovine serum albumin	30 mg
	DMEM	150ml
Solution 3	Bovine serum albumin	500 mg
	Trypsine inhibitor	500 mg
	Neurobasal medium	50 ml

2.2.3 Quantification of neuronal viability

2.2.3.1 Trypan blue exclusion test

Cellular viability was determined by the trypan blue exclusion method which identified damaged neurons on the basis of membrane leakage. The hydrophilic dye trypan blue (Fig. 6) is unable to permeate intact cellular membranes.

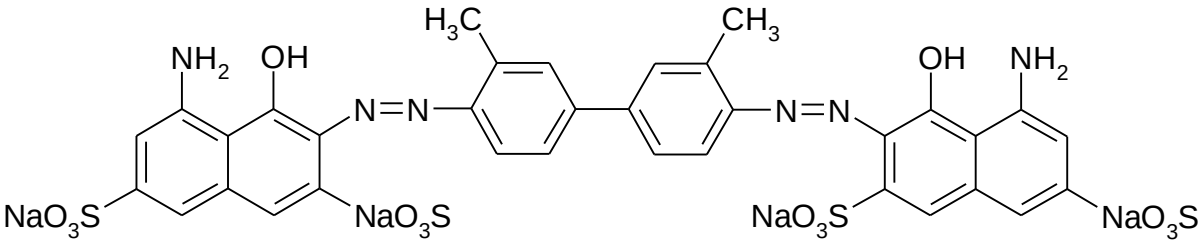


Fig. 6. Structure of the hydrophilic conjugated azo-dye trypan blue

Upon damaging conditions the outer membranes are disrupted and the dye can permeate the cell and stain the cytosol. For this purpose, cultures were incubated for 10 min with an 0.4% solution of the hydrophilic dye trypan blue. Then the cells were washed three times with PBS and fixed with methanol. After 24 h of treatment the number of stained and unstained neurons was counted in 12 randomized sub-fields of three different flasks containing approximately 80 neurons per sub-field. Neuronal damage was expressed as percent ratio of trypan blue stained cells versus the total number of cells.

PBS-buffer pH 7.4 (Phosphate buffered saline)	KH_2PO_4	1.06 mM
	NaCl	154 mM
	Na_2HPO_4 x 7 H_2O	4.47 mM
	pH adjusted with NaOH (1 M)	
Trypan blue solution	Trypan blue	400 mg
	NaCl	810 mg
	K_2HPO_4	60 mg
	Distilled water	ad 100 ml

2.2.3.2 Lactate dehydrogenase test (LDH-test)

Cell viability was determined by the release of the cytosolic enzyme lactate dehydrogenase (LDH) into the extracellular space. This enzyme is to hydrophilic to leave its intracellular localization to the extracellular medium. Upon membrane damage LDH is released to the culture medium and the amount of LDH-release can be correlated with the amount of cellular damage. LDH is quantified due to its ability to catalyze the following reaction:

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-Lactate} + NAD^+ \ (A_{365})$$

NAD^+ can be determined photometrically at 365 nm. After exposure to staurosporine for 24 h, the intracellular and extracellular LDH activity was measured by determination of the culture medium content and the total LDH content of the lysed cells using a commercial LDH assay kit (n = 5 culture flasks per group). LDH-reagent A and LDH-reagent B were mixed (25:1) and 1 ml reagent mix was incubated with 40 µl probe for 30 seconds. After transferring the solution to a glass cuvette the absorption at 365 nm was measured after 30 s, 1 min, 2 min and 3 min. LDH release was calculated as the percent ratio of changes in the extinction per minute at a wavelength of the extracellular versus the total LDH activity. Controls were taken as 100%.

Reagent A	Phosphate buffer pH 7.5	54 mM
	NADH	0194 mM
Reagent B	Pyruvate	16.2 mM

2.2.4 Determination of neuronal apoptosis

After fixing the cells in methanol for 5 minutes, they were washed twice by a methanolic solution of the DNA fluorochrome Hoechst 33258 (10 µg/ml) and then incubated with fresh Hoechst solution for 15 min at 37 ºC. This lipophilic cationic dye (Fig. 7) can easily permeate membranes of intact and degenerated cells and stain the nuclear DNA by binding to the minor groove of DNA at AT-rich sequences. Afterwards, the cells were washed twice with methanol and then stored in ice-cold PBS buffer. Nuclear morphology was observed under a fluorescence microscope (Axiovert 100, Zeiss, Germany) or laser scanning microscope (LSM 510, Zeiss, Germany) at an excitation wavelength of 350 nm and an emission wavelength of 450-520 nm. Cells which exhibited reduced nuclear size, chromatin condensation (visible as an intense fluorescence) and nuclear fragmentation were considered to be apoptotic neurons. The number of cells with apoptotic features and total cell number was counted in 8 randomized sub-fields of two different culture flasks containing approximately 60 neurons per sub-field. Neuronal apoptosis was expressed as percent ratio of cells with apoptotic features versus the total number of cells. Photomicrographs were taken for visual demonstration of the observed effects.

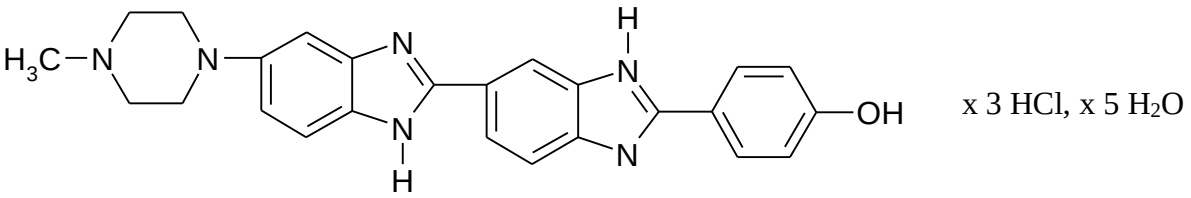


Fig. 7. Chemical structure of the nuclear dye Hoechst 33258

2.2.5 Determination of reactive oxygen species content

2.2.5.1 Intracellular measurement

ROS were measured using the lipophilic non-fluorescent dye dihydrorhodamine 123 which accumulates in mitochondria and is oxidized by ROS to the positively charged fluorescent rhodamine 123. Upon oxidation the dye gains an extended conjugated electron system which moves its absorption maximum towards a longer absorption wavelength of 490 nm (Fig. 8).

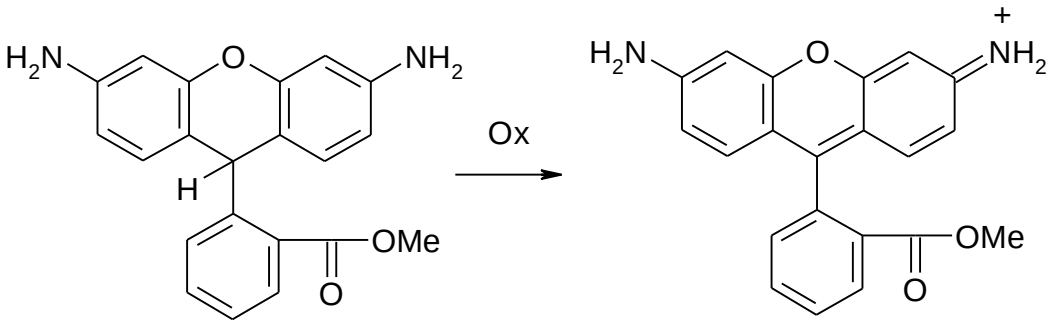


Fig. 8. Structure of dihydrorhodamine 123 and its oxidation to rhodamine in the presence of oxidative conditions, such as ROS.

To record fluorescence, cells which were cultured on 25 mm glass cover slips were stained with 5 µM dihydrorhodamine 123 for 15 min and then washed three times with PBS. The glass coverslips were taken out carefully and placed in a special plastic incubation chamber with PBS which allowed continuous exchange of the supernatant fluid (Fig. 9). Digital video imaging of rhodamine 123 fluorescence was conducted using a fluorescence microscope with attenuated UV illumination from a 75 W xenon lamp. Fluorescence intensity was measured with an excitation wavelength of 490 nm and at an emission wavelength of 510 nm. An electronic shutter which opened during image acquisition only, minimized photobleaching and phototoxicity. Images were taken by a CCD camera and were digitalized as 256x256 pixels. Before measurement of fluorescent values, a background picture was taken that was later subtracted from the images. Data were analyzed using Argus 50 software and saved as TIFF-files on a computer (DECpc 422 dxLP, Digital, München) (Fig. 10). Fluorescence intensities were given as arbitrary fluorescence units (Fl.U.).

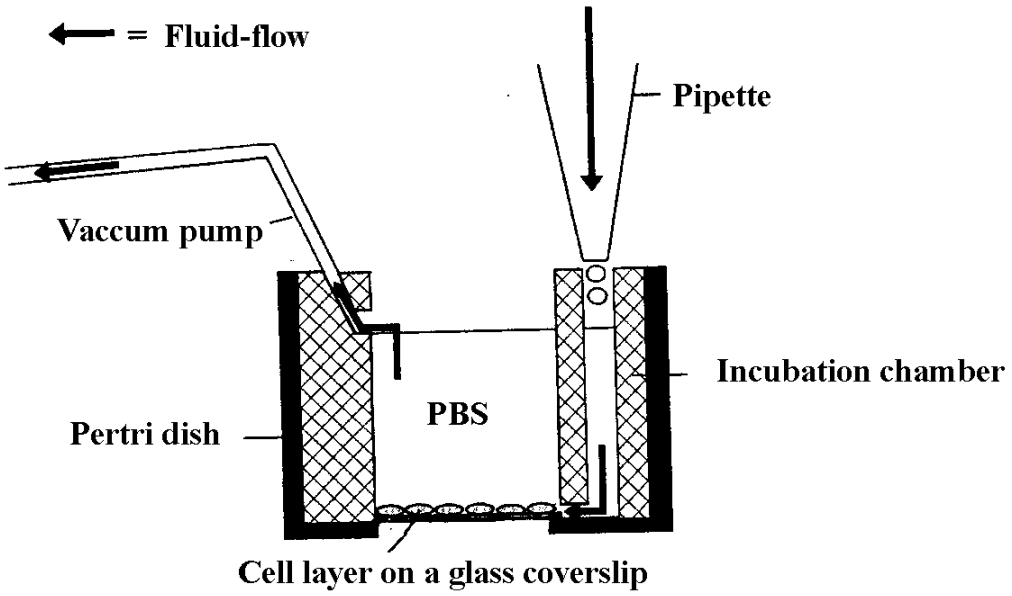


Fig.9 Incubation chamber for measurement of ROS and mitochondrial membrane potential

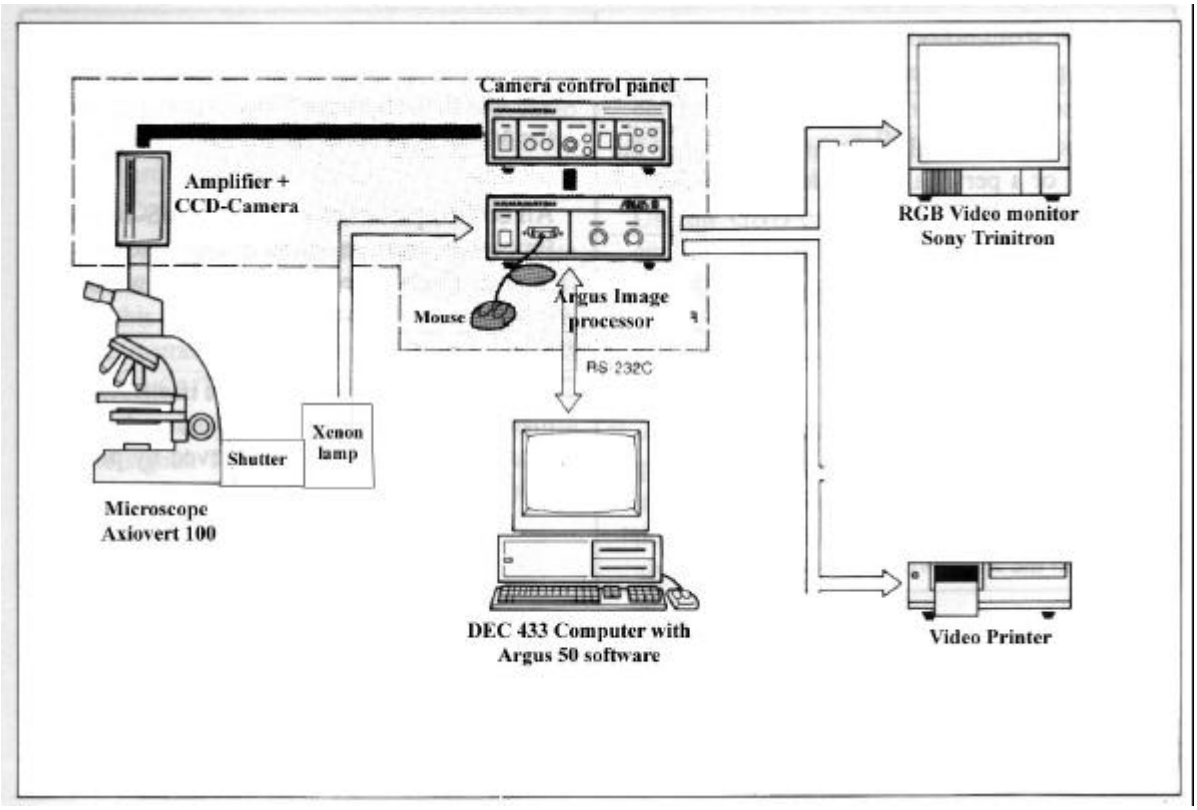


Fig.10 Arrangement of fluorescence microscopy measurement

2.2.5.2 Measurement of ROS in cellular extracts

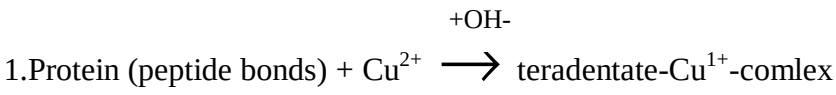
Cells were incubated with 5 µM dihydrorhodamine 123 for 5 minutes. Afterwards, cells were washed with Locke`s solution and suspended in 300 µl NaCl (0.9%) solution. An aliquot of the probe was directly measured with a fluorescence plate reader at an excitation wavelength of 520 nm. Another aliquot was used for the determination of total cellular protein content by the BCA kit. Results are expressed as fluorescence intensity / protein content (Fl.U./µg protein).

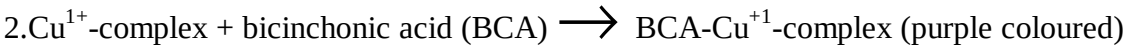
Locke`s buffer pH 7.4	NaCl	154 mM
	KCl	5.6 mM
	$CaCl_2$	2.3 mM
	$MgCl_2$	1 mM
	$NaHCO_3$	3.6 mM
	HEPES	5 mM
	Glucose	20 mM

2.2.6 Protein Measurement

Protein concentration for equal loading of gels for western blotting and EMSA was measured by the BCA kit. At first, an albumin protein standard was used (2mg/ml) which was diluted to concentrations ranging from 1 µg/ml to 80 µg/ml (1, 3, 5, 10, 20, 40, 80 µg/ml). Standards and probes (6 µl) were mixed with working solution of the BCA kit containing a mixture of 7.2 ml reagent B 0.3 ml reagent C and 7.5 ml reagent A. After incubation for 1 h at 60 ºC, 100 µl of each probe or standard was transferred to a microtiter-plate and the absorbance was measured at 562 nm using a photometer. A standard curve was calculated by Winstat and corresponding probe protein levels were calculated by Microsoft Exel.

Principle of BCA-protein quantification:





Reagent A	Na_2CO_3	
	$NaHCO_3$	
	Sodium tartrate	
	NaOH	0.2 N
Reagent B	Aqueous solution of bicinchoninic acid	4 %
Reagent C	$CuSO_4$	4 %
Albumin standard	BSA	2. mg/ml
	NaCl 0.9 %	
	NaN_3 0.05 %	

2.2.7 Immunostaining

Hippocampal cells that were cultured on 25 mm glass coverslips cells were fixed with methanol at −20°C for 20 min and then incubated with Hoechst 33258 as described to identify nuclear localization. Then the cells were exposed to blocking buffer for 30 min to avoid unspecific binding. Thereafter, primary monoclonal anti-mouse p65 NF-kB-antibody was added overnight at 4°C. After intensive washing with PBS, cells were exposed to secondary rhodamine-conjugated anti-rabbit Ig G (1:400) in the dark for 30 min at room temperature. After washing with PBS, the cells were incubated with primary polyclonal anti-rabbit neurofilament-antibody to differentiate neuronal and glial localization of NF-kB immunoreactivity. Four h the cells were washed and exposed to secondary fluorescein-isothiocyanate (FITC)-conjugated anti-mouse IgG in the dark for 30 min at room temperature. Then the glass coverslips were removed, turned on a slide, moistured with PBS and fixed with varnish. Immunostaining was evaluated using confocal laser scanning microscopy which allowed precise localization of intracellular immunostaining, especially nuclear translocation of p65. This very modern approach facilitated the monitoring of subcellular compartments and minimized bleeding effects of the used triple staining with concomitant identification of three different dyes. The principles of confocal laser scanning microscopy are illustrated in figure 11.

Cells were excited by 3 different lasers, Helium/Neon (rhodamine; 540 nm), Argon (FITC; 490 nm and UV-laser (Hoechst 22358; 350 nm). Data and images were analyzed by Zeiss Image Browser software. Negative controls were performed by omitting the primary antibody.

Blocking buffer pH 7.4	Bovine serum albumin	0.1 g
	Distilled water	ad 10 ml
Antibody incubation buffer pH 7.4	Bovine serum albumin	1 g
	Milk powder	5 g
	Distilled water	ad 100 ml

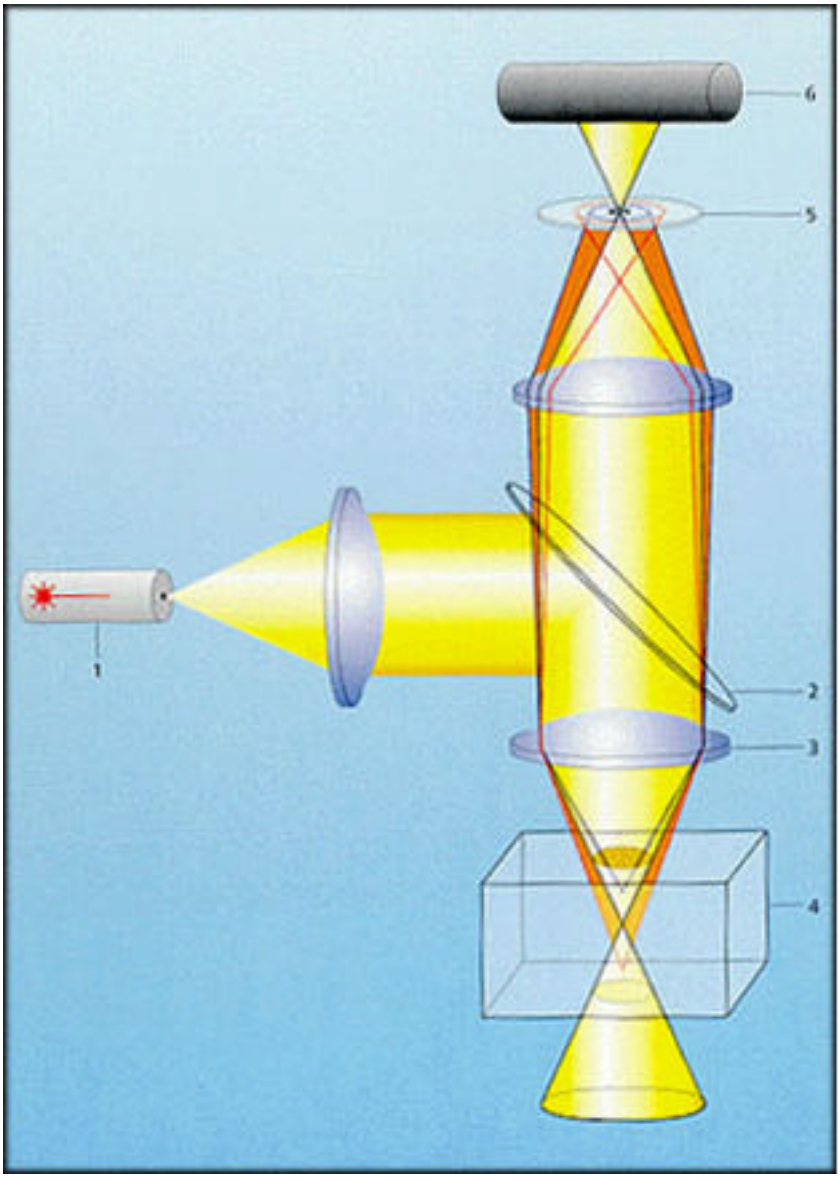


Fig. 11. Principles of confocal laser scanning microscopy

1 - light source (laser); 2 - dichroic mirror; 3 – objective lens; 4 – sample; 5 – pinhole; 6 - detector

The big advantage of confocal microscopy is the possibility to collect light exclusively from a single plane. A pinhole sitting conjugated to the focal plane (i.e. confocal) excludes light from the detector that is reflected/emitted from others than the focal plane. The laser scanning microscope scans the sample sequentially point by point and line by line and assembles the pixel information to one image. By moving the focus plane single images (optical slices) can be put together to create a three dimensional stack that can be digitally processed afterwards.

2.2.8 Western blotting analysis

Cell cultures were washed once with PBS, collected at 4°C and then lysed on ice in an extraction buffer. The cells were gently aspirated 5 times through a 25 G needle and centrifuged at 15 000 x g for 15 min at 4°C. The supernatants were collected on ice and were measured for protein using the BCA protein assay kit. Proteins (20 µg/lane) were incubated for 5 min in sample buffer at 95°C and were equally loaded and separated on SDS-polyacrylamide gels with varying acryl amide concentration (10%-20%). After electrophoresis, the gels were washed in blotting buffer III, nitro-cellulose membranes were incubated in blotting buffer II and filter papers were wetted in blotting buffer I,II and III. Then the proteins were blotted onto nitro-cellulose membranes (Protran BA 83, Schleicher & Schüll, Dassel, Germany) at 250 mA for 15 min. Equal and complete protein transfer was monitored by membrane staining with Ponceau s. After washing with PBS the membranes were incubated in blocking buffer for 3 h at 20°C. Incubation with primary polyclonal rabbit anti-I-kB-α antibodies (1:1000) SOD-1 or SOD-2 was performed overnight at 4°C in blocking buffer. The next day, the blots were washed twice in washing buffer and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit Ig G antibody (1:2000) at 20°C in blocking buffer. Afterwards, the membranes were washed again. For detection, the membranes were exposed to enhanced chemiluminescence solution for 1 min, dried, placed on a glass-plate. The blots were then exposed to a film in the dark room. Exposure time varied depending on the signal intensity. Films were then developed in developer solution for 5 min., washed for 5 min in water, exposed to fixative solution, washed again and dried.

The optical density of the I-kB-α or SOD-1 and SOD-2 signals were semi-quantified by an image program (Scanalytics, MWG Biotech, Germany). Controls were arbitrarily defined as 100% intensity.

Extraction buffer	Glycerol	1 ml
	SDS 10%	3 ml
	Tris 0.5 M (ph 6.8)	2.5 ml
	PMSF	1 mM
	Calpain-inhibitor	1 µM
	Trypsin-inhibitor	7 µg/ml

	Distilled water	ad 10 ml
Loading buffer 5x	Glycerol	1 ml
	SDS 10 %	3 ml
	2-mercaptoethanol	2.5 ml
	Bromphenolblue 0.05%	2.5 ml
	Tris 0.5 M pH 6.8	2.5 ml
15 % SDS-polyacrylamide gel	Glycerol 50 %	0.42 ml
	Lower Tris 4x	5 ml
	Acrylamide	10 ml
	Ammonium persulphate (APS) 10 %	75 µl
	N,N,N`,N`-tetramethyl-ethylen-diamine (TEMED)	7.5 µl
	Distilled water	4.5 ml
Lower Tris 4x pH 8.8	Tris	363.4 g
	SDS 10 %	80 ml
	Distilled water	ad 2 l
Collecting gel	Tris-HCl (0.5 M, pH 6.8)	1.25 ml
	Acrylamide	0.65 ml
	SDS 10 %	50 µl
	APS 10 %	25 µl
	TEMED	5 µl
	Distilled water	3.05 ml
Electrophoresis buffer	Tris	3 g
	Glycine	14.4 g
	SDS	1 g
	Distilled water	ad 1 l

Blotting buffer I	Tris	18.17 g
	Methanol	100 ml
	Distilled water	ad 500 ml
Blotting buffer II	Tris	1.82 g
	Methanol	100 ml
	Distilled water	ad 500 ml
Blotting buffer III	Tris	1.51 g
	Methanol	100 ml
	Amino-n-caproic acid	2.62 g
	Distilled water	ad 500 ml
Washing buffer	Tween 20	0.1 g
	PBS	ad 1 l
Blocking buffer	Tween 20	0.1 ml
	BSA	2 g
	Dry milk powder	5 g
	PBS	ad 100 ml

2.2.9 Preparation of nuclear extracts

Cells were harvested, washed twice with ice-cold TBS, centrifuged for 10 s at 10000 rpm and the pellet was re-suspended in 400 µl of cold buffer A. The cells were allowed to swell on ice for 10 min and then vortexed with 25µl of Nonidet P40. After centrifugation for 30 s at 15,000 rpm; the nuclear pellet was dissolved in 50 µl of buffer C. After vigorous rocking at 4 C^0 on a shaking platform the nuclear extract was centrifuged at 15000 rpm for 4 min and the supernatant was frozen in aliquots at –80 C^0 after analyzing the protein concentration by using the BCA kit.

TBS (Tris-buffered saline) pH 7.6	Tris HCl	1.26 g
	Tris	0.24 g
	NaCl	6.8 g
Buffer A pH 7.9	HEPES	20 mM
	EDTA	0.1 mM
	EGTA	1 mM
	KCl	10 mM
	Dithiothreitol	1 mM
	PMSF	0.5 mM
Buffer C pH 7.9	HEPES	20 mM
	Glycerol	25 %
	EDTA	1 mM
	EGTA	1 mM
	NaCl	400 mM
	Dithiothreitol	1 mM
	PMSF	1 mM

2.2.10 Electrophoretic mobility shift assay (EMSA)

EMSA was performed using a double-stranded 22 base pair oligonucleotide (5-AGTTGAGGGGACTTTCCCAGGC-3) containing the decameric NF-kB consensus sequence. Double-stranded oligonucleotides were end-labeled with -[32P]ATP (3000 Ci/mmol; Amersham) and T4 polynucleotide kinase. The whole procedure was performed within two days. On the first day, a kinase reaction was started to construct the radioactive-labeled oligonucleotide. Therefore, the oligonucleotide was incubated with kinase buffer containing ^{32}P-ATP for 45 min at 37ºC in a thermo-mixer (Eppendorf, Hamburg) and stored overnight at –20 ºC. Then, non-denaturing polyacrylamide gels (4%) of 2 mm width were prepared in a 20x20 cm electrophoresis chamber and stored overnight at –4 ºC. At the following day, for binding reaction, the nuclear extract (10 µg/5µl protein) was incubated in a total volume of 20 µl loading mix for 30 min at room temperature. DNA-protein complexes were loaded on the pre-electrophoresed polyacrylamide gels in electrophoresis buffer for 1.5 h at 200 V. The gel was removed and exposed to a whatman paper which was subsequently dried under vacuum (Gel dryer 583, Biorad, Munich; vacuum pump, Uni Equip, Munich). The whatman paper containing the radioactive signals was then exposed to a film in an incubation chamber at –20 ºC for varying times and developed using a developer.

Labeling buffer	Ligase solution 10x	5 µl
	Oligonucleotide	25 ng
	T4-Polynucleotide-kinase (PNK)	1 µl
	γ^{32}P-ATP (β-radiation)	5 µL
	Distilled water	38 µl
Polyacrylamide-gel 4% (non-denaturating)	Acrylamide 30 %	10 ml
	TBE 10 x	3.5 ml
	APS 10 %	400 µl
	TEMED	40 µl
	Distilled water	60 ml
Buffer D^+ pH 7.9	HEPES	20 mM

	Glycerin	20 %
	KCl	100 mM
	EDTA	0.5 mM
	Nonidet P40	0,25 %
	Dithiothreitol	2 mM
	PMSF	0.1 mM
Ficoll 5x	Ficoll 400	20 %
	HEPES	20 mM
	KCl	100 mM
	PMSF	0.1 mM
Loading Mix	BSA 1 %	2 µl
	dldC 1µg/ml	2 µl
	Ficoll 5x	4 µl
	Buffer D^+	2 µl
	^{32}P-labelled oligonucleotide	1 µl
	Nuclear extract (probes)	5 µl
	Distilled water	4 µl
Electrophoresis buffer	Tris	22.3 mM
	Boric acid	22.3 mM
	EDTA	0.5 mM
	Distilled water	ad 1 L

2.2.11 Quantification of mitochondrial membrane potential changes

Mitochondrial membrane potential was determined by a well established methods employing fluorescence microscopy with rhodamine 123, a cationic dye that is readily sequestered by active mitochondria depending on their transmembrane potential as described by Mattson et al. (1997). Briefly, neuronal cultures were incubated with 5 µM rhodamine 123 for 15 min and then washed 3 times with PBS. Digital video imaging of rhodamine 123 fluorescence was

conducted using a fluorescence microscope (Axiovert 100, Zeiss, Germany) with attenuated UV illumination from a 75 W xenon lamp. Fluorescence intensity was measured with an excitation wavelength of 490 nm and at an emission wavelength of 510 nm. An electronic shutter which opened during image acquisition only, minimized photobleaching and phototoxicity. Images were taken by a CCD camera (C 2400-87, Hamamatsu, Germany) and were digitalized as 256 x 256 pixels. Data were analyzed using Argus 50 software (Hamamatsu, Germany). Fluorescence intensities were given as arbitrary fluorescence units (Fl.U.).

2.2.12 Statistics

All values were given as means ± standard deviation (S.D.). For all data one way analysis of variance (ANOVA 1) with subsequent Scheffé test was employed. Data were calculated by statgraphics or winstat.

3 Results

3.1 Characterization of reactive oxygen species generation and neuronal damage by different stimuli

3.1.1 Influence of the camera sensitivity on the linearity of fluorescence microscopy measurements

For the measurement of fluorescence intensities the camera sensitivity of the fluorescence microscope could be varied. This was a necessary step to adjust the intensity of the signals and therefore facilitate comparable results. During the entire experiment the camera sensitivity was always kept at constant levels to determine relative changes in fluorescence signals. Nevertheless, it was still unclear if a linear enhancement of dye concentration was paralleled by a linear rise in measured fluorescence intensities. To clarify this point, increasing concentrations of the fluorescent dye fluoresceine were measured at different camera sensitivities in a cell-free system. This revealed that measurable fluorescence intensities could be registered from sensitivity 2 to sensitivity 5. As demonstrated by their correlation coefficients, the sensitivities revealed appropriate linear correlation with an optimum at sensitivity 4 (Fig. 12). For this reason, in all following experiments the used sensitivity ranged from 3 to 5, depending on the control intensities which were always adjusted at first and then remained constant during the entire experiment.

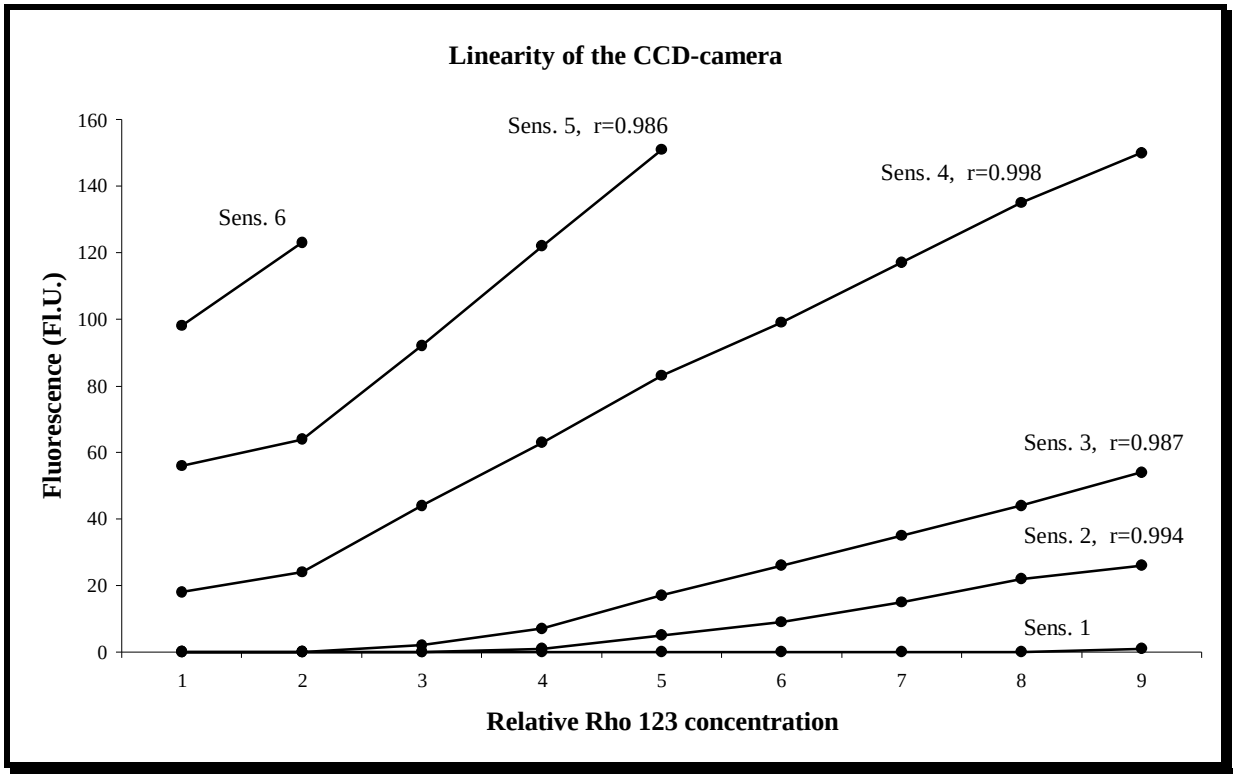
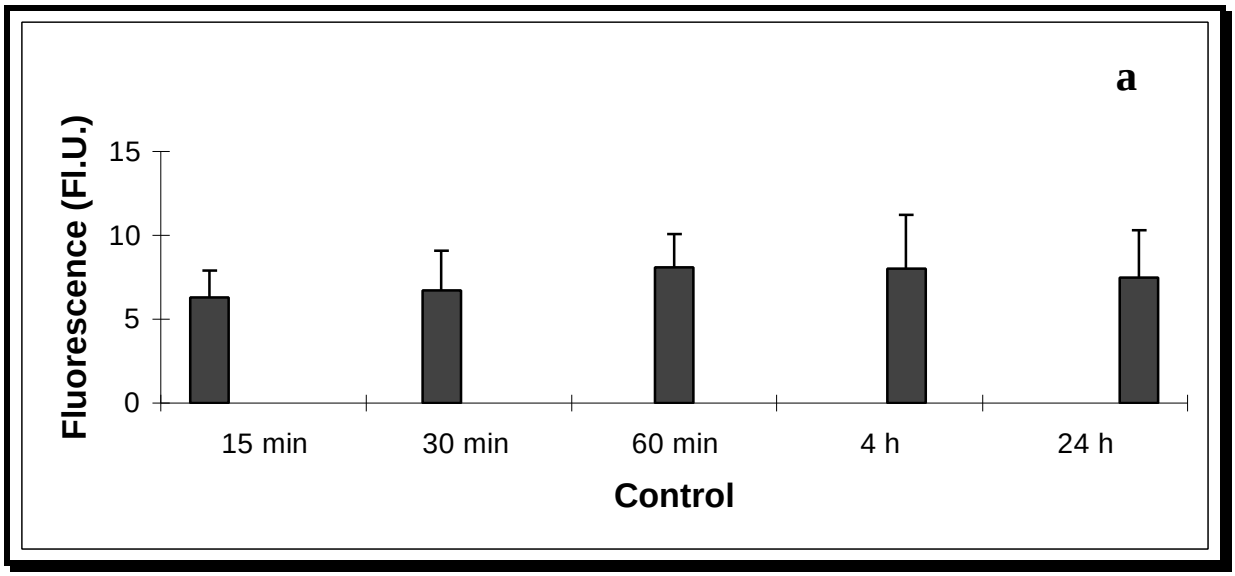
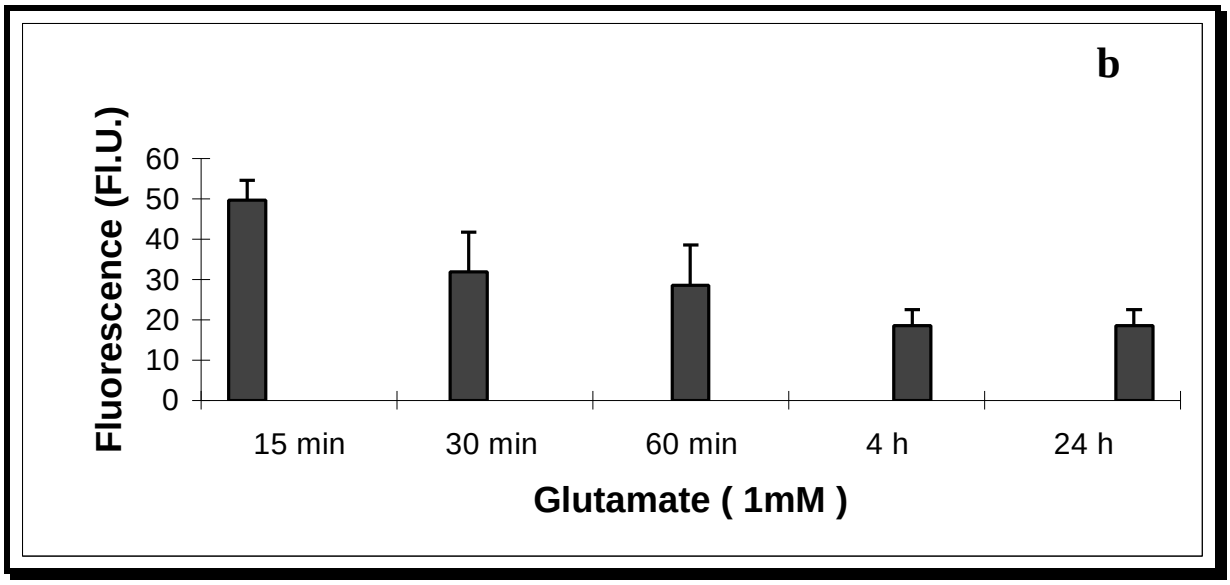


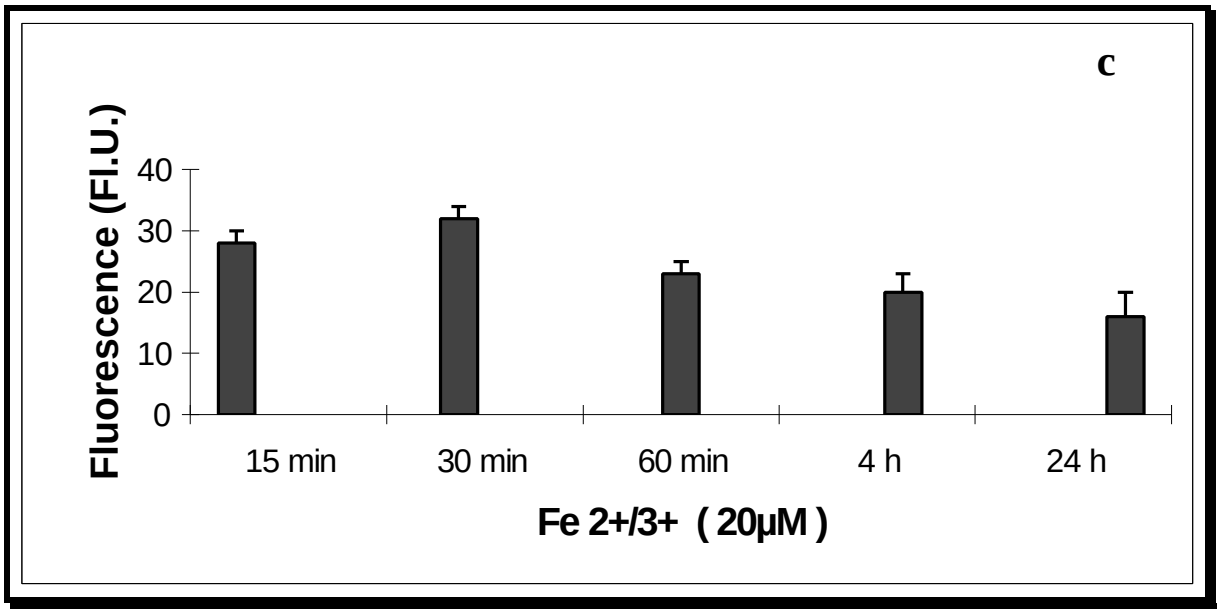
Fig. 12. Determination of camera linearity*. In a cell-free system increasing concentrations of fluoresceine in PBS were measured. Nine different concentrations were subsequently monitored at different camera sensitivities (1-6). The correlation coefficients (r) were calculated by statgraphics software.*

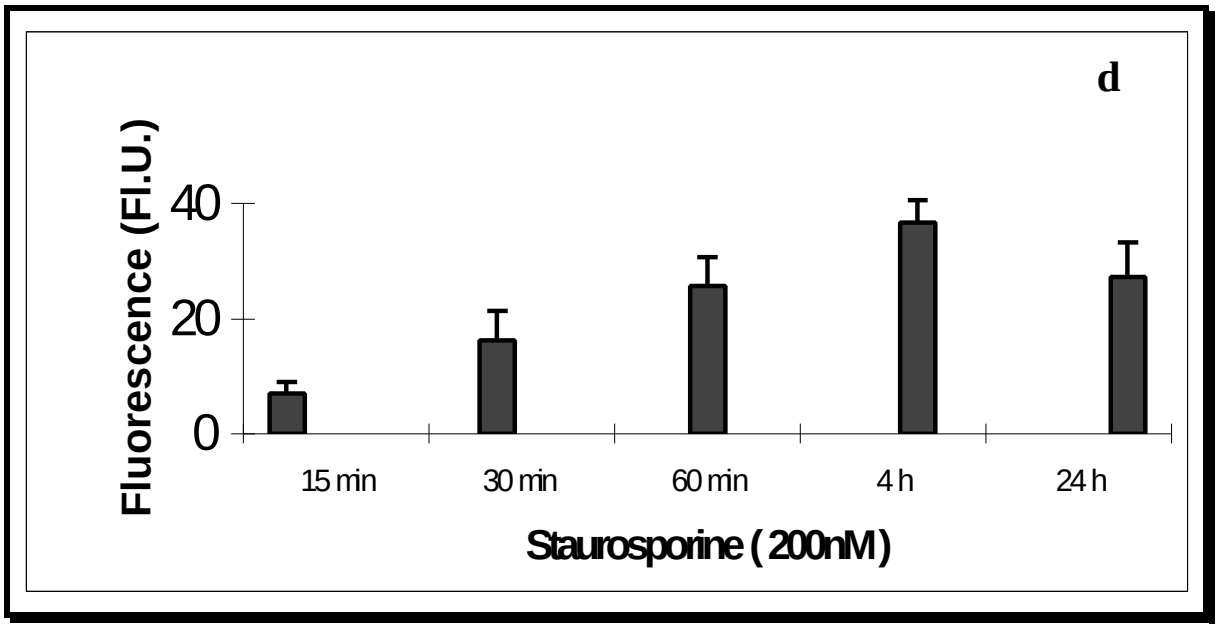
3.1.2 Time course of reactive oxygen species generation after stimulation with damaging agents

To establish a damaging model of ROS-induced neuronal apoptosis or necrosis it was necessary to determine the alterations of intracellular ROS-species content after exposure to different agents. Therefore, the time course of glutamate-, staurosporine-, $FeSO_4$- and serum deprivation-induced ROS levels was monitored for 24 h. Treatment with 200 nm staurosporine resulted in a delayed formation of ROS reaching a maximal effect after 4 h and remained up to 24 h (Fig. 13a). In contrast, glutamate and Fe^{2+} entailed a rapid, transient peak of ROS which subsided gradually until 24 h (Figs 13b and 13c). As the effect of serum-deprivation on the alteration of intracellular ROS was rather marginal (data not shown) this condition was not used to investigate the effect of drugs on ROS-induced neuronal damage. However, serum deprivation to study drug effects on neuronal apoptosis as moderate cell death was detectable after serum deprivation.


a
Fluorescence (Fl.U.)
15
10
5
0
15 min
30 min
60 min
4 h
24 h
Control


b
Fluorescence (Fl.U.)
60
50
40
30
20
10
0
15 min
30 min
60 min
4 h
24 h
Glutamate (1mM)


c
Fluorescence (FI.U.)
40
30
20
10
0
15 min
30 min
60 min
4 h
24 h
Fe 2+/3+ (20µM)


d
Fluorescence (FI.U.)
40
20
0
15 min
30 min
60 min
4 h
24 h
Staurosporine (200nM)

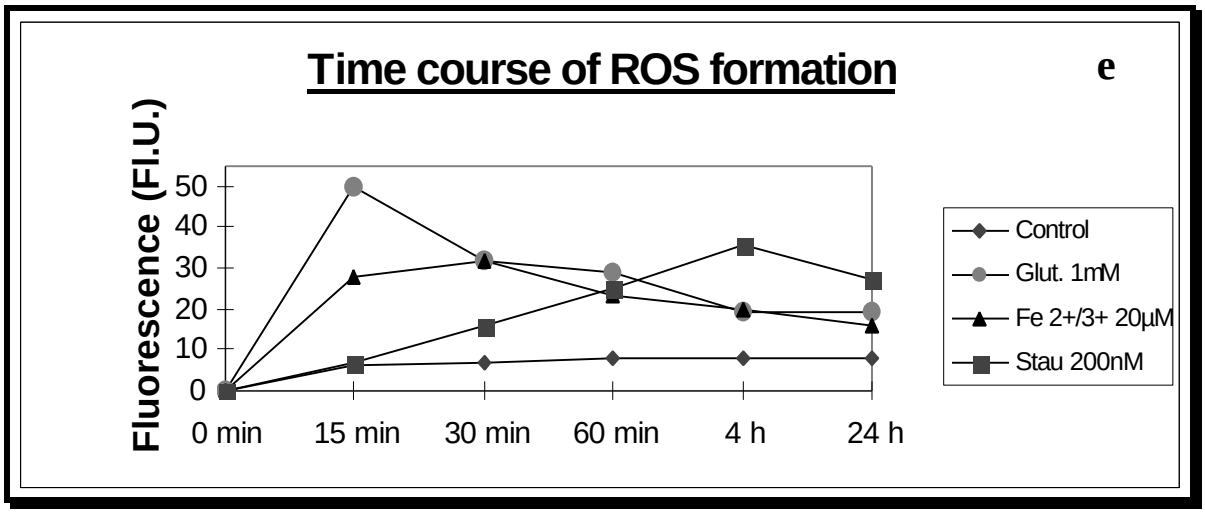


Fig. 13. Time course of reactive oxygen species formation. *The time course of (a) controls, (b) glutamate (1 mM)-, (c) $Fe^{2+/3+}$ (20 µM)- and (d) staurosporine (200 nM)-induced intracellular ROS levels in neurons from chick embryonic telencephalons was monitored for 24 h. ROS time-course correlation of all investigated agents is illustrated in (e). To determine ROS alterations, cells were incubated with 5 µM of the non–fluorescent dye dihydrorhodamine 123 for 15 min. Intracellular fluorescence intensities of the oxidized rhodamine 123 were recorded by single cell analysis and expressed as arbitrary fluorescence units (Fl.U.). Values are given as means ± S.D. for n = 5 – 7 neurons in 6 – 8 separate experiments.*

3.2 Inhibition of oxidative stress by drugs with ROS-scavenging properties

3.2.1 Effect of ACE-inhibitors

3.2.1.1 The ACE-inhibitors enalapril and moexipril protect against glutamate-induced necrosis

The protective effect of the ACE-inhibitors enalapril and moexipril against glutamate (1 mM; 1 h)-induced neurotoxicity was determined by trypan blue exclusion. In glutamate-treated cultures the percentage of trypan blue-stained neurons increased from 7.5% (controls) to 34.9%. Enalapril and moexipril significantly reduced the percentage of damaged neurons in a concentration-dependent manner (Figs. 14 and 15). Relatively high concentrations of the ACE-inhibitors used in a micromolar range were required to achieve neuroprotection. As increasing concentrations enhanced the neuroprotective efficacy it is assumable that the

observed effect was not receptor-dependent but rather due to direct chemical interaction. Enalapril or moexipril alone had no effect on neuronal viability (data not shown).

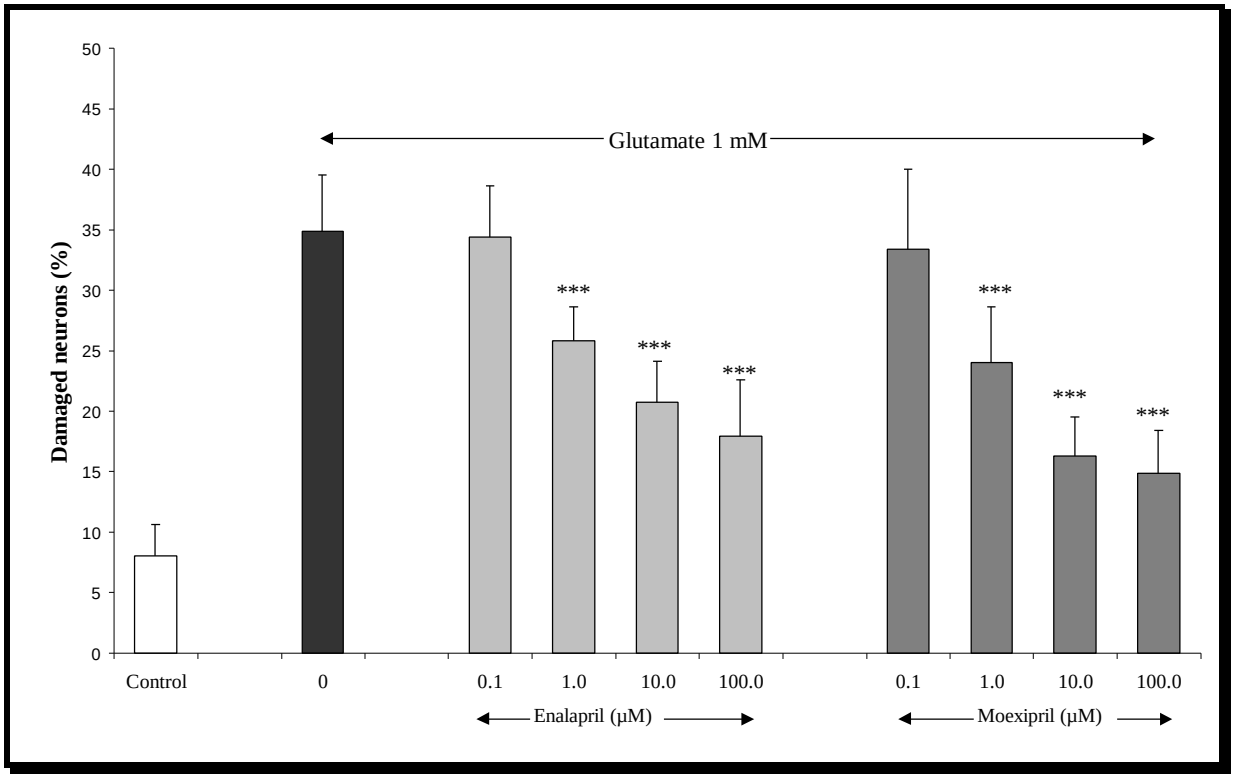
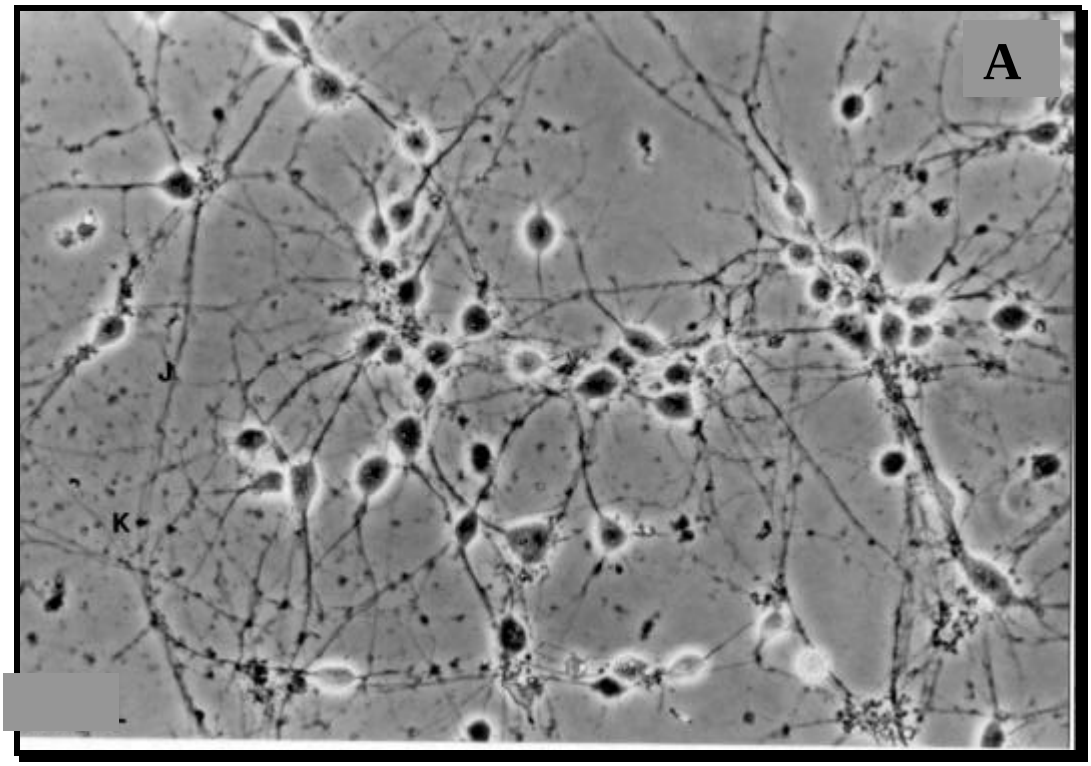
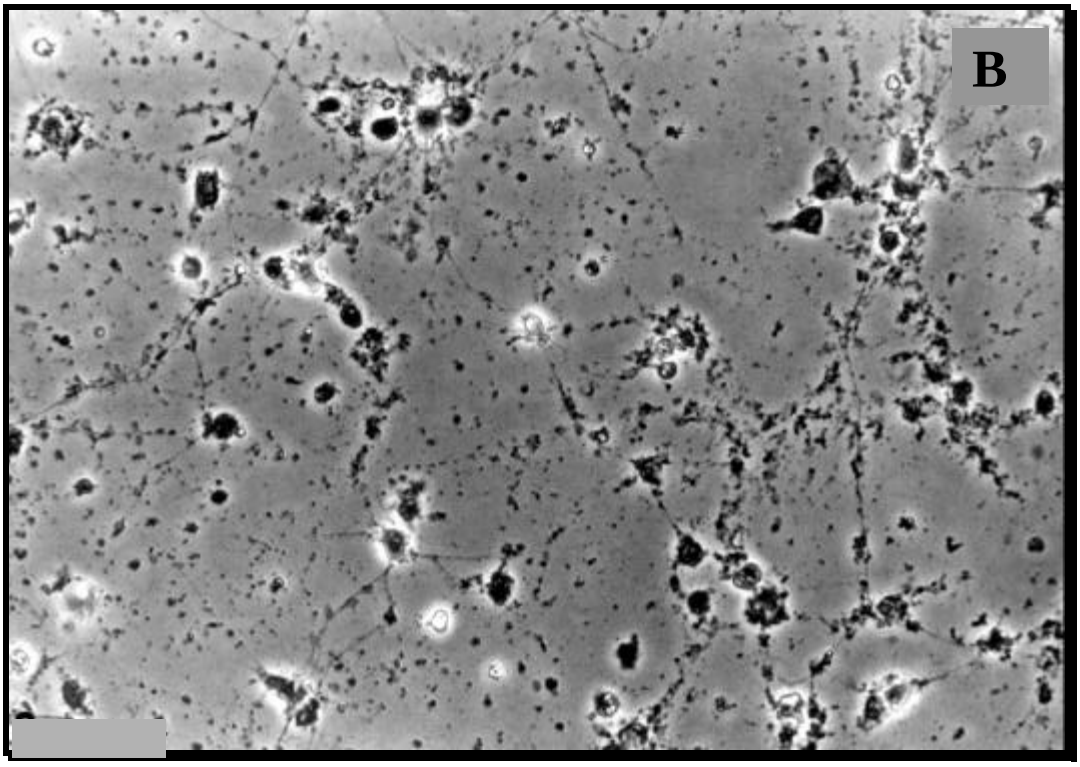


Fig. 14. Effect of enalapril and moexipril on glutamate-induced neuronal damage. *After 5 d in culture neurons from chick embryo telencephalons were treated with glutamate (1 mM; 1 h). Cellular viability was determined 24 h after glutamate treatment by trypan blue exclusion. Moexipril and enalapril were added simultaneously with glutamate and were also present 24 h after treatment. Values are given as means ± S.D. of n=8 experiments. Differences between glutamate treated cultures in the presence and absence of enalapril or moexipril: ***P<0.001 using one way analysis of variance with subsequent Scheffé test.*


A
J
K


B

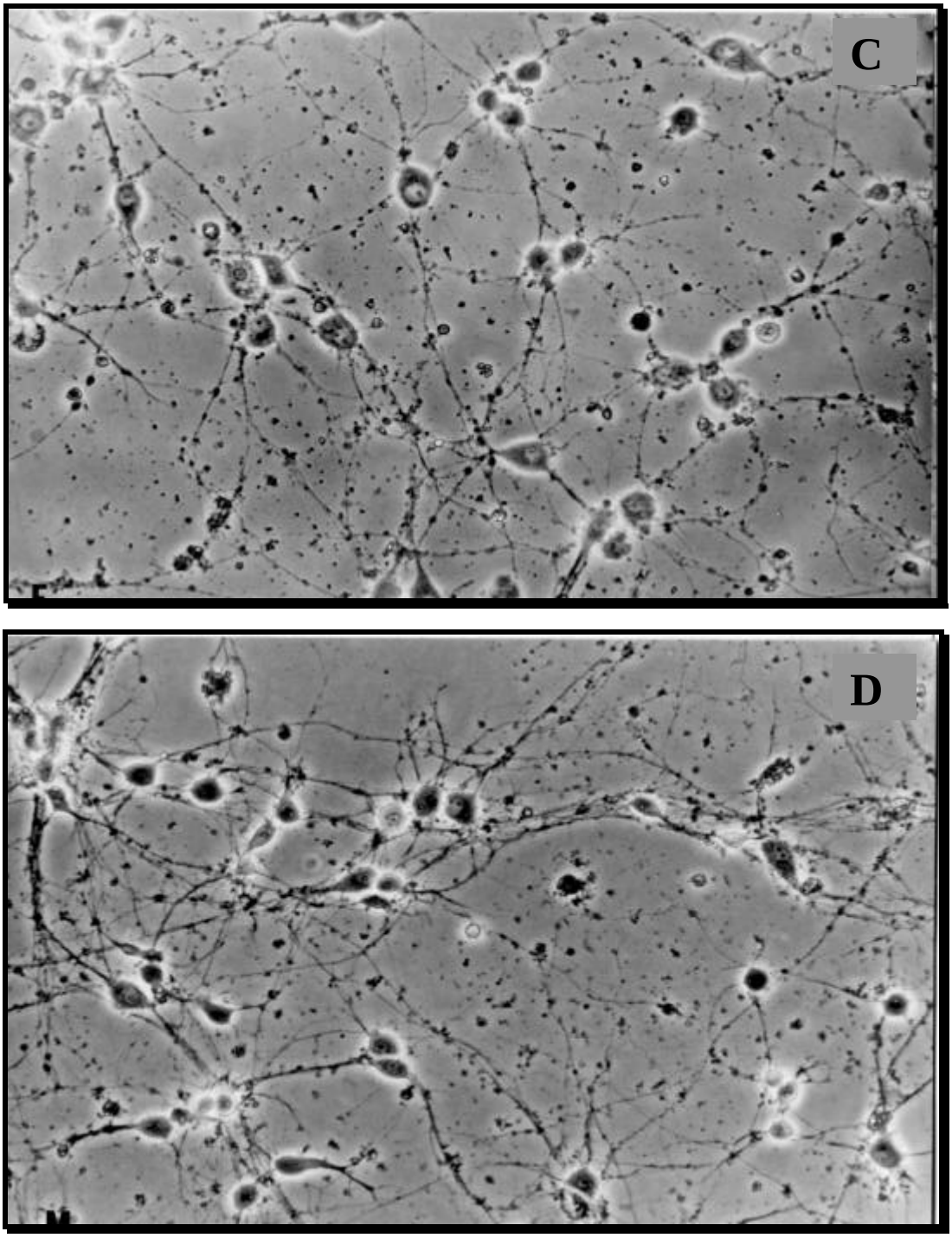


Fig. 15. Effect of enalapril and moexipril on glutamate-induced neuronal damage*. After 5 d in culture neurons from chick embryo telencephalons were treated with glutamate (1 mM; 1 h). Moexipril and enalapril were added simultaneously with glutamate and were also present 24 h after treatment. Cellular viability is demonstrated by representative phase-contrast photomicrographs showing controls (A) and cultures treated with glutamate in the absence (B) and in the presence of 10 µM moexipril (C) or 10 µM enalapril (D). Note the prevention of axonal loss, membrane disruption and cellular debris in ACE-inhibitor-treated cultures as compared with mere glutamate treatment.*

3.2.1.2 The ACE-inhibitors enalapril and moexipril reduced Fe^{2+}-mediated neuronal damage

The protective effect of enalapril and moexipril against $Fe^{2+/3+}$ -induced neurotoxicity (20 µM $FeSO_4$ and 20 µM $FeCl_3$; 24 h) was determined by trypan blue exclusion. $Fe^{2+/3+}$ exacerbated neuronal damage to a level of 25.8% trypan blue-stained neurons as compared to 5.5% in controls. $Fe^{2+/3+}$-induced neurotoxicity was significantly attenuated by enalapril or moexipril to maximally 18.2% and 16.9%, respectively (Fig. 16). Enalapril or moexipril alone had no effect on neuron viability (data not shown).

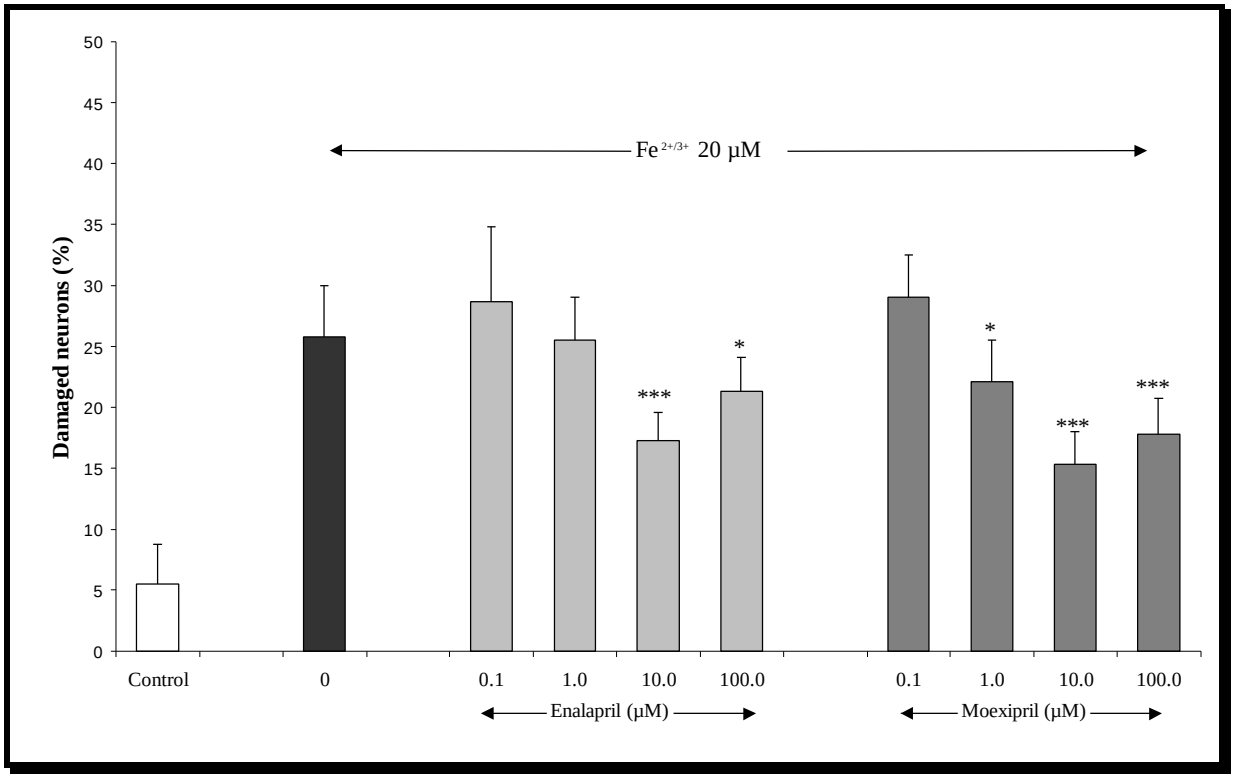


Fig. 16. Effect of enalapril and moexipril on $Fe^{2+/3}$ -induced neuronal damage*. After 5 d in culture neurons from chick embryo telencephalons were treated with $Fe^{2+/3+}$ (20 µM; 24 h). Cellular viability was determined 24 h after Fe $^{2+/3+}$-treatment by trypan blue exclusion method. Moexipril and enalapril were added simultaneously with $Fe^{2+/3+}$ and were also present 24 h after treatment. Values are given as means ± S.D. of n=8 experiments. Differences between $Fe^{2+/3+}$-treated cultures in the presence and absence of enalapril or moexipril: *P<0.05; ***P<0.001 using one way analysis of variance with subsequent Scheffé test.*

3.2.1.3 The ACE-inhibitors enalapril and moexipril attenuated neuronal apoptosis caused by staurosporine or by serum deprivation

To investigate the influence of enalapril and moexipril on neuronal apoptosis, the cultures were treated with 200 nM staurosporine for 24 h. Staurosporine markedly increased the percentage of neurons with apoptotic features from 12.2% in controls to 61.4%. Enalapril and moexipril did not cause significant changes in the percentage of apoptotic neurons when added under control conditions (data not shown) but exerted a concentration-dependent anti-apoptotic effect when administered simultaneously with staurosporine (Figs .17 and 18). Already 0.1 µM of moexipril was sufficient to achieve significant protection whereas 1 µM enalapril was minimally required.

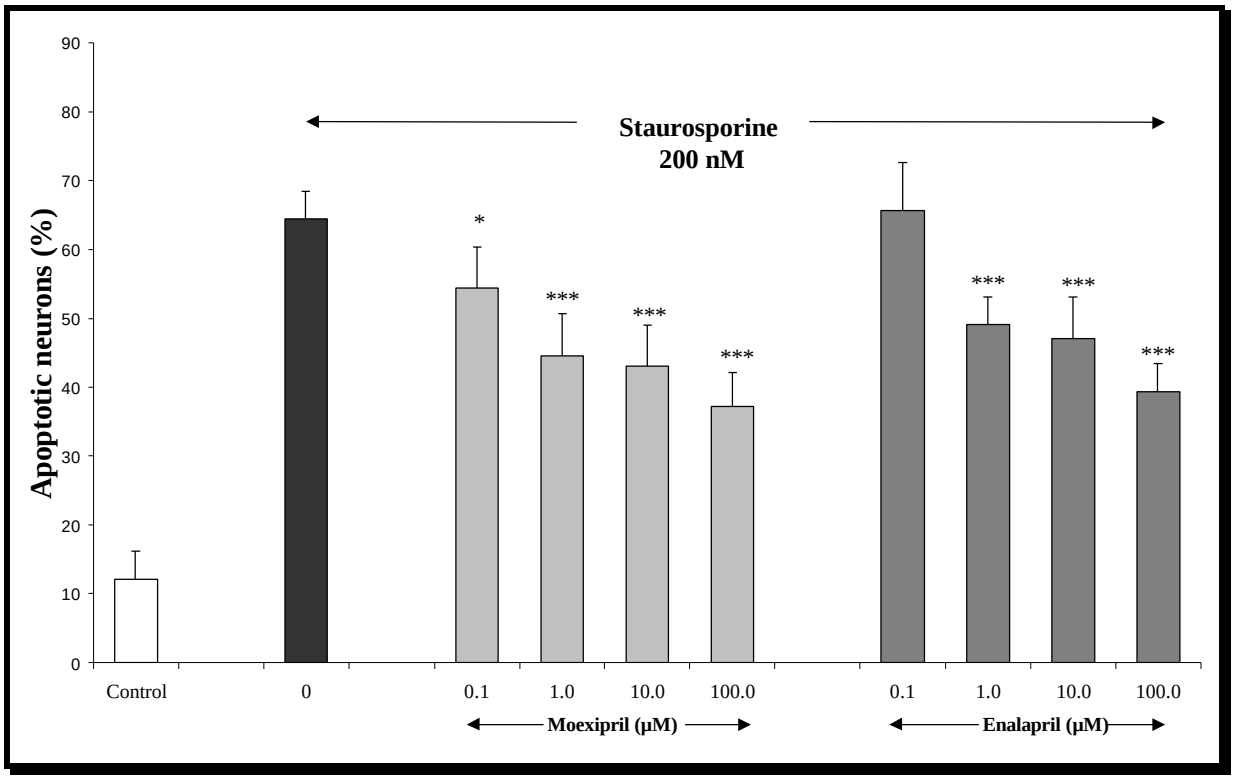
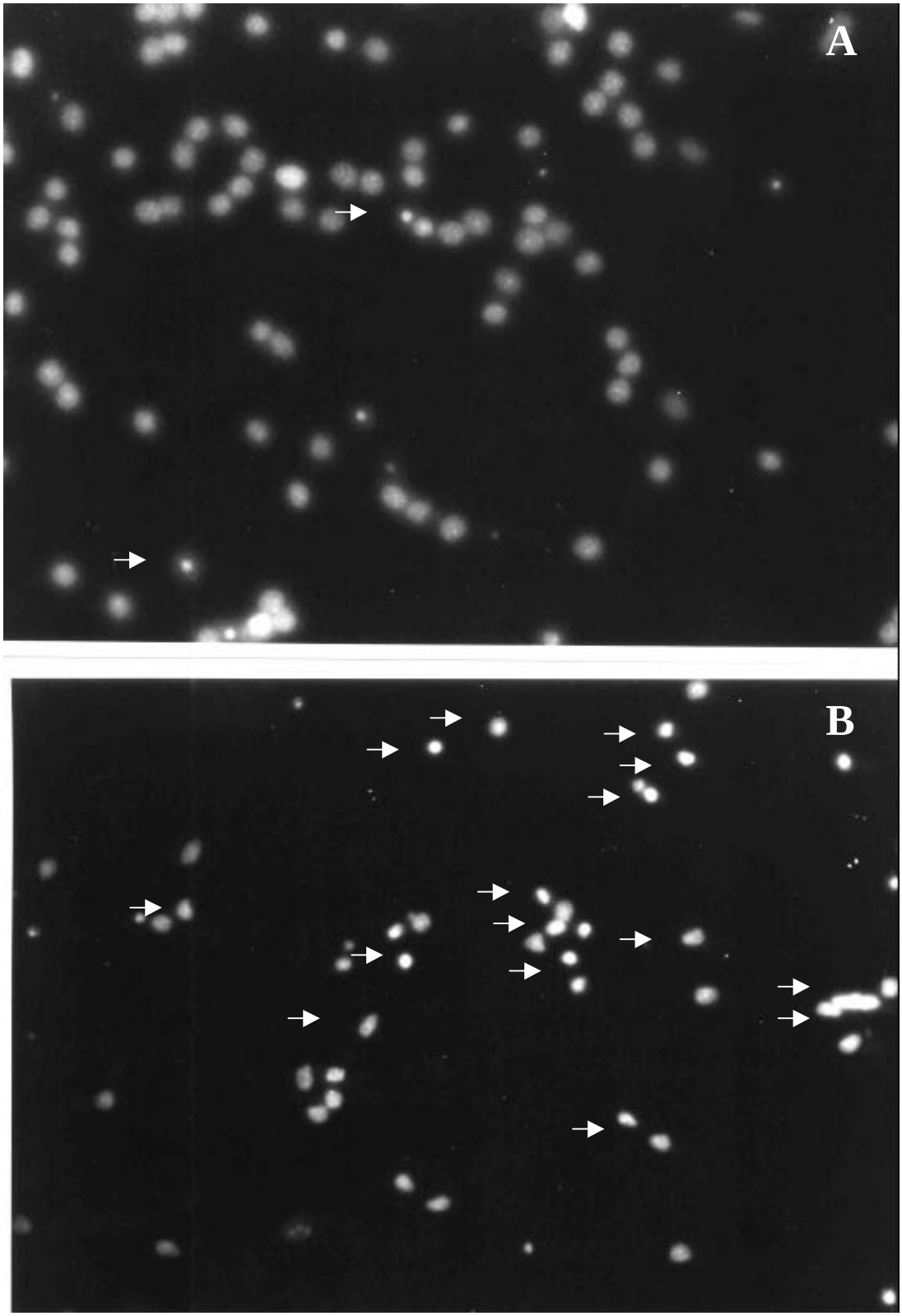


Fig. 17. Effect of enalapril and moexipril on staurosporine-induced neuronal damage. *After 5 d in culture neurons from chick embryo telencephalons were treated with staurosporine (200 nm; 24 h). Neuronal apoptosis was identified by nuclear staining with Hoechst 33258, 24 h after staurosporine treatment. Moexipril and enalapril were added simultaneously with staurosporine and were also present 24 h after treatment. Values are given as means ± S.D. of n=8 experiments. Differences between staurosporine treated cultures in the presence and absence of enalapril or moexipril: *P<0.05; ***P<0.001 using one way analysis of variance with subsequent Scheffé test.*


A
B

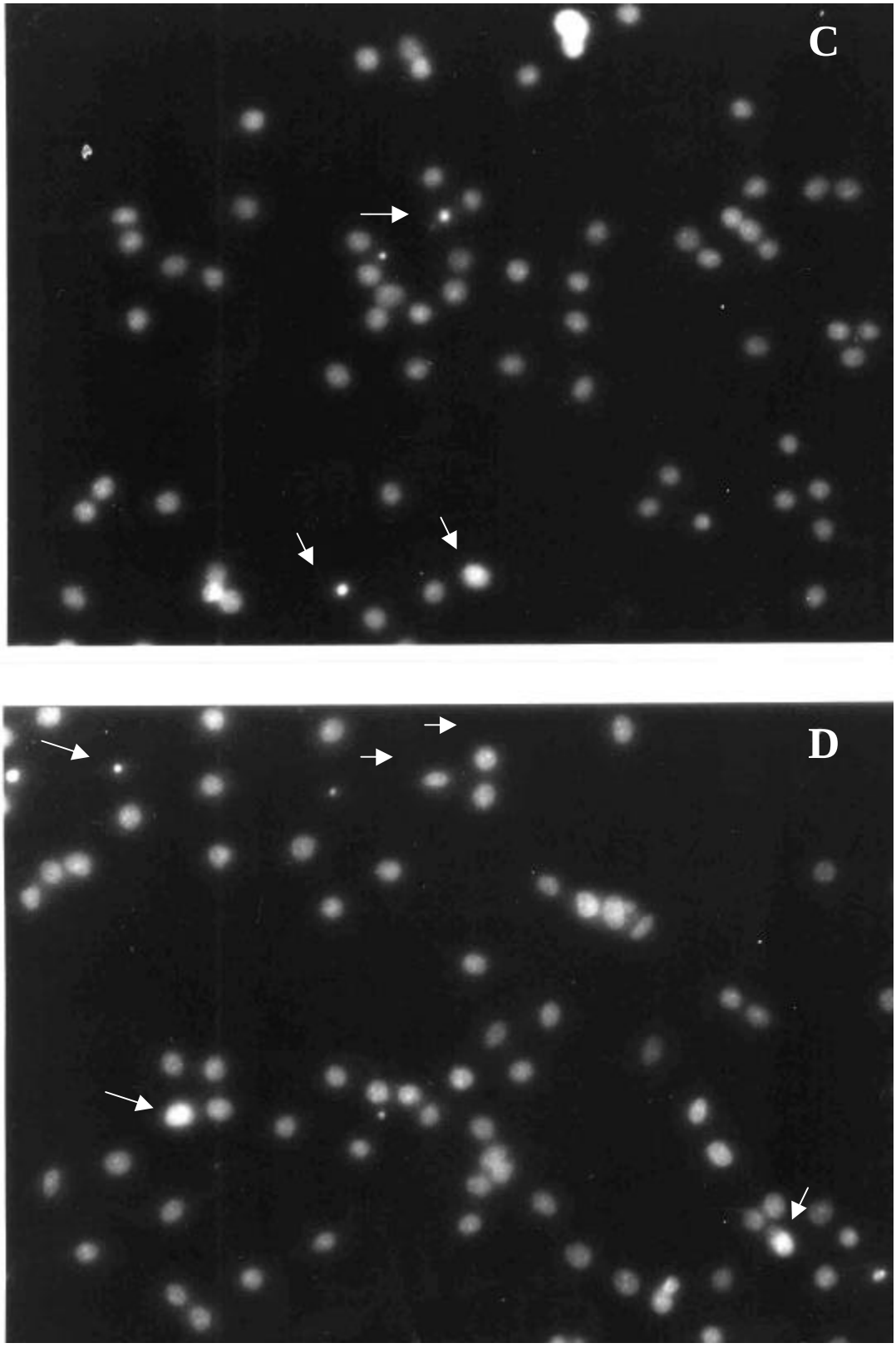


Fig. 18. Nuclear staining of chick embryonic neurons with Hoechst 33258. *Representative photomicrographs showing controls (A), staurosporine-treated cultures (B) and staurosporine-treated cultures in the presence of 10 µM enalapril (C) or 10 µM moexipril (D) are demonstrated. Reduced*

nuclear size, chromatin condensation (visible as an intense fluorescence) and DNA-fragmentation are characteristics of apoptosis

Serum deprivation for 24 h produced a rather mild apoptotic cell death as compared to staurosporine-exposure. Serum-deprivation increased the number of apoptotic neurons to 32%. Concomitant treatment with enalapril or moexipril effectively reduced neuronal cell death at concentrations of 10 µM or 100 µM (Fig. 19). The effect was comparable to vitamin E (10 µM), a potent antioxidant that was used as a positive control. The NMDA-antagonist MK 801 that was also applied as positive control had no significant effect on serum deprivation-induced neuronal apoptosis.

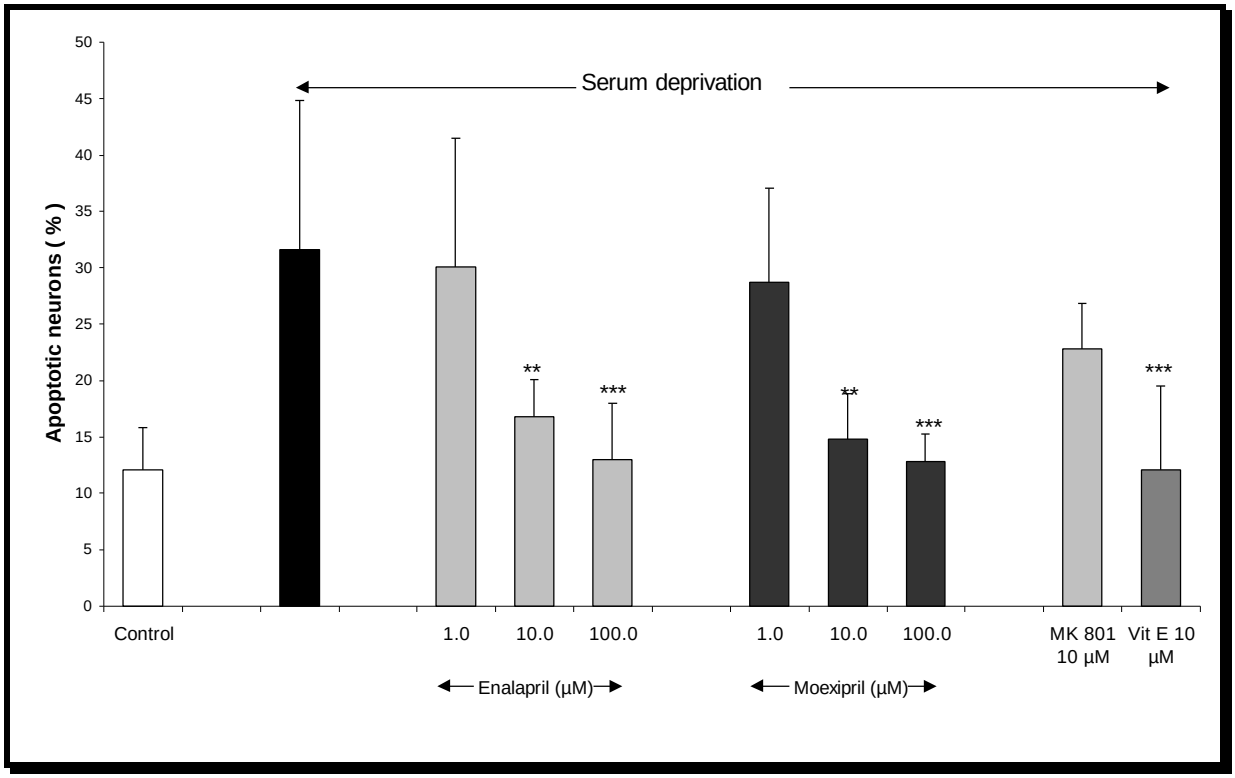


Fig. 19. Effect of enalapril and moexipril on serum deprivation-induced neuronal damage. *After 5 d in culture the serum was removed from neuronal cultures by medium exchange. Neuronal apoptosis was identified by nuclear staining with Hoechst 33258, 24 h after serum deprivation. Moexipril and enalapril were added immediately after serum removal. Values are given as means ± S.D. of n=8 experiments. Differences between staurosporine-treated cultures in the presence and absence of enalapril or moexipril: **P<0.01; ***P<0.001 using one way analysis of variance with subsequent Scheffé test.*

3.2.1.4 Neuronal reactive oxygen species burst was prevented by enalapril and moexipril

To monitor the formation of ROS a fluorescence microscope combined with a digital video imaging system was used which allowed measurement of ROS-fluorescence within single neurons. After exposing the cultures to $Fe^{2+/3+}$ (20 µM $FeSO_4$ and 20 µM $FeCl_3$) for 3 h there was an increase in fluorescence from 10 Fl.U. in controls to 57 Fl.U. Addition of enalapril or moexipril decreased the $Fe^{2+/3+}$-induced elevation of mitochondrial fluorescence to maximally 19 Fl.U. and 29 Fl.U., respectively (Table 1 and Fig. 20). Similar radical scavenging effects could be observed when cultures were exposed to 1 mM glutamate for 1 h or 200 nM staurosporine for 3 h which resulted in a burst of oxygen radical generation in each case. Again, enalapril as well as moexipril significantly reduced ROS-fluorescence (Table 1)

			Enalapril (µM)			
Treatment	Control	0	0.1	1.0	10.0	100.0
			Fluorescence (Fl.U.)			
$Fe^{2+/3+}$ (20 µM; 3 h)	10 ± 2	57 ± 11	45 ± 5	34 ± 7[b]	38 ± 9[c]	19 ± 3[c]
Glutamate (1mM; 1 h)	5 ± 1	36 ± 7	32 ± 5	25 ± 4[b]	21 ± 7[b]	6 ± 3[c]
Staurosporine (200 nM; 3 h)	7 ± 1	40 ± 4	37 ± 2	26 ± 4[b]	19 ± 3[c]	17 ± 2[c]

			Moexipril (µM)			
Treatment	Control	0	0.1	1.0	10.0	100.0
			Fluorescence (Fl.U.)			
$Fe^{2+/3+}$ (20 µM; 3 h)	10 ± 2	57 ± 11	45 ± 9	29 ± 6[c]	30 ± 5[c]	30 ± 7[c]
Glutamate (1mM; 1 h)	5 ± 1	36 ± 7	32 ± 5	23 ± 3[c]	15 ± 5[c]	10 ± 2[c]
Staurosporine (200 nM; 3 h)	7 ± 1	40 ± 4	39 ± 5	27 ± 4[b]	17 ± 3[c]	15 ± 3[c]

Table 1 Effect of the ACE-inhibitors enalapril and moexipril on $Fe^{2+/3+}$-, glutamate- or staurosporine-induced formation of reactive oxygen species*. Five days after seeding primary cultures of chick neurons were treated with $Fe^{2+/3+}$(20 µM; 3 h), glutamate (1 mM; 1 h) or staurosporine (200 nM; 3 h). The ACE-inhibitors enalapril and moexipril were added simultaneously.*

To determine the formation of ROS, cells were incubated with 5 µM of the non–fluorescent dye dihydrorhodamine 123 for 15 min. Fluorescent intensities of the oxidized rhodamine 123 are expressed as arbitrary units (Fl.U.). Values are given as means ± S.D. for n = 5 – 7 neurons in 6 – 8 separate experiments. Differences between $Fe^{2+/3+}$, glutamate or staurosporine treated cultures in the presence and absence of enalapril or moexipril: $^{b}P < 0.01$, $^{c}P < 0.001$.

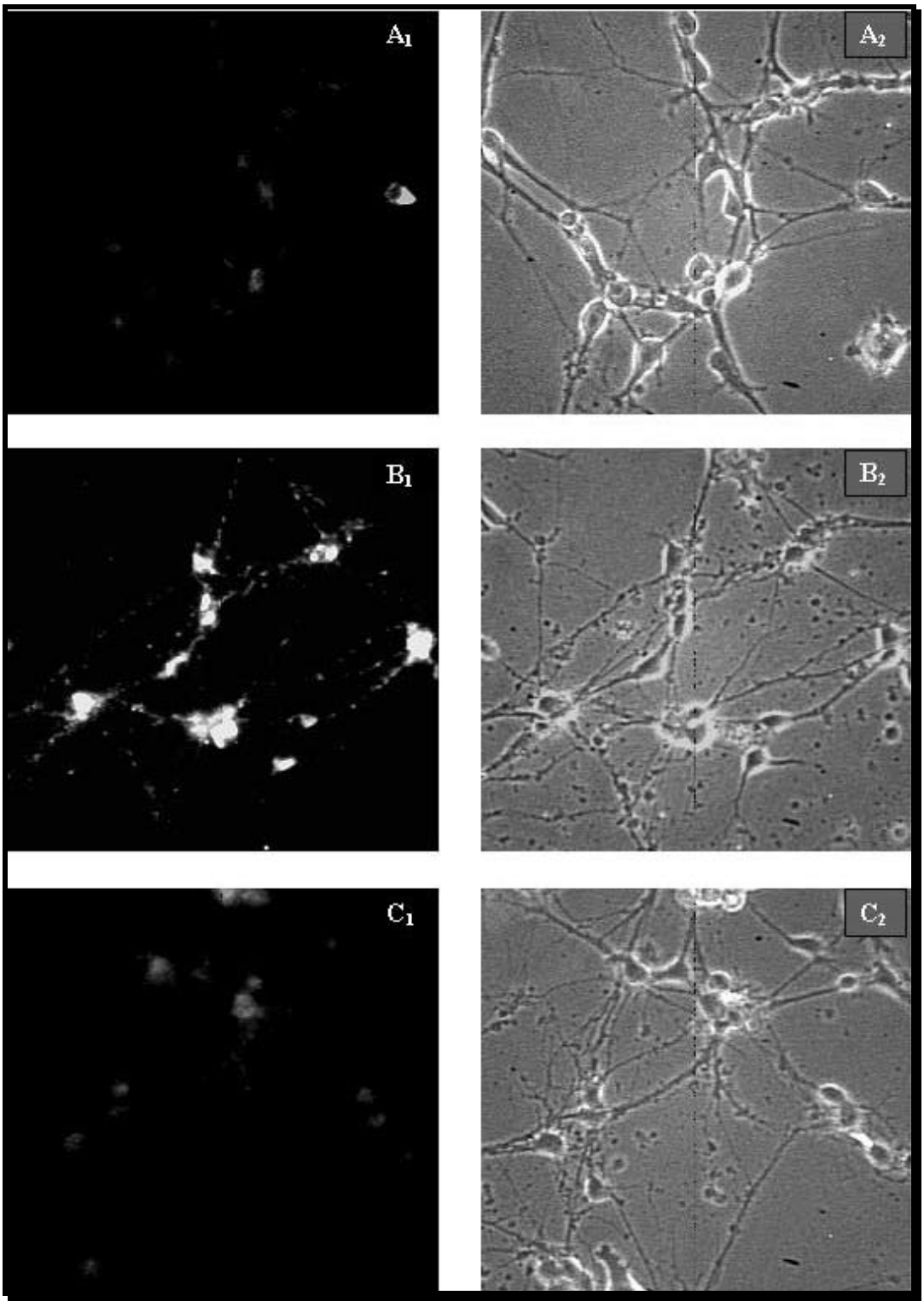


Fig. 20. Effect of the ACE-inhibitors enalapril and moexipril on $Fe^{2+/3+}$-induced formation ROS. *Five days after seeding primary neuronal cultures were treated with 20 µM $Fe^{2+/3+}$ for 3 h. Enalapril and moexipril were added simultaneously with $Fe^{2+/3+}$. Representative rhodamine 123 fluorescence microscopy images of controls (A_1) and cultures treated with 20 µM $Fe^{2+/3+}$ in the absence (B_1) and in the presence of 10 µM enalapril (C_1) or 10 µM moexipril (D_1) are demonstrated. Correlating phase-bright images are shown in A_2, B_2, C_2 and D_2.*

3.2.2 Effect of estrogens

3.2.2.1 The estrogens 17-ß-estradiol and 2-OH-estradiol reduced $FeSO_4$-mediated neuronal damage

Primary neuronal cultures from embryonic chick telencephalons were damaged by an incubation with 100 µM $FeSO_4$ for 24 h to a level of 42.8 ± 4.0% trypan blue-stained neurons as compared to 13.3 ± 3.9% in control cultures. Neurotoxicity, induced by Fe^{2+}, was significantly attenuated by 17ß-estradiol (1-10 µM) (Fig. 21a). In a second series of experiments, concentrations of 0.01–1 µM 2-OH-estradiol reduced the percentage of damaged neurons from 43% in Fe^{2+}-treated cultures to maximally 22%. In contrast, concentrations of 100 µM 17ß-estradiol showed no neuroprotective effect and 10 µM 2-OH-estradiol markedly enhanced the Fe^{2+}-induced neuronal damage (Figs. 21a, 21b). 17ß-estradiol (0.001-100 µM) and 2-OH-estradiol (0.001-1 µM) alone did not influence neuronal viability while 2-OH-estradiol concentrations of 10 µM and higher markedly enhanced the percentage of trypan blue-stained neurons.

3.2.2.2 Receptor-independent neuroprotective effect of estrogens

The unspecific estrogen-receptor antagonist tamoxifen (1 µM) added simultaneously with the estrogens did not block the effects of 17ß-estradiol and 2-OH-estradiol against neuronal damage (Figs. 21a, 21b). Tamoxifen (1 µM) alone had no influence neuronal viability.

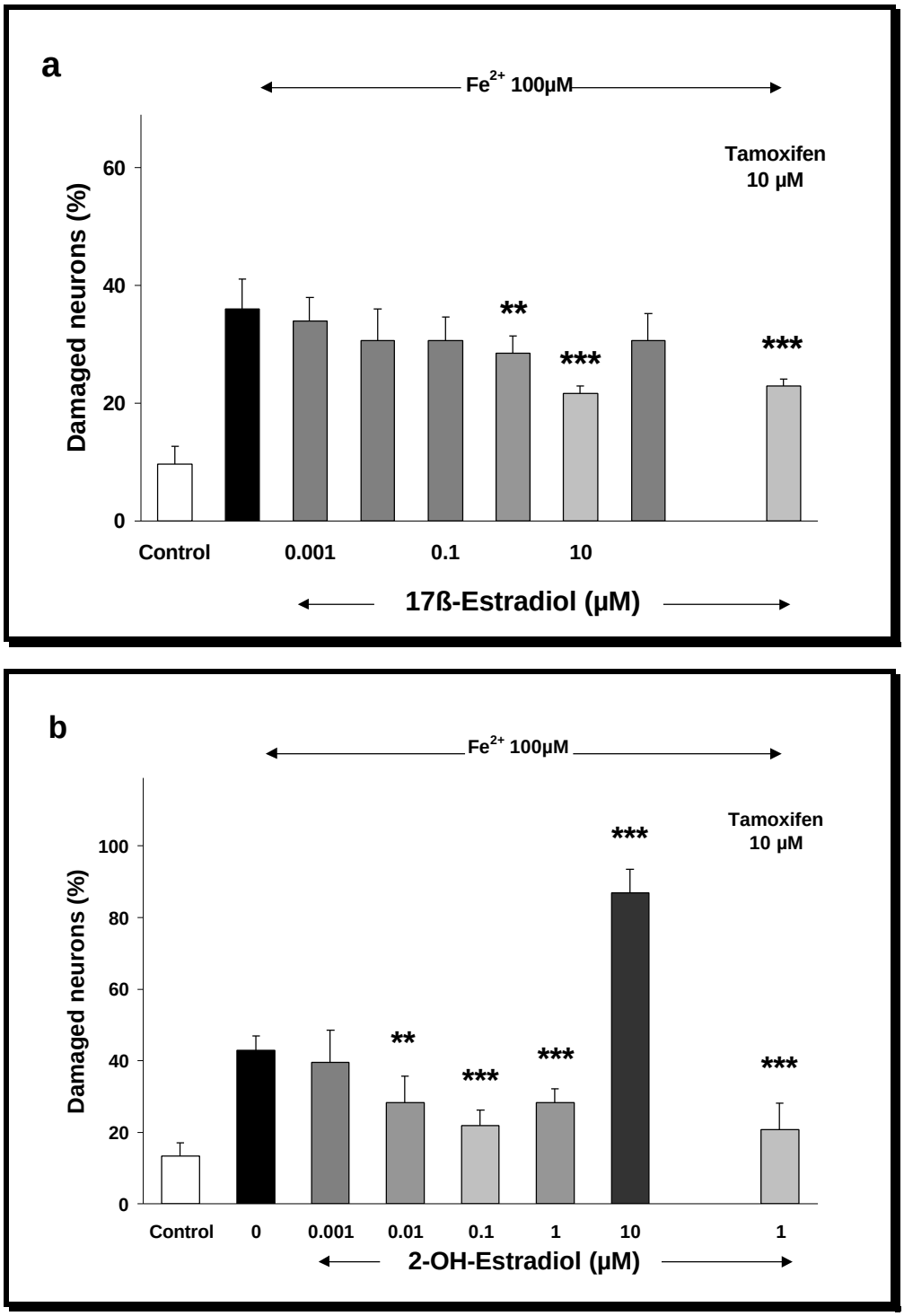
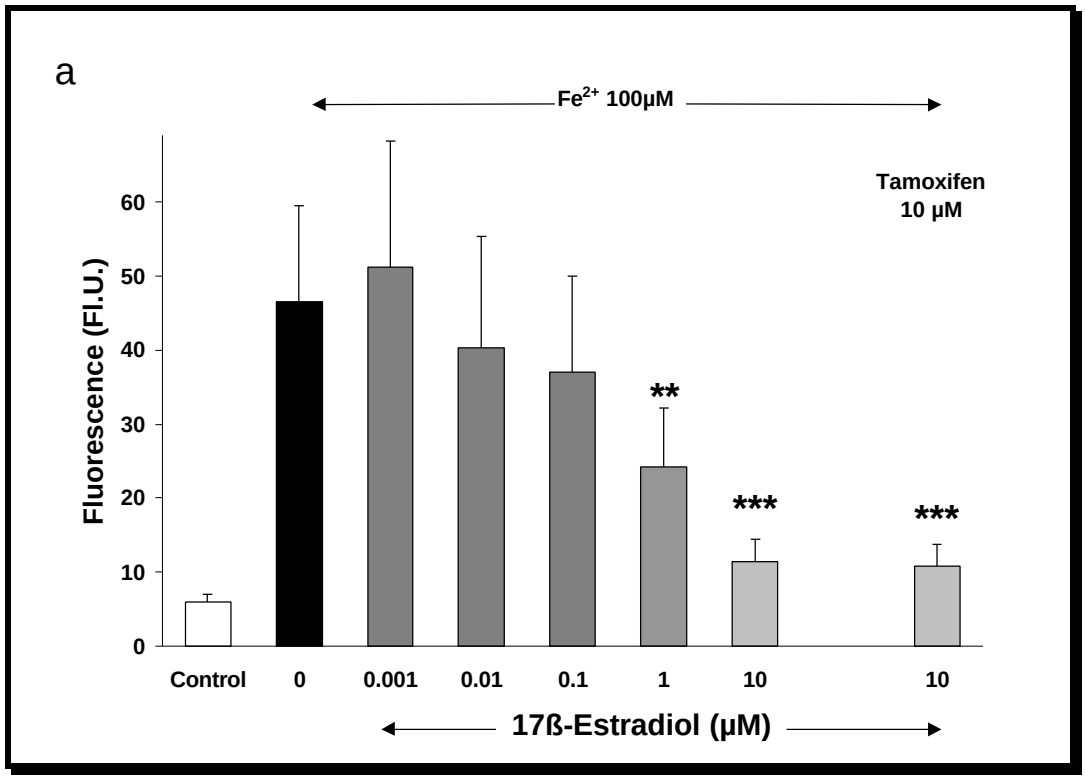


Fig. 21. Effect of the estrogens 17ß-estradiol and 2-OH-estradiol on Fe^{2+}-induced neuronal damage. *Chick embryonic neurons were treated with 100 µM $FeSO_4$. Cellular viability was determined 24 h after Fe^{2+}-treatment by trypan blue exclusion method. 17ß-estradiol (a) and 2-OH-estradiol (b) were added simultaneously with Fe^{2+}. Values are given as means ± S.D. of 8 experiments. Different from Fe^{2+}-treated cultures: **P<0.01; ***P<0.001 using one way analysis of variance with subsequent Scheffé test.*

3.2.2.3 Fe^{2+}-induced elevation of intracellular ROS content was reduced by estrogens

ROS content was quantified using intraneuronal fluorescence microscopy. After exposing the cultures to 100 µM Fe^{2+} for 3 h there was an increase in fluorescence from 6 Fl.U. in controls to 47 Fl.U. (Fig. 22a). Both, 17ß-estradiol and 2-OH-estradiol decreased the Fe^{2+}-induced ROS-fluorescence in a concentration-dependent manner. The lowest concentration of 17ß-estradiol required to significantly diminish ROS-fluorescence was 1 µM (Figs. 22a and 23). A significant reduction of ROS-fluorescence by 2-OH-estradiol was achieved already at 0.01 µM (Figs. 22b and 23). Tamoxifen (1 µM) did not block the effect of the estrogens on the production of ROS after Fe^{2+}-treatment. Tamoxifen (1 µM), 17ß-estradiol (0.001-10 µM) or 2-OH-estradiol (0.001-10 µM) alone did not affect ROS-fluorescence as compared to controls (data not shown).



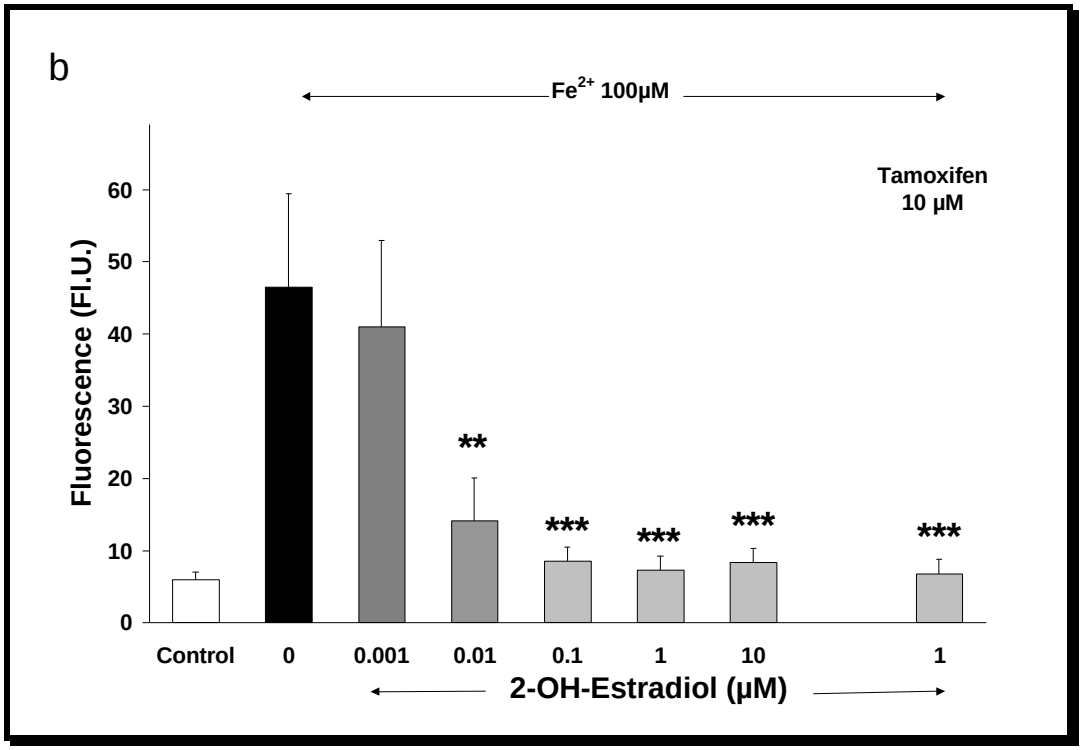


Fig. 22. Effect of 17ß-estradiol and 2-OH-estradiol on Fe^{2+}-induced elevation of reactive oxygen species*. Chick embryonic neurons were treated with Fe^{2+} (100 µM; 3 h). 17ß-estradiol (**a**) and 2-OH-estradiol (**b**) were added simultaneously with Fe^{2+}. ROS were determined by intracellular fluorescence microscopy. Values are given as means ± S.D. for n = 5 – 7 neurons in 6 – 8 separate experiments. Statistics were performed by one-way analysis of variance with subsequent Scheffé test. Differences between Fe^{2+} treated cultures in absence and presence of 17ß-estradiol or 2-OH-estradiol: $^{**}P < 0.01$, $^{***}P < 0.001$.*

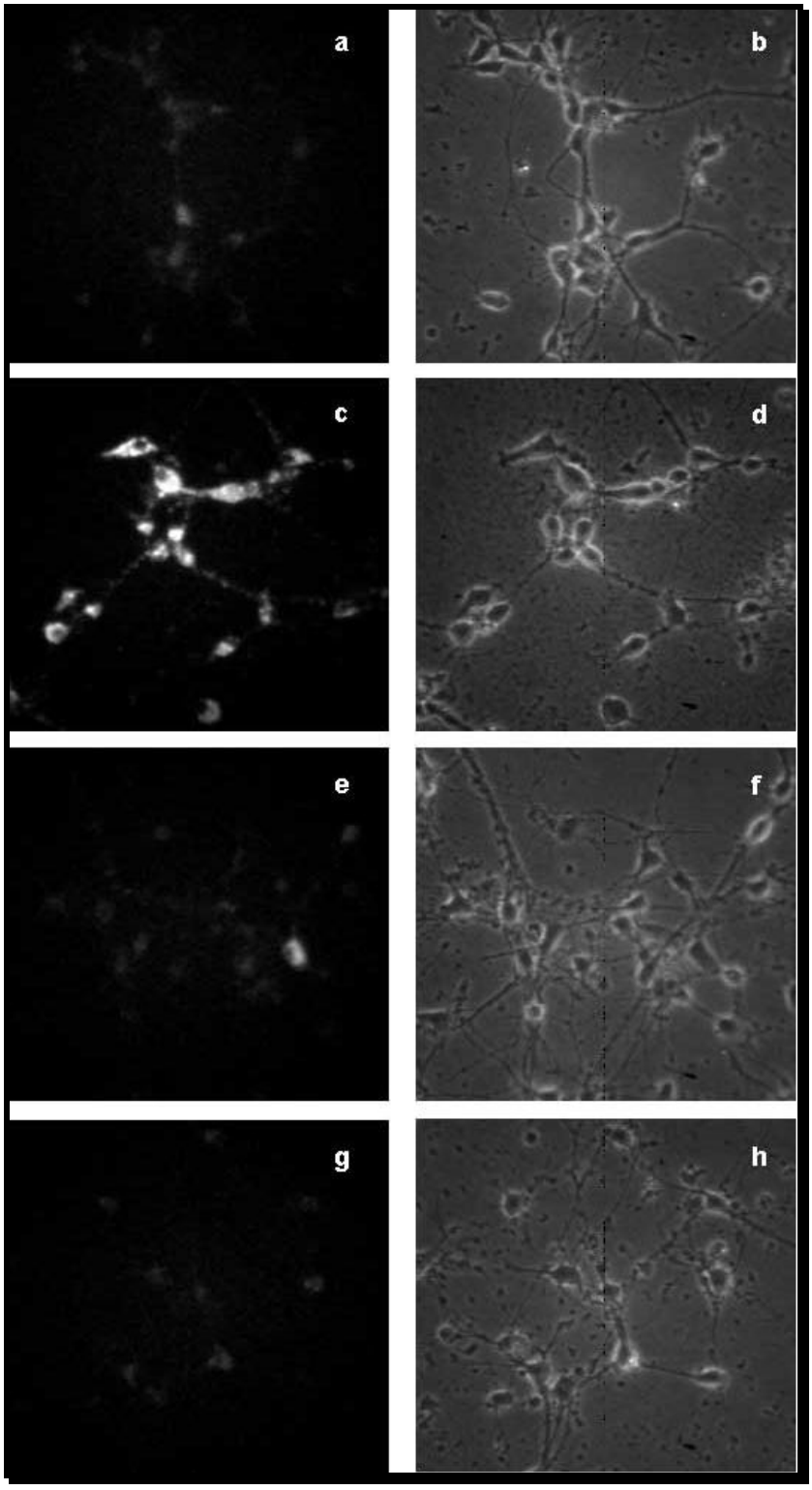


Fig. 23. Effect of 17ß-estradiol and 2-OH-estradiol on Fe^{2+}-induced enhancement of reactive oxygen species. *Representative photomicrographs show rhodamine 123 fluorescence of controls (**a**) and cultures treated with Fe^{2+} in the absence (**c**) and in the presence of 1 µM 17ß-estradiol (**e**) or 0.01 µM 2-OH-estradiol (**g**) are demonstrated. Correlating transmission-light images are shown in **b, d, f** and **h**.*

3.3 Reactive oxygen species as mediators of neuroprotection and signal transduction

3.3.1 Characterization of mild ROS stimulation by xanthine/xanthine oxidase or $FeSO_4$

3.3.1.1 Concentration- and time-dependency of xanthine/ xanthine oxidase- and Fe^{2+}-induced ROS generation

Although high amounts of oxygen radicals have been shown to exert severe neurotoxicity in the investigated cell culture models there has been evidence that moderate amounts of ROS could have beneficial or indispensable regulatory effects on neurons. Two treatment models were established to investigate the effect of mild ROS-stimulation on neuronal function, integrity and ROS-induced intracellular response. One model was the concomitant treatment of xanthine with xanthine oxidase (X/XO) converting xanthine to uric acid. This enzymatic reaction is accompanied by the generation of superoxide anion radicals ($O_2^{\cdot-}$). The other agent was ferrous sulfate (Fe^{2+}) which was known to stimulate predominantly the generation of hydroxyl-radicals ($OH^{\cdot}$) in the presence of metal cations (Fenton`s reaction). To distinguish between the effect of severe ROS-stimulation and moderate exogenous ROS-generation on neuronal functions it was primarily necessary to determine the dose-response-curve of X/XO and Fe^{2+}-induced ROS formation. Treatment of cultured neurons with X/XO for 15 min revealed that a concentration of X/XO (10µM / 0.1 mU x ml^{-1}) did not rise ROS whereas X/XO (10 µM/0.5 mU x ml^{-1}) entailed a mild enhancement of ROS content from 18 Fl.U. in controls to 36 Fl.U. (Fig.24). Higher concentrations of X/XO (500µM / 5 mU x ml^{-1}) produced a marked ROS-burst which was four times higher than control level. In the following experiments 15 min treatment with X/XO (10 µM/0.5 mU x ml^{-1}) = X/XO (pre) was used to precondition cells with a mild superoxide anion radical-stimulus.

For mild ROS-stimulation with $FeSO_4$ a similar time-dependency was observed. As marked ROS accumulation was already found after 15 min with 100 µM $FeSO_4$ (fluorescence. raised to 24 Fl.U. as compared to 7 Fl.U. in controls) this window was used to treat the cells with moderate amounts of hydroxyl-radicals (data not shown).

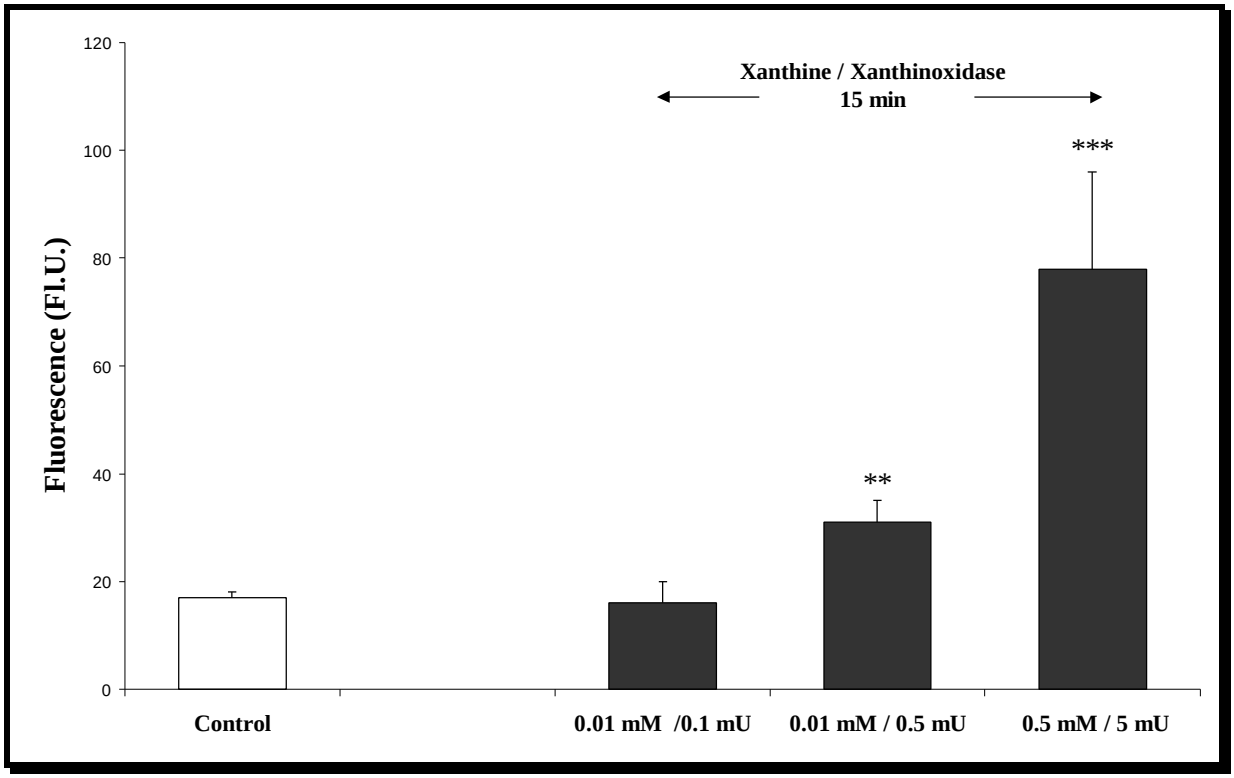


Fig. 24. Dose-dependency of xanthine/xanthine oxidase (X/XO)-mediated stimulation of reactive oxygen species. *Primary cultures of chick neurons were simultaneously exposed to increasing concentrations of X/XO (10 µM /0.1 mU x ml^{-1}; 10 µM /0.5 mU x ml^{-1}; 500 µM /5 mU x ml^{-1}). To determine reactive oxygen species, cells were incubated with 5 µM of the non–fluorescent dye dihydrorhodamine 123 for 15 min. Fluorescent intensities of the oxidized rhodamine 123 are expressed as arbitrary units (Fl.U.). Values are given as means ± S.D. for n = 5 – 7 neurons in 6 – 8 separate experiments. Differences between X/XO-treated cultures and controls: $^{**}P < 0.01$, $^{***}P < 0.001$.*

To further define the ROS-mediated neuronal damage by severe ROS-administration, the strong ROS-elevating concentration of X/XO (500µM / 5 mU x ml^{-1}) was also investigated for time-dependent ROS-response. Neuronal ROS-content markedly increased up to 1h after exposure but declined after 4 h (Fig. 25). Therefore, 1 h treatment with X/XO (500µM / 5 mU x ml^{-1}) was selected to cause ROS-induced neuronal damage (X/XO (dam)).

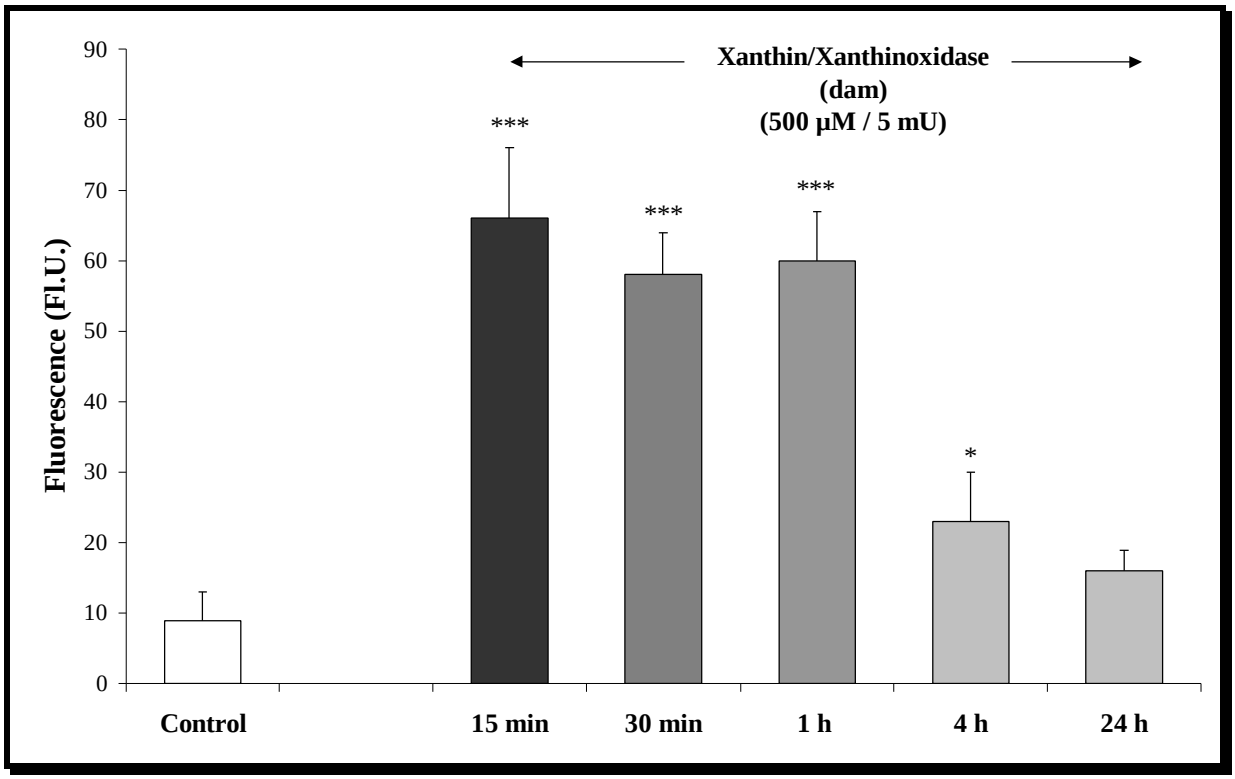


Fig. 25. Time-dependency of xanthine/xanthine oxidase (X/XO)-mediated stimulation of reactive oxygen species. *Primary cultures of chick neurons were simultaneously exposed to a constant concentration of X/XO (500 µM /5 mU x ml^{-1}) and measured after different time-points (15 min, 30 min, 1h, 4 h, 24 h). Reactive oxygen species content was determined by fluorescence microscopy. Fluorescent intensities of the oxidized rhodamine 123 are expressed as arbitrary fluorescence units (Fl.U.). Values are given as means ± S.D. for n = 5 – 7 neurons in 6 – 8 separate experiments. Differences between X/XO-treated cultures and controls: $^{**}P < 0.01$, $^{***}P < 0.001$.*

3.3.1.2 Incubation-response of Fe^{2+} and xanthine/xanthin oxidase on neuronal viability

To find the appropriate conditions of mild ROS-treatment it was important to determine the toxic border incubation range and therefore differentiate between beneficial and harmful ROS-exposure. Whereas incubation with 100 µM Fe^{2+} for 15 min or 1 h did not produce any toxicity, exposure for 4 h or more significantly damaged cultured neurons up to 85%. (Fig. 26a). A similar time-response was demonstrated with X/XO (pre), causing significant toxicity after 8 h (Fig. 26b). As the maximal rate of neuronal damage upon X/XO (pre) treatment raised to only 40% after 24 h, X/XO (pre) was considered to be the milder ROS-treatment as compared to 100 µM Fe^{2+}.

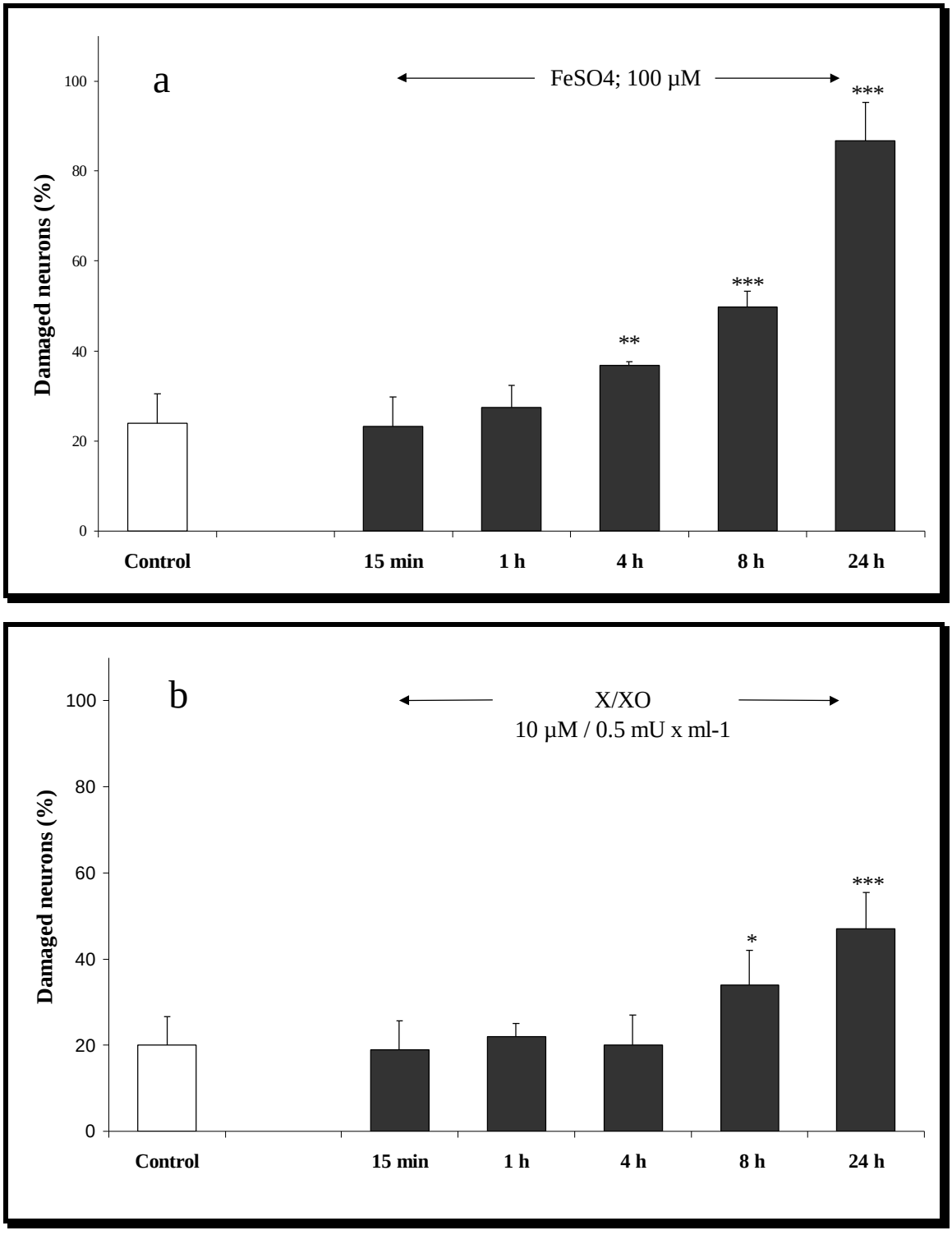


Fig. 26. Time-dependency of X/XO (pre) or Fe^{2+}-mediated effects on neuronal survival. *Primary cultures of chick neurons were simultaneously exposed 100 µM $FeSO_4$ (**a**) or 10 µM / 0.5 mU x ml^{-1} X/XO (**b**). Neuronal damage was determined by the trypan blue exclusion method after different time-points (15 min, 1h, 4 h, 8 h, 24 h). Values are given as means ± S.D. for n = 5 – 7 neurons in 6 – 8 separate experiments. Statistics were performed by one-way analysis of variance with subsequent Scheffé test. Differences between X/XO-treated cultures and controls: $^{**}P < 0.01$, $^{***}P < 0.001$.*

3.3.2 Preconditioning by moderate ROS stimulation protects against subsequent damage

Conversely to their damaging properties, ROS have been recently discussed to play an important role for intracellular signal transduction. Especially short and moderate treatment of neurons with potentially damaging agents protected against subsequent insults. Such phenomenon which was called preconditioning was discussed to employ ROS for the mediation of signaling pathways that mediated neuroprotection.

3.3.2.1 Experimental arrangement of ROS-mediated preconditioning

Chick neurons were exposed to the preconditioning stimuli X/XO (pre) or 100 µM $FeSO_4$ for 15 min, followed by 24 h recovery. Measurement of ROS was performed 5 min, 10 min, 15 min and 24 h after the preconditioning stimulus was added.

In order to cause neuronal injury, the cells were incubated at day 6 with either 1 mM glutamate for 1 h or with xanthine/xanthine oxidase (500 µM / 5 mU x ml^{-1}; = X/XO (dam)) for 1 h followed by 23 h of recovery in serum-free medium. Apoptotic cell damage was induced by incubating the cells with 200 nM staurosporine in serum-free medium for 24 h. Neuronal viability was assessed at day 7 (24 h after the induction of the injury). The experimental protocol is illustrated in Fig. 27.

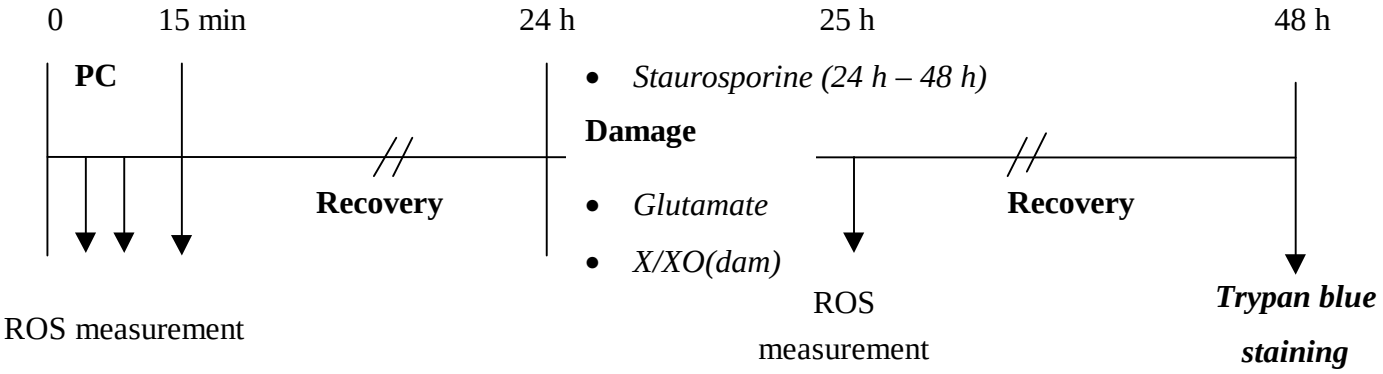


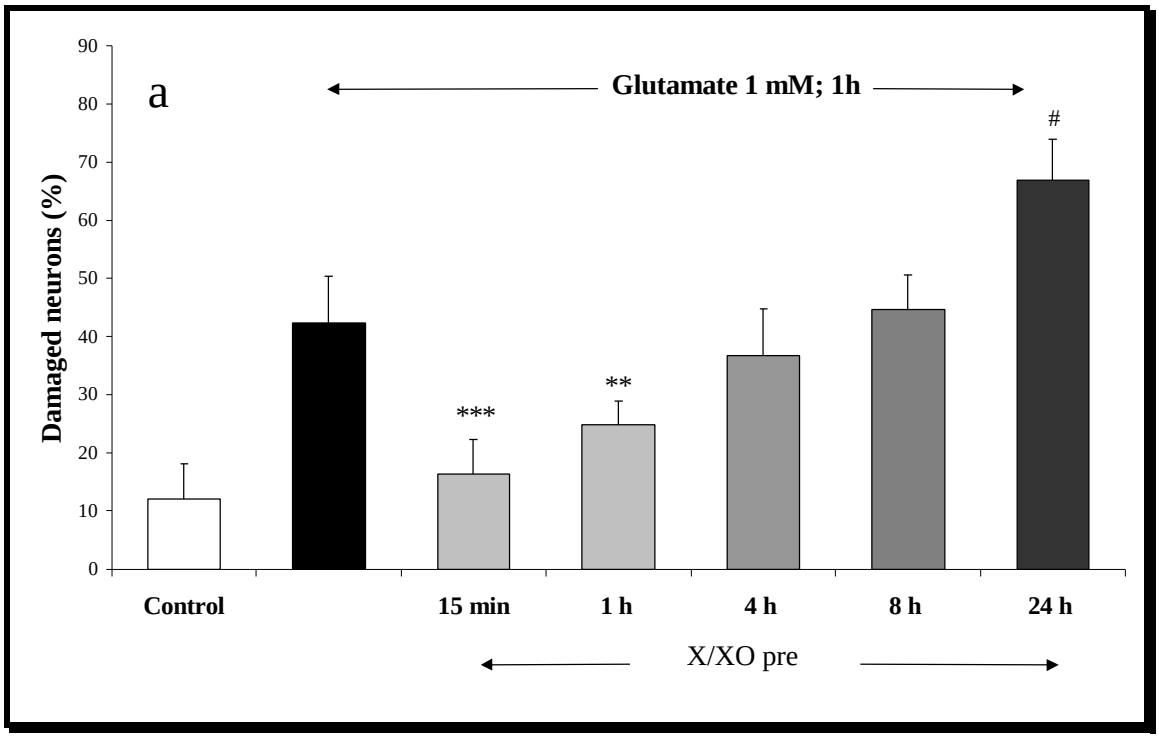
Fig. 27. Experimental design to precondition neuronal cells against subsequent insults by different damaging agents

3.3.2.2 Preconditioning by mild ROS-stimulation with Fe^{2+} or X/XO exerted a time-dependent neuroprotection against glutamate-induced necrosis

The protective effect of neuronal preconditioning with either $FeSO_4$ (100 µM) or X/XO (pre) against subsequent excitotoxic or oxidative damage was determined by trypan blue exclusion. When the neurons were exposed to glutamate (1 mM; 1 h) the percentage of trypan blue-stained neurons increased from 11% (controls) to 42%. Preconditioning with X/XO (pre) for 15 min most effectively reduced neuronal damage to 16% (Fig. 28a). The protective effect was still achieved by 1 h incubation but the efficacy of protection was already lower. ROS-incubation for 4 h and 8h had no protective effect any more whereas 24 h X/XO (pre)-treatment aggravated the glutamate-induced neuronal damage.

A similar window of protection was observed when Fe^{2+} was used to precondition the cells showing a maximal reduction of glutamate toxicity from 40% to 19% at 15 min incubation time (Fig 28b). Prolonged duration of exposure to Fe^{2+} caused a severe and time-dependent enhancement of neuronal damage. The turn-round incubation time which revoked neuroprotection into neuronal damage was found between 1h and 4 h ROS stimulation.

As the most effective protection was observed at 15 min ROS-exposure by X/XO (pre) as well as by 15 min Fe^{2+} (100 µM), this treatment regimen was used to investigate the effect of mild ROS-stimulation in further experiments.



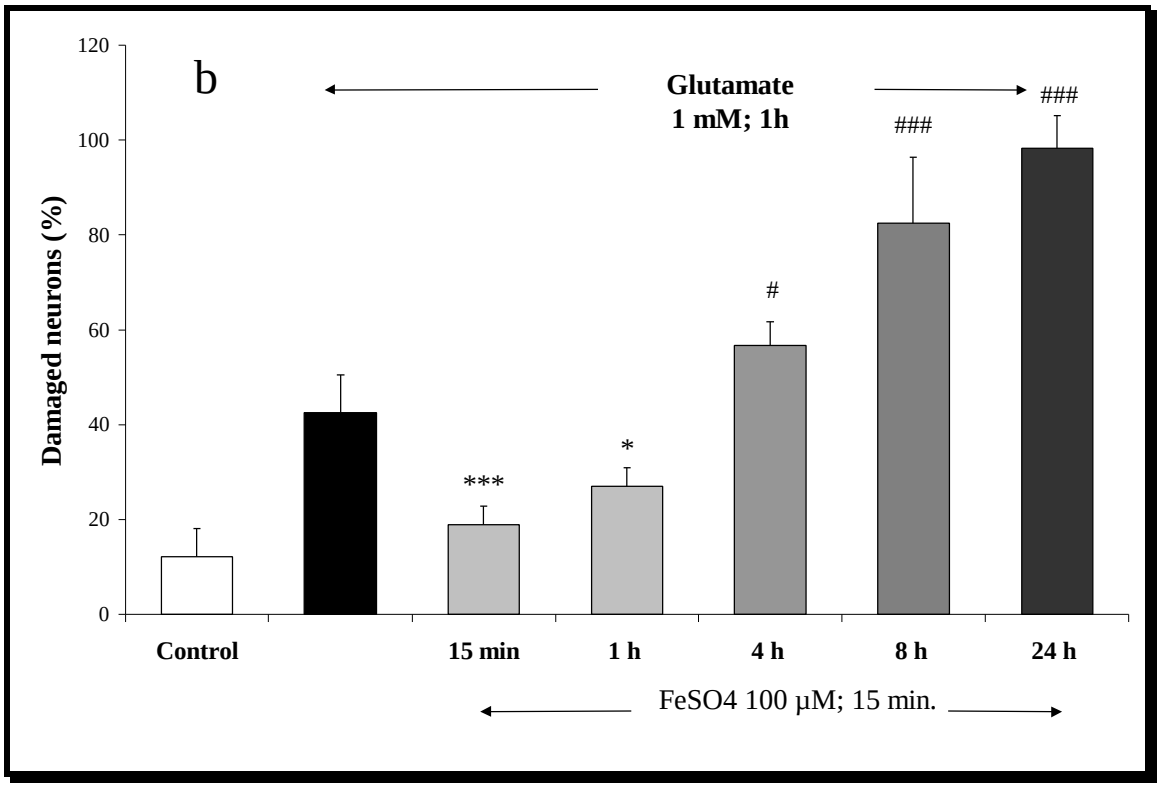


Fig. 28. Time-dependency of X/XO (pre) or Fe^{2+}-mediated preconditioning on glutamate-induced neuronal damage*. After 5 d in culture neurons from chick embryo telencephalons were preconditioned with X/XO (pre) (**a**) or Fe^{2+} (100 µM; 15 min) (**b**). Twenty-four h later, cells were treated at day 6 with glutamate (1 mM; 1 h). Cellular viability was determined after an additional 24 h recovery period at day 7 by the trypan blue exclusion method. Values are given as means ± S.D. of n=8 experiments. Different from non-preconditioned cultures, treated with glutamate: $^{*}P < 0.01$, $^{**}P < 0.01$, $^{***}P < 0.001$ using one way analysis of variance with subsequent Scheffé test*

3.3.2.3 Preconditioning with X/XO (pre) or Fe^{2+} mediates protection against X/XO (dam)-induced oxidative damage and against staurosporine-induced neuronal apoptosis

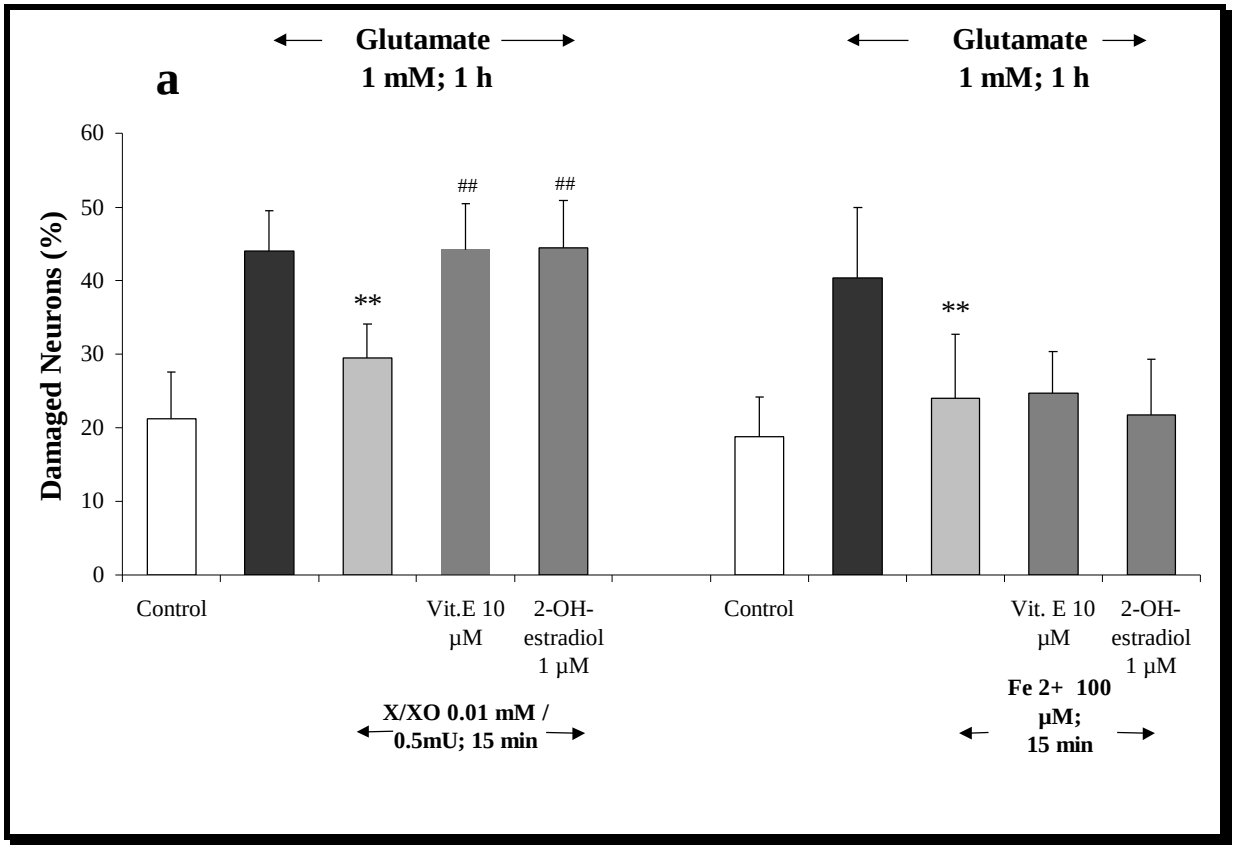
After exposure to X/XO (dam) the percentage of trypan blue-stained neurons increased from 21% (controls) to 47%. Preconditioning with X/XO (pre) effectively reduced neuronal damage to 19% (Fig. 29a). A protective effect was also observed when Fe^{2+} was used to precondition the cells showing a reduction of X/XO (dam) toxicity to 24%.

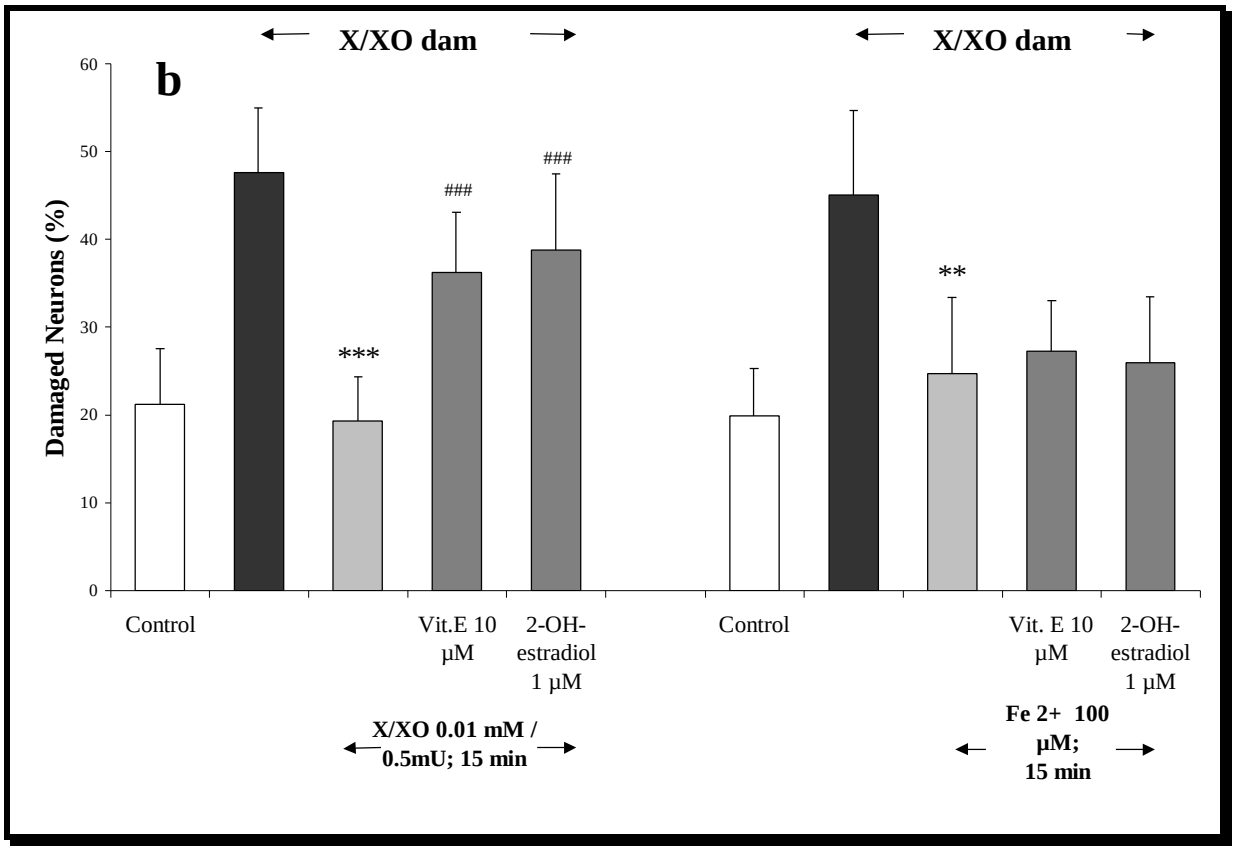
To investigate the influence of preconditioning on neuronal apoptosis, the cultures were treated with 200 nM staurosporine for 24 h which is well known to induce programmed cell death. Staurosporine markedly increased the percentage of neurons with apoptotic features

from 11.2% in controls to 48.6%. When this staurosporine treatment was preceded by a 15 min preconditioning with either X/XO (pre) or Fe^{2+}, apoptosis was reduced to 28.3% or 27.5%, respectively. (Figs. 29c and 30). In the absence of the damaging agents preconditioning stimuli alone had no effect on neuronal viability (data not shown).

3.3.2.4 The radical scavengers vitamin E and 2-OH-estradiol blocked the X/X (pre)-mediated neuroprotection

The X/XO (pre) -induced protection against subsequent insult with glutamate, X/XO (dam) or staurosporine was abolished when the radical scavengers vitamin E (10 µM) or 2-OH-estradiol (1 µM) were administered simultaneously with the preconditioning stimulus X/XO (pre) (Figs. 29). This finding strongly suggests that the observed neuroprotection was mediated by ROS. In contrast to X/XO (pre)-mediated preconditioning, the amelioration of neuronal viability by Fe^{2+} could not be blocked by antioxidants being present during the 15 min preconditioning (Figs X). To investigate this discrepancy, the formation kinetics of X/XO (pre)- and Fe^{2+}-induced ROS-generation under preconditioning conditions was further studied (Fig. 31).




b
X/XO dam
X/XO dam
Damaged Neurons (%)
60
50
40
30
20
10
0
***
###
###
**
Control
Vit.E 10 µM
2-OH-estradiol 1 µM
Control
Vit. E 10 µM
2-OH-estradiol 1 µM
X/XO 0.01 mM / 0.5mU; 15 min
Fe 2+ 100 µM; 15 min

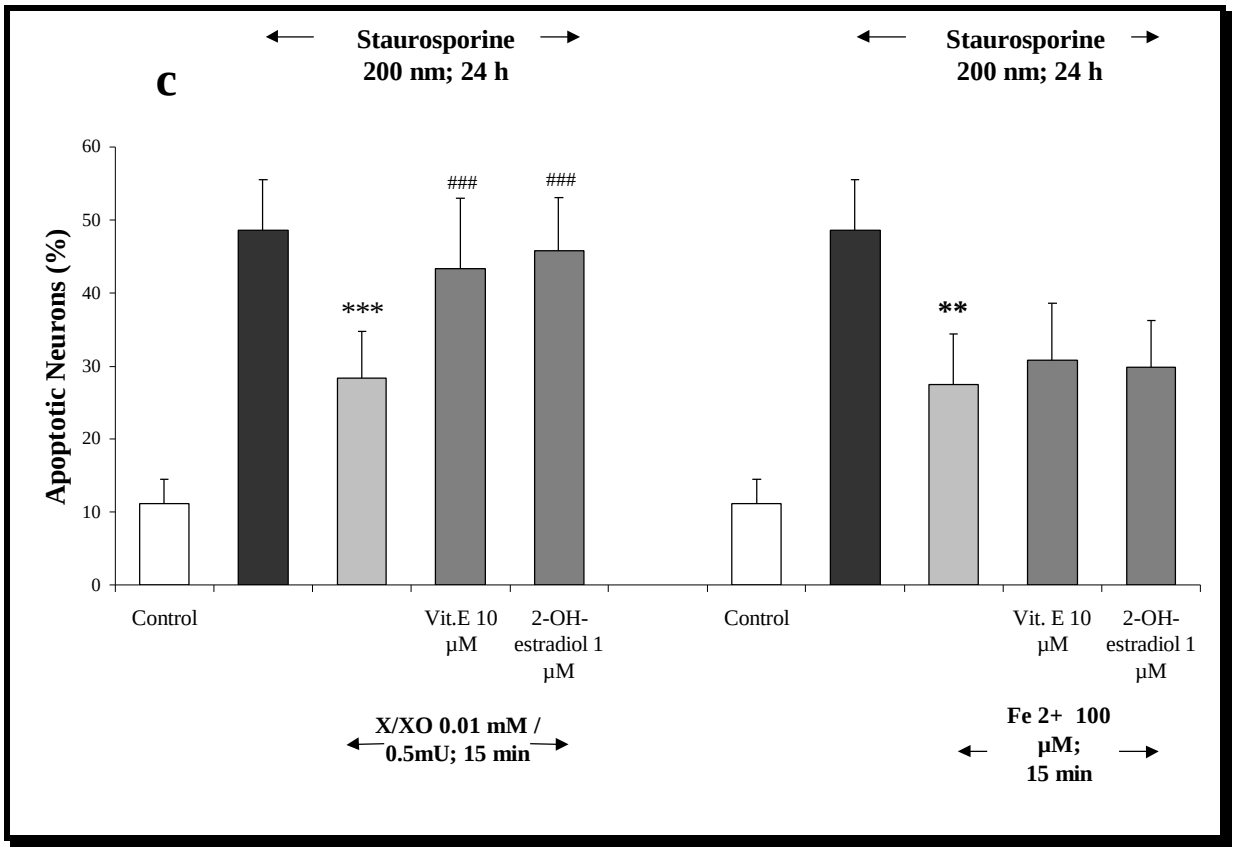

c
Staurosporine 200 nm; 24 h
Staurosporine 200 nm; 24 h
Apoptotic Neurons (%)
60
50
40
30
20
10
0
***
###
###
**
Control
Vit.E 10 µM
2-OH-estradiol 1 µM
Control
Vit. E 10 µM
2-OH-estradiol 1 µM
X/XO 0.01 mM / 0.5mU; 15 min
Fe 2+ 100 µM; 15 min

Fig. 29. Effect of X/XO (pre) or Fe^{2+} preconditioning on glutamate-, X/XO (dam)- or staurosporine-induced neuronal damage. *After 5 d in culture neurons from chick embryo telencephalons were preconditioned with Fe^{2+} (100 µM; 15 min) or X/XO (pre). The radical-scavengers vitamin E (10 µM) or 2-OH-estradiol (1 µM) were added together with the damaging agents. Twenty-four h later, cells were treated at day 6 with glutamate (1 mM; 1 h) (**a**), X/XO (dam) (**b**) or staurosporine (200 nM; 1 h) (**c**). Cellular viability was determined after an additional 24 h recovery period at day 7 by the trypan blue exclusion method. Neuronal apoptosis was identified by nuclear staining with Hoechst 33258. Values are given as means ± S.D. of n=8 experiments. Different from non-preconditioned cultures, treated with glutamate, X/XO (dam) or staurosporine: $^{**}P < 0.01$, $^{***}P < 0.001$. Different from X/XO (pre)-preconditioned cultures, treated with glutamate, X/XO (dam) or staurosporine: $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ using one way analysis of variance with subsequent Scheffé test*

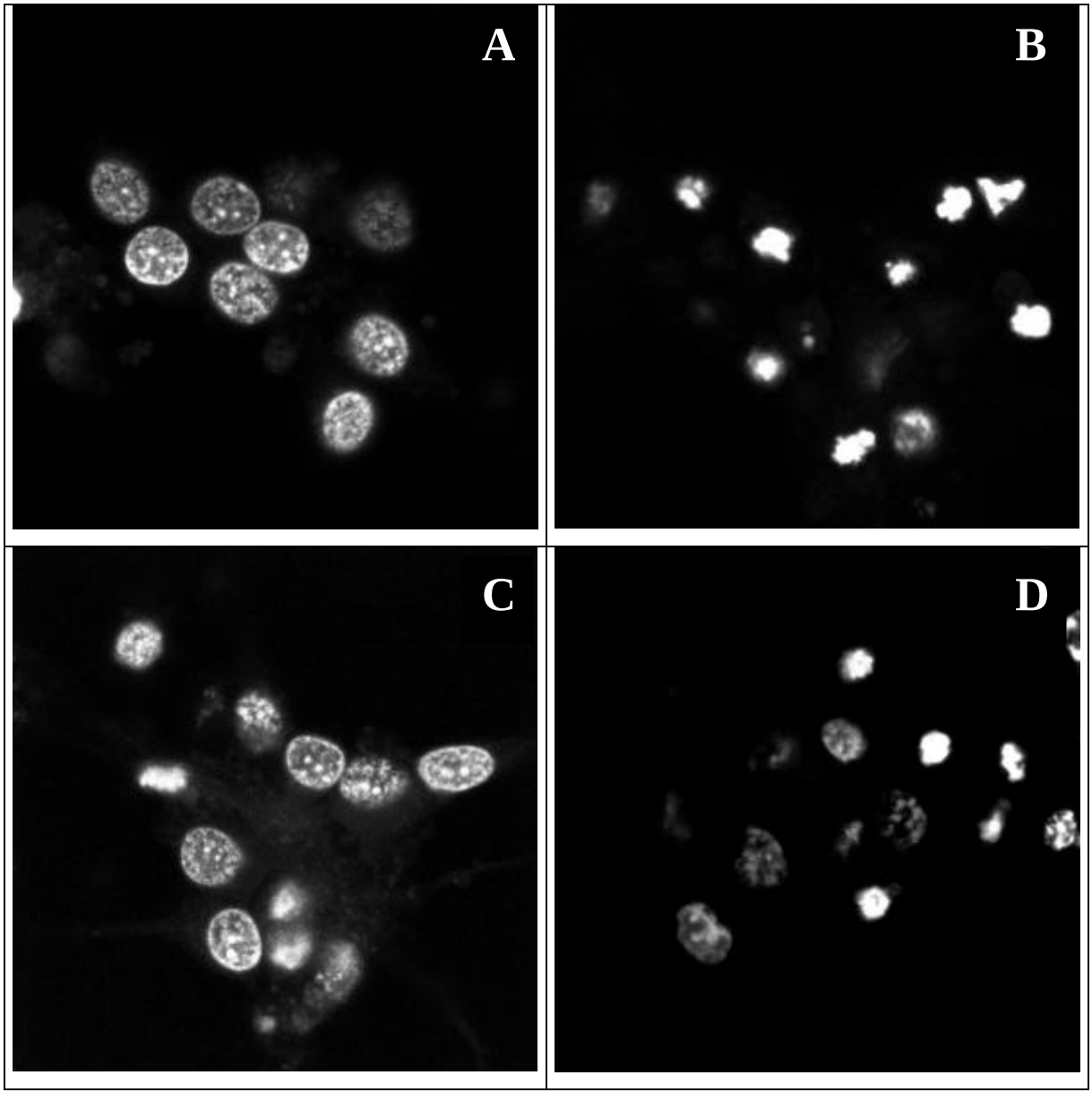
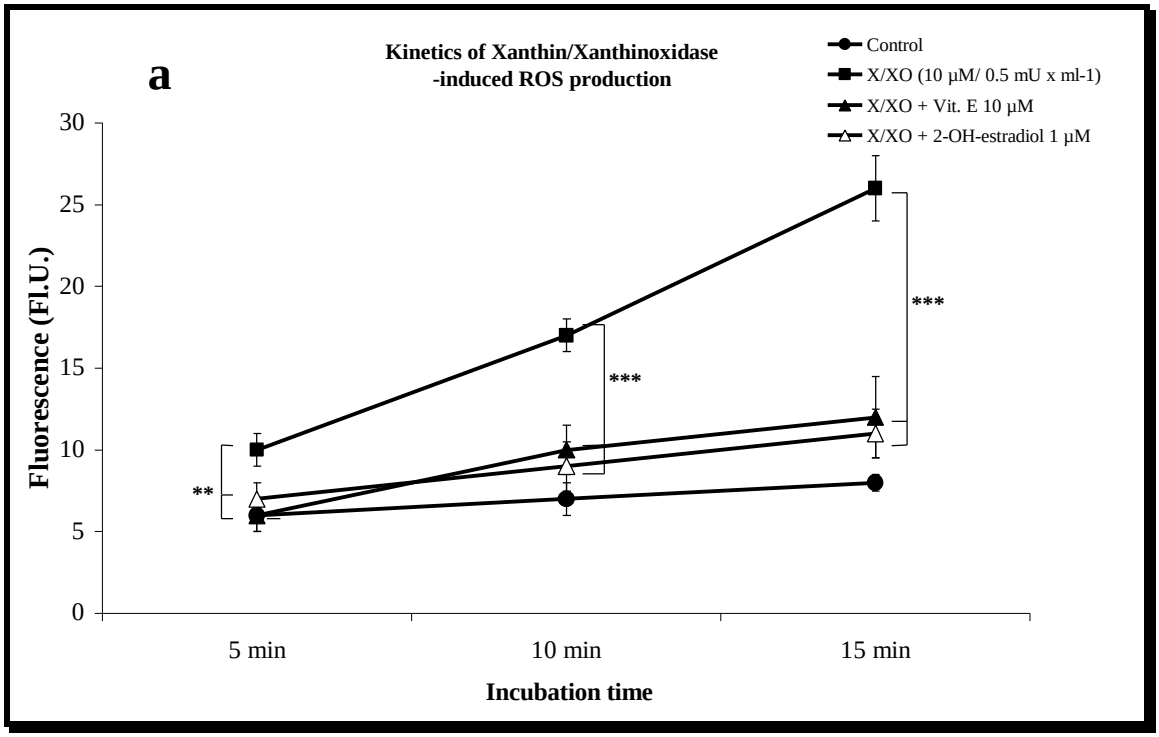


Fig. 30. Nuclear staining of chick embryonic neurons with Hoechst 33258. *Representative laser-scanning microscope images showing controls (**A**), staurosporine-treated cultures (**B**) and staurosporine-treated cultures preconditioned with X/XO (pre) in the absence (**C**) or presence of the radical-scavenger vitamin E (10 µM) (**D**) are demonstrated. Reduced nuclear size, chromatin condensation (visible as an intense fluorescence) and DNA-fragmentation are characteristics of apoptosis.*

3.3.2.5 Immediate and permanent suppression of reactive oxygen species is required to block the ROS-mediated neuroprotection by radical scavengers

Measurement 5, 10 and 15 min after incubation with X/XO (pre) revealed a linear and moderate enhancement of neuronal ROS content which could be significantly abolished with 1 µM 2-OH-estradiol and 10 µM vitamin E. These doses went out to be the most effective concentrations at all measured time points (Fig. 31a). In contrast, the Fe^{2+}-induced rise of ROS could only be blocked by the antioxidants after 15 min but not during the first 10 min of the preconditioning time period (Fig. 31b). Interestingly, this initial insensitivity of Fe^{2+} against radical scavenging was also evident when higher antioxidant concentrations or different pre-treatment patterns with vitamin E or 2-OH-estradiol were performed.



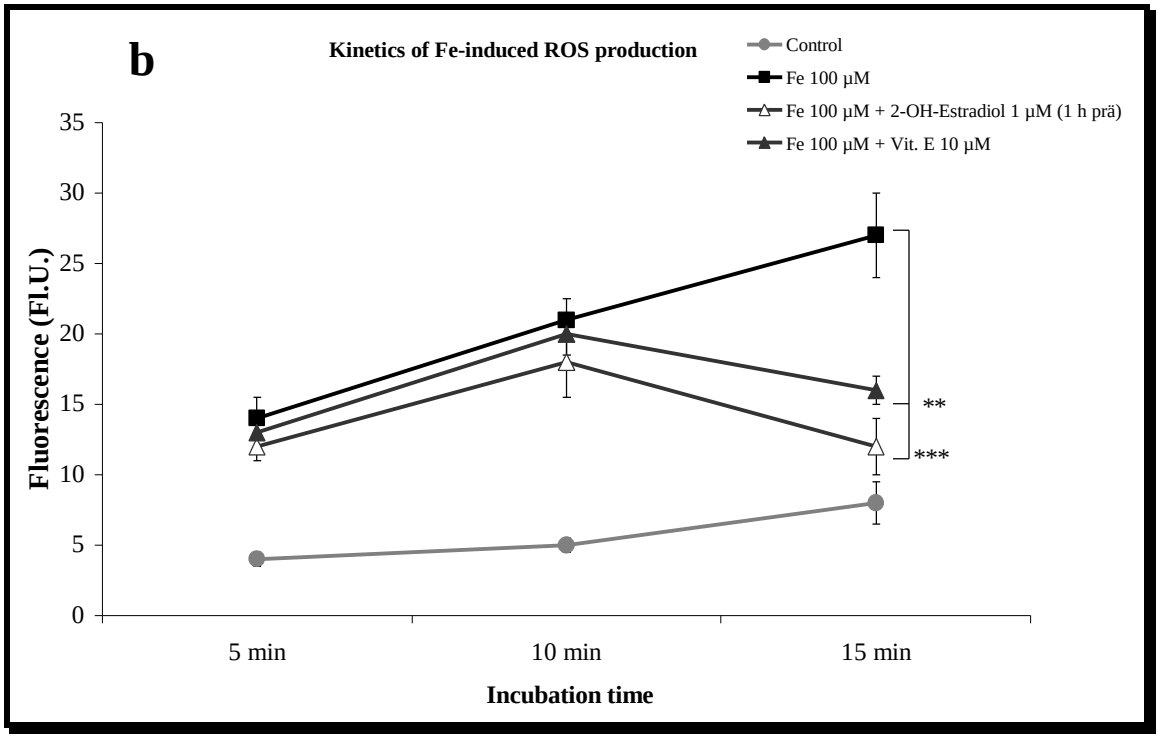


Fig. 31. Kinetics of X/XO (pre) or Fe^{2+}-induced ROS formation during the 15 min preconditioning time period. *Five days after seeding, primary cultures of chick neurons were treated with X/XO (pre) (**a**) or with Fe^{2+} (100 µM) (**b**). The radical-scavengers vitamin E (10 µM) or 2-OH-estradiol (1 µM) were added simultaneously. To determine ROS, intranneuronal fluorescence microscopy was employed. Fluorescence intensities of the oxidized rhodamine 123 were measured 5, 10 and 15 min after incubation and expressed as arbitrary fluorescence units (Fl.U.). Values are given as means ± S.D. of 6 – 8 separate experiments. Statistics were performed by one-way analysis of variance with subsequent Scheffé test. Differences between Fe^{2+} or X/XO (pre)-treated cultures and cultures treated with Fe^{2+} or X/XO (pre) in the presence of the antioxidants vitamin E (10 µM) or 2-OH-estradiol (1 µM): **P < 0.01, ***P < 0.001.*

To confirm the insensitivity of initial Fe^{2+}-mediated ROS generation towards radical scavengers, the structurally different antioxidants pyrolidine dithiocarbamate (PDTC), N-acetylcysteine or ascorbic acid were investigated to determine ROS alterations. Therefore, a model was used which measured total cellular rhodamine fluorescence. This was determined by lysing the cells after 15 min DHR 123 exposure with subsequent measurement of cellular fluorescence in a plate reader and determination of total protein content. Again, the antioxidants could not prevent the initial ROS-burst after Fe^{2+}-administration indicating the ineffectiveness of the scavenging treatment and proving the reliability of the intracellular ROS measurement (Fig. 32).

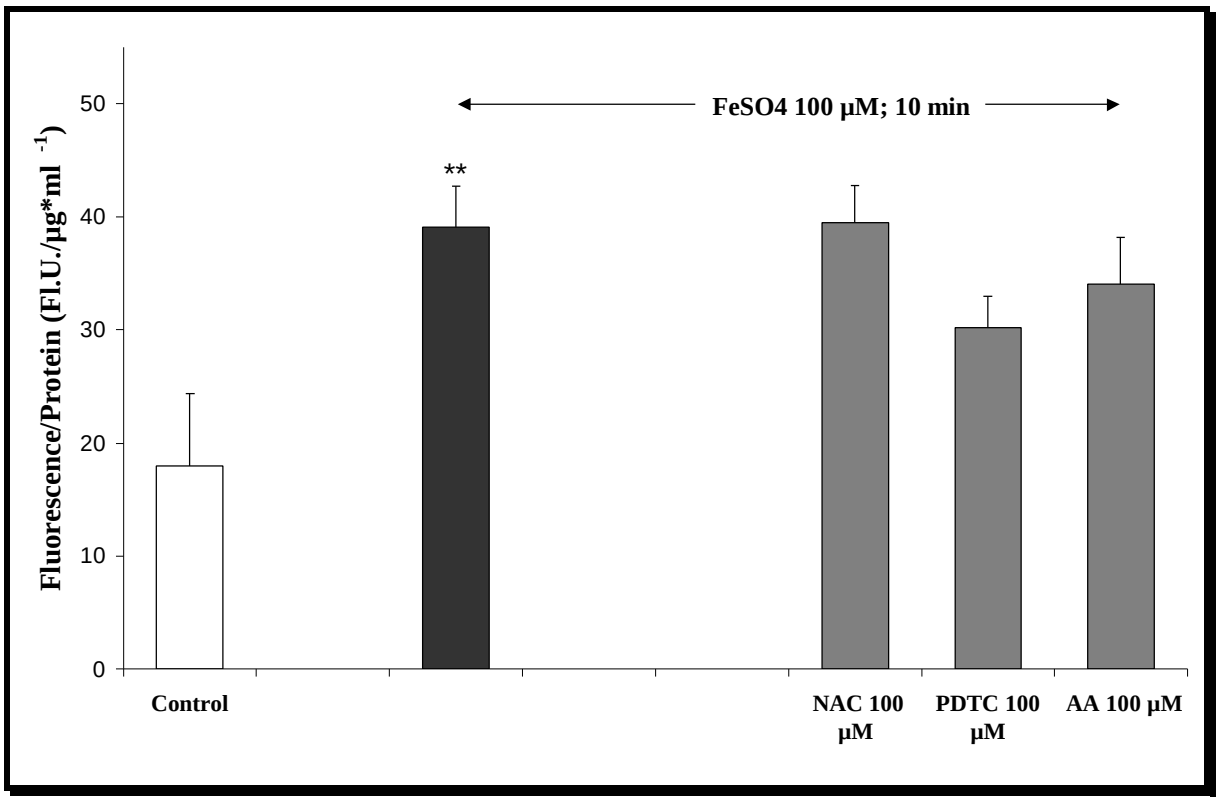


Fig. 32. Effect of alternative radical scavengers on the transient Fe^{2+}-mediated induction of reactive oxygen species. *Primary neuronal cultures form chick embryonic telencephalons were exposed to Fe^{2+} for 10 min. 100 µM N-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC), and ascorbic acid (AA) were administered immediately prior to Fe^{2+}. ROS were quantified by measurement of total cellular fluorescence from protein extracts. Therefore, cells were incubated with 5 µM dihydrorhodamine 123 for 5 minutes. Afterwards, cells were washed with Locke`s solution and suspended in 300 µl NaCl (0.9%) solution. An aliquot of the probe was directly measured with a fluorescence plate reader at an excitation wavelength of 520 nm. Another aliquot was used for the determination of total cellular protein content by the BCA kit. Results are expressed as fluorescence intensity / protein content (Fl.U. / µg protein).*

3.3.2.6 The preconditioning effect of mild reactive-oxygen species is mediated by preventing the subsequent glutamate, X/XO (dam) or staurosporine-induced oxygen radical burst

ROS content was monitored immediately after a 1 h incubation with the damaging agents glutamate, X/XO (dam) or staurosporine within single neurons. All agents caused a severe elevation of ROS production which was markedly reduced when the neurons were

preconditioned with X/XO (pre) or Fe^{2+}. When X/XO (pre) was used, the suppression of the subsequent oxygen radical burst could be abolished by the presence of the antioxidants vitamin E or 2-OH-estradiol during the preconditioning period. The same antioxidants were not able to block the Fe^{2+}-mediated reduction of ROS generation (Table 2 and Fig. 33).

The initial ROS-stimulation seems to initiate an intracellular response which makes neurons more resistant to oxidative stress. The mechanisms of this phenomenon were further investigated in the current thesis.

	Fluorescence (Fl.U.)		
Preconditioning Treatment			
Control	7 ± 2	10 ± 3	9 ± 2
	Glutamate (1 mM; 1 h)	**X/XO (dam)**	**Staurosporine (200 nM; 1h)**
	45 ± 5	68 ± 11	46 ± 5
X/XO (pre)	18 ± 3[a]	22 ± 4[a]	20 ± 3[a]
X/XO (pre) + Vit. E (10 µM)	40 ± 6[b]	63 ± 5[b]	43 ± 4[b]
X/XO (pre) + 2-OH-estradiol (1 µM)	44 ± 4[b]	61 ± 4[b]	47 ± 4[b]
Fe^{2+}	21 ± 2[a]	25 ± 2[a]	22 ± 2[a]
Fe^{2+} + Vit. E (10 µM)	19 ± 6[a]	26 ± 4[a]	26 ± 4[a]
Fe^{2+} + 2-OH-estradiol (1 µM)	24 ± 6[a]	21 ± 3[a]	23 ± 1[a]

Table 2. ROS generated by X/XO (pre) or Fe^{2+} mediated preconditioning against glutamate-, X/XO (dam)- or staurosporine-induced elevation of oxygen radicals. *Five days after seeding, primary cultures of chick neurons were preconditioned with Fe^{2+} (100 µM; 15 min) or X/XO (pre). The radical-scavengers vitamin E (10 µM) or 2-OH-estradiol (1 µM) were added simultaneously with the preconditioning agents. Twenty-four h later cells were treated with glutamate (1 mM; 1 h), X/XO (dam) or staurosporine (200 nM; 24 h). Single cell-measurement of ROS was performed immediately after this 1 h treatment. Fluorescence intensities of the oxidized rhodamine 123 are expressed as arbitrary fluorescence units (Fl.U.). Values are given as means ± S.D. of 6 – 8 separate experiments. Different from non-preconditioned cultures, treated with glutamate, X/XO (dam) or staurosporine: [a]P*

< 0.01. Different from X/XO (pre)-preconditioned cultures, treated with glutamate, X/XO (dam) or staurosporine: $^{b}P < 0.01$.

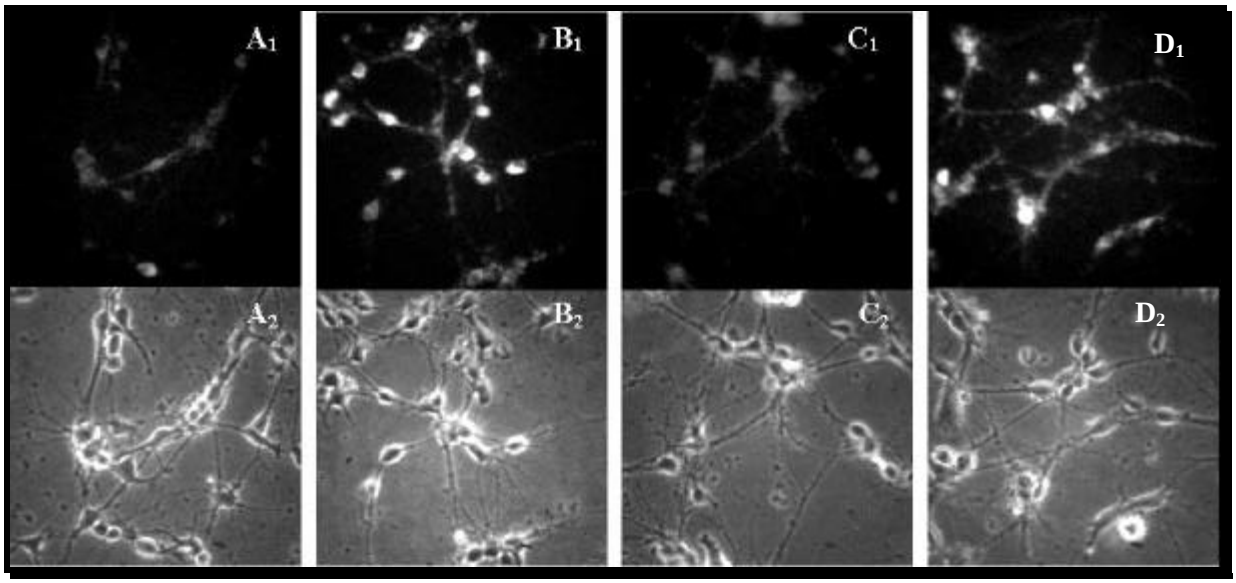


Fig. 33. Effect of X/XO (pre) preconditioning on X/XO (dam)-induced formation of ROS. *Five days after seeding, primary cultures of chick neurons were preconditioned with X/XO (pre). The radical-scavenger vitamin E (10 µM) was added simultaneously with X/XO (pre). Twenty-four h later, cells were treated with X/XO (dam). Representative rhodamine 123 fluorescence images of controls (**A_1**), cultures treated with X/XO (dam) (**B_1**) and X/XO (dam)-treated cultures preconditioned with X/XO (pre) in the absence (**C_1**) or presence of the radical-scavenger vitamin E (10 µM) (**D_1**) are demonstrated. Correlating phase-bright images are shown in **A_2**, **B_2**, **C_2** and **D_2**.*

3.3.3 Neuroprotection by mild reactive oxygen species treatment is mediated by the transcription factor NF-kB

3.3.3.1 ROS-mediated preconditioning is abolished by antioxidants, cycloheximide and inhibitors of NF-kB.

To investigate the mechanism of the observed neuroprotection by moderate ROS-stimulation, primary hippocampal cultures from postnatal rats were used. The protective effect of neuronal preconditioning with either $FeSO_4$ (100 µM) or X/XO (pre) against subsequent staurosporine (200 nM)-induced apoptotic damage (p 24 h) was determined by LDH-release (p. 48 h). The current study has demonstrated that within the preconditioning period of 15 min quantitative measurement of DHR-fluorescence 5, 10 and 15 min after incubation with X/XO (pre) or Fe^{2+} revealed a linear and moderate enhancement of neuronal ROS content. This transient ROS-generating treatment also protected cultured hippocampal neurons against subsequent staurosporine-induced damage (Fig. 34). The X/XO (pre)-mediated protection against the subsequent insult was abolished when the antioxidants vitamin E (10 µM) or 2-OH-estradiol (1 µM) were administered simultaneously with the preconditioning stimulus X/XO (pre) (Fig 34a). The NF-kB-inhibiting agent PDTC (1 µM) and the proteasome-inhibitor lactacystine (0.1 µM) blocked the preconditioning effect as well. Neuroprotection was also reversed by concomitant application of the protein synthesis inhibitor cycloheximide (1µM) indicating that de novo protein generation could be involved in the observed neuroprotection (Fig. 34a). A similar protective effect was observed when Fe^{2+} was used to precondition the cells showing a reduction of staurosporine toxicity from 40% to 24%. The amelioration of neuronal viability by Fe^{2+} could be blocked by the NF-kB-inhibitors PDTC and lactacystine or by the protein synthesis inhibitor cycloheximide but not by the antioxidants 2-OH-estradiol or vitamin E (Fig 34b). In the absence of the damaging agents, preconditioning stimuli, antioxidants, lactacystine or cycloheximide alone had no effect on neuronal viability.

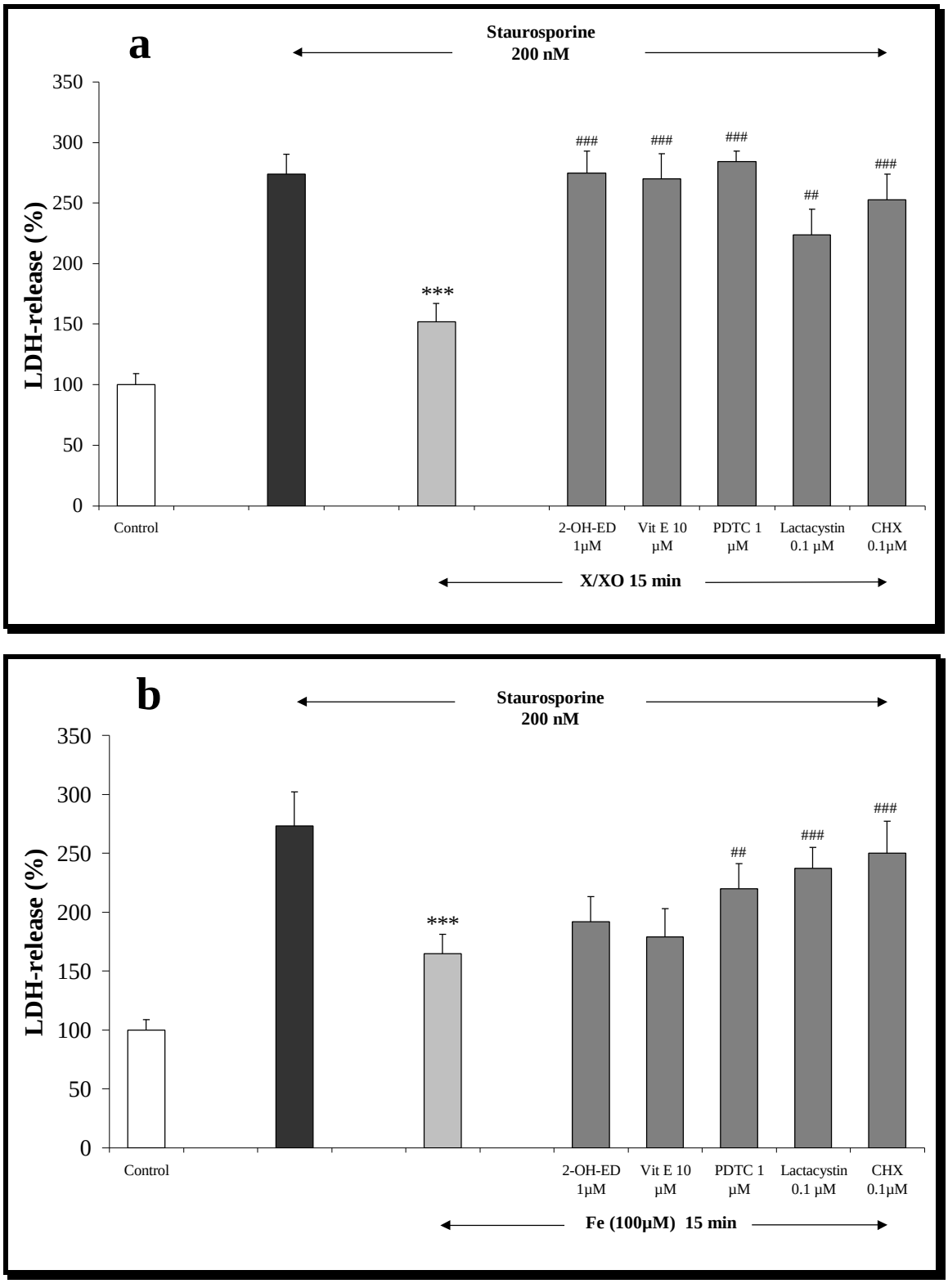


Fig. 34. The protective effect of X/XO (pre) (pre) or Fe^{2+} preconditioning against staurosporine-induced damage is blocked by inhibitors of NF-kB. *Ten days after seeding, primary hippocampal cultures of postnatal rats were incubated for 15 min with X/XO (pre) (a) or with 100 µM Fe^{2+} (b) The radical-scavengers vitamin E (10 µM) or 2-OH-estradiol (1 µM) were added simultaneously with the preconditioning agents and removed together with Fe^{2} and X/XO (pre). PDTC (1 µM), the*

proteasome–inhibitor lactacystine (0.1 µM) and cycloheximide (CHX; 0.1 µM) were also administered simultaneously with Fe^{2+} and X/XO (pre) and re-administered after the preconditioning stimuli had been removed. After a recovery period of 24 h, cells were treated with staurosporine (200 nM; 1 h) at day 11. Cellular viability was determined after an additional 24 h by lactate dehydrogenase (LDH)-release. LDH release was calculated as percent ratio of the extra- vs. the intracellular LDH activity. Controls were taken as 100 percent. Values are given as means ± S.D. of n=6 experiments. Different from non-preconditioned cultures, treated with staurosporine: $^{***}P < 0.001$. Different from X/XO (pre) or Fe^{2+}-preconditioned cultures, treated with staurosporine: $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ using one way analysis of variance with subsequent Scheffé test.

3.3.3.2 Neuroprotection against staurosporine-mediated apoptosis is blocked by the NF-kB-decoy-DNA.

To investigate the influence of specific NF-kB inhibition by an NF-kB consensus sequence containing oligonucloitde decoy, another series of experiments was performed using nuclear staining with Hoechst 22358 to identify alterations in the amount of apoptosis. When staurosporine treatment which increased apoptosis to 66% was preceded by a 15 min preconditioning with either X/XO (pre) or Fe^{2+}, the number of apoptotic nuclei was reduced to 39% or 42%, respectively (Fig 35). The attenuation of neuronal apoptosis by X/XO (pre) or Fe^{2+}-induced preconditioning was inhibited by decoy which was present from 2 h before the onset of preconditioning until 24 h afterwards. A non-sense-oligonucleotide containing one altered base-pair in the consensus sequence of the decoy-oligonucleotide did not block neuroprotection proving the specificity of the inhibition. Decoy and non-sense alone had no effect on neuronal viability (Fig 35).

The results strongly indicate that the neuroprotective effect of mild ROS stimulation was mediated by an activation of NF-kB.

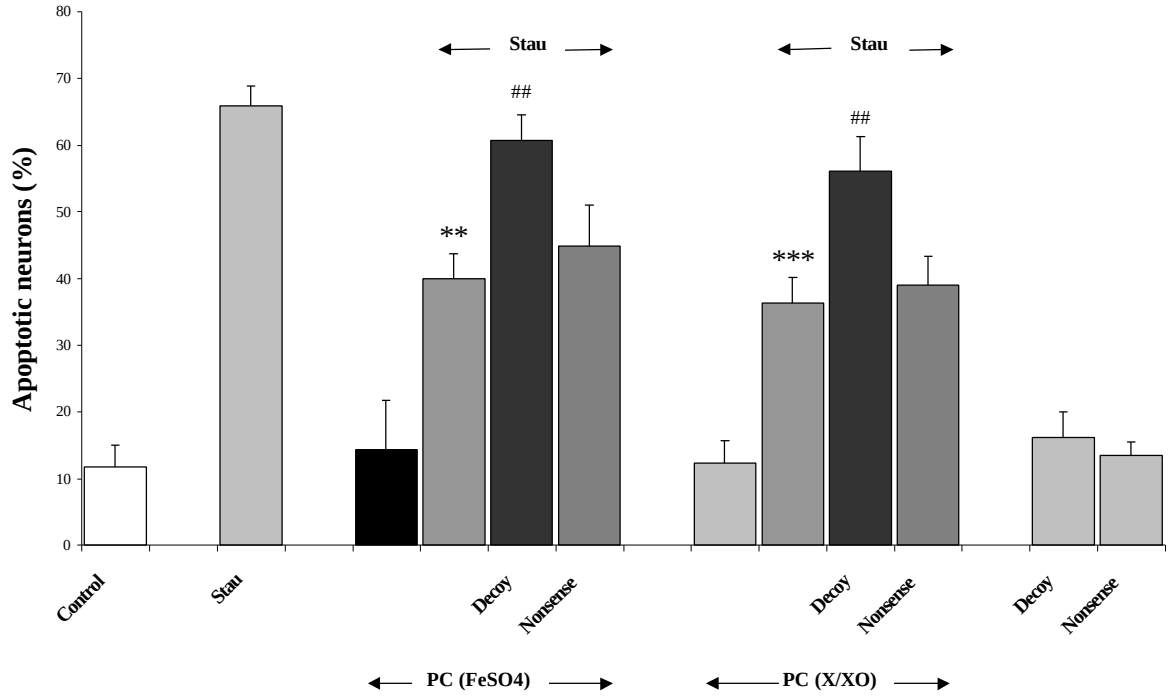


Fig. 35. Specific inhibition of NF-kB by decoy abolished the protective effect of X/XO (pre) or Fe^{2+} preconditioning against staurosporine-induced apoptosis. *Ten days after seeding, primary hippocampal cultures of postnatal rats were incubated for 15 min with 100 µM Fe^{2+} or with X/XO (pre). Decoy and nonsense-oligonucleotides were administered after the preconditioning stimuli had been removed by medium exchange and were present for 24h. At day 11, cells were treated with staurosporine (200 nM; 1 h). Cellular viability was determined after an additional 24 h by nuclear staining with Hoechst 22558. Cells with reduced nuclear size, chromatin condensation (visible as an intense fluorescence) and DNA-fragmentation were considered apoptotic. Values are given as means ± S.D. of n=8 experiments. Different from non-preconditioned cultures, treated with staurosporine: $^{**}P < 0.01$, $^{***}P < 0.001$. Different from X/XO (pre) or Fe^{2+}-preconditioned cultures, treated with staurosporine: $^{\#\#}P < 0.01$ using one way analysis of variance with subsequent Scheffé test.*

3.3.3.3 Preconditioning with ROS also decreased the staurosporine-induced oxygen radical burst *in hippocampal neurons*.

ROS content was also monitored after a 4 h incubation with the apoptosis-inducing agent staurosporine within single hippocampal neurons. Staurosporine caused a severe elevation of ROS production which was markedly reduced when the neurons were preconditioned with X/XO (pre) or Fe^{2+}. When X/XO (pre) was used, the suppression of the subsequent oxygen radical burst could be abolished by the presence of the antioxidants vitamin E or 2-OH-

estradiol during the preconditioning period (Table 3). The same antioxidants were not able to block the Fe^{2+}-mediated reduction of ROS generation. As already demonstrated in cultured chick neurons, this phenomenon may be explained by a different ROS formation kinetics within the first 15 min due to an initial insensitivity of Fe^{2+} against radical scavenging (see Fig. X).

	Fluorescence (Fl.U.)
Treatment	
Control	14 ± 5
Staurosporine (200 nM; 4 h)	46 ± 4
Staurosporine (200 nM; 4 h) + X/XO (pre)	18 ± 2[a]
Staurosporine (200 nM; 4 h) + X/XO (pre) + Vitamin E (10 µM)	39 ± 5
Staurosporine (200 nM; 4 h) + X/XO (pre) + 2-OH-estradiol (1 µM)	42 ± 4
Staurosporine (200 nM; 4 h) + Fe^{2+}	24 ± 4[b]
Staurosporine (200 nM; 4 h) + Fe^{2+} + Vitamin E (10 µM)	28 ± 6[b]
Staurosporine (200 nM; 4 h) + Fe^{2+} + 2-OH-estradiol (1 µM)	27 ± 3[b]

Table 3. Preconditioning with X/XO (pre) or Fe^{2+} reduced the staurosporine-induced oxygen radical burst in hippocampal neurons. *Ten days after seeding, primary hippocampal cultures of postnatal rats were incubated for 15 min with X/XO (pre) or with 100 µM Fe^{2+} The radical-scavengers vitamin E (10 µM) or 2-OH-estradiol (1 µM) were added simultaneously with the preconditioning agents and removed together with Fe^{2} and X/XO (pre). ROS were quantified after 4 h by fluorescence microscopy. Values are given as arbitrary fluorescence units (Fl.U.) of n=6 experiments. Different from non-preconditioned cultures, treated with staurosporine: $^{***}P < 0.001$. Different from staurosporine-treated cultures: $^{a}P < 0.001$, $^{b}P < 0.01$ using one way analysis of variance with subsequent Scheffé test.*

3.3.3.4 Preconditioning by mild ROS-stimulation induced a nuclear translocation of NF-kB which was blocked by ROS-scavengers

While strong evidence from the demonstrated viability studies suggested the involvement of NF-kB in the ROS-mediated neuroprotection, it remained to be clarified if NF-kB activation really occurred under ROS-stimulating conditions. Therefore, cultures were tested for NF-kB activation by immunostaining with a monoclonal antibody for the NF-kB p65 subunit. In controls, most immunoreactivity was found in the cytosol (Fig. 36A) while stimulation with X/XO (pre) for 15 min (Fig. 36B) or Fe^{2+} 15 min (Fig. 36C) resulted in a marked translocation of NF-kB into the nucleus. This effect which appeared after 1 h, 4h, 8h and 24 h was maximal after 4 h. The X/XO (pre)-mediated translocation could be blocked by the antioxidant vitamin E indicating the requirement of ROS for the observed X/XO (pre)-mediated NF-kB activation (Fig. 36D). The Fe^{2+}-mediated translocation could not be reduced by antioxidants. An activation of NF-kB was found in neurons as well as in astrocytes as observed by concomitant neurofilament or GFAP-staining (data not shown)

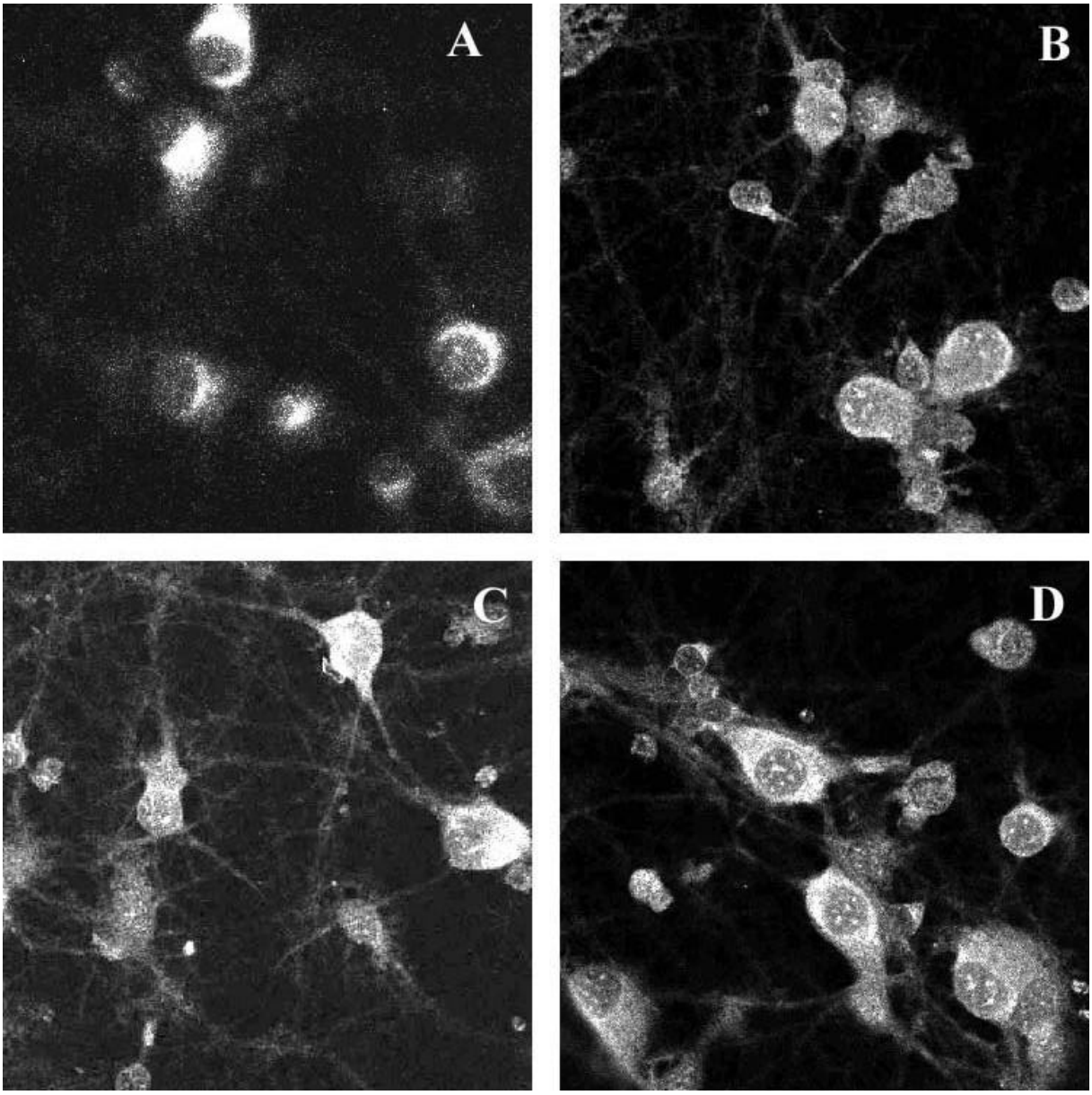


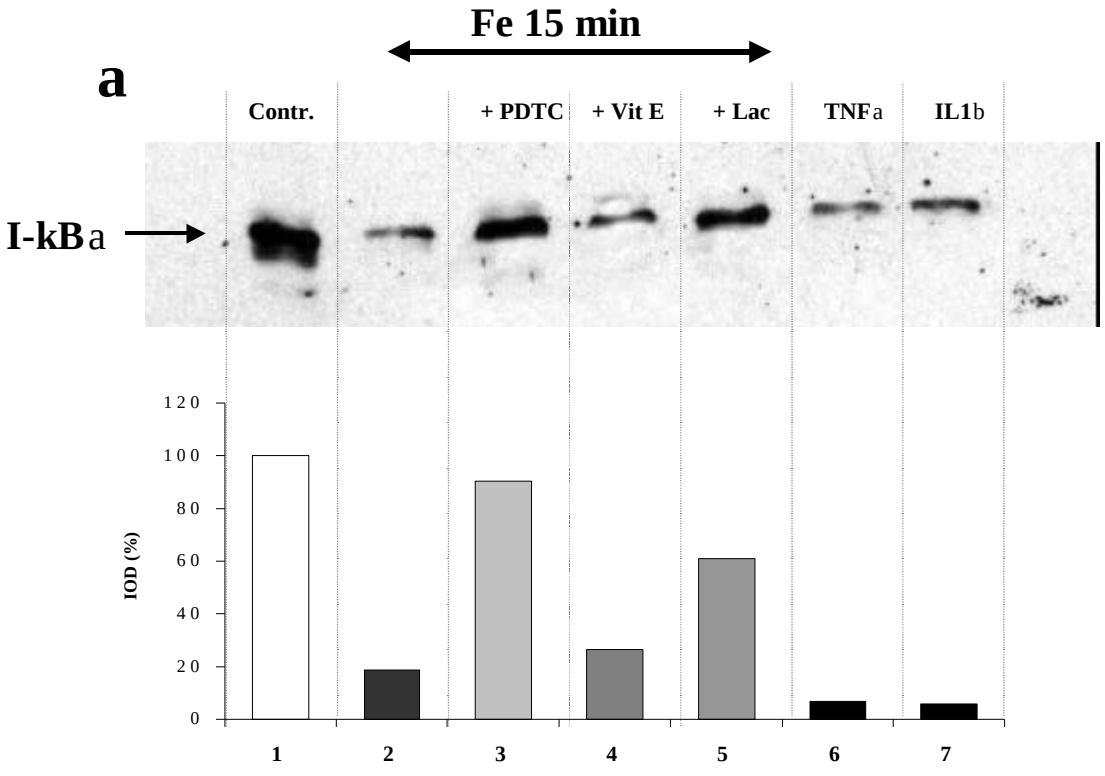
Fig. 36. Stimulation with X/XO (pre) or Fe^{2+} induced nuclear translocation of the NF-kB subunit p65 into the nucleus*. Primary hippocampal cultures of postnatal rats were incubated for 15 min with 100 µM Fe^{2+} or with X/XO (pre). The radical-scavenger vitamin E (10 µM) was added simultaneously with X/XO (pre). After 4 h the cells were fixed in methanol, and immunostaining was performed by over-night incubation (4 ºC) with a monoclonal p65 antibody which was succeeded by treatment with a secondary rhodamine-conjugated anti-mouse antibody. Representative images of p65 staining obtained by laser scanning microscopy are showing controls (A), cultures treated with X/XO (pre) (B), Fe^{2+} (C), or X/XO (pre) in the presence of vitamin E (D).*

3.3.3.5 Preconditioning with moderate amounts of reactive oxygen species decreased I-kB-a-expression

Upon activation of NF-kB the inhibitory subunit I-kBα is downregulated due to its degradation by the proteasome. Western Blot analysis revealed a marked decrease of I-kBα by the preconditioning stimuli X/XO (pre) and Fe 4 h after administration. This degradation of I-kBα indirectly confirms the activation of NF-kB. IL-1ß and TNF-α which were used as positive controls for NF-kB activation also reduced I-kBα signals. The X/XO (pre)-induced downregulation was reversed by the antioxidants vitamin E and PDTC as well as by the proteasome inhibitor lactacystine (Fig. 37a) confirming the activation of NF-kB by ROS.

A similar effect was observed with Fe^{2+}-treated cultures except that vitamin E did not abolish the decreased expression of I-kBα (Fig. 37b).

EMSA was used to confirm the results of I-kB-α western blotting and to ensure that the degradation of I-kBα was accompanied by an increased DNA-binding activity. Preconditioning treatment with X/XO entailed enhanced NF-kB activity which was blocked by the antioxidants and by the proteasome-inhibitor (Fig. 38).



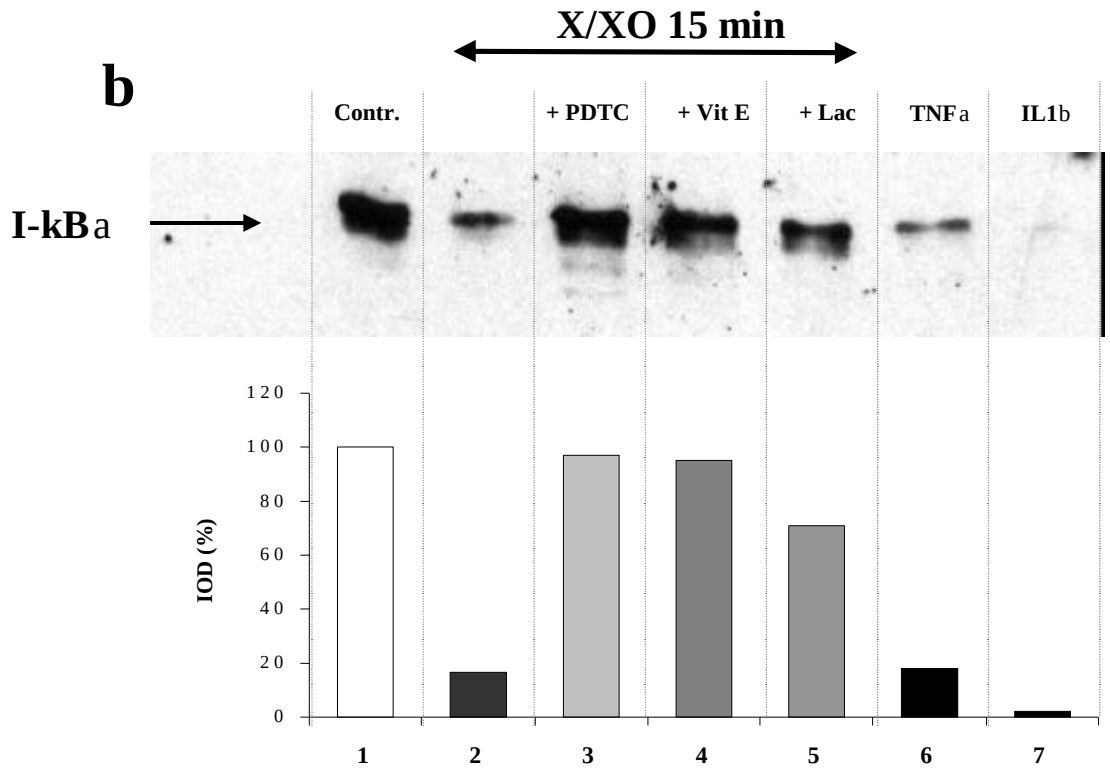


Fig. 37. Moderate incubation with ROS decreased the I-kBa-expression which was blocked by inhibitors of NF-kB. *Primary hippocampal cultures of postnatal rats (day 10) were incubated for 15 min with 100 µM Fe^{2+} (a) or with X/XO (pre) (b). The radical-scavenger vitamin E (10 µM) was added simultaneously with the preconditioning agents and removed together with Fe^{2} and X/XO (pre). PDTC (1 µM), the proteasome–inhibitor lactacystine (0.1 µM) (Lac) as well as TNFa and ILb which were used as positive controls remained in the culture medium until the cells were harvested. To determine the expression of I-kBa the cells were collected in lysis buffer after 4 h and protein alterations were analyzed by western blot using a polyclonal antibody against I-kBa. Semi-quantitative analysis was performed by determination of the integrated optical density (IOD) using scion image software to support the visual impression. Controls were arbitrarily expressed as 100%.*

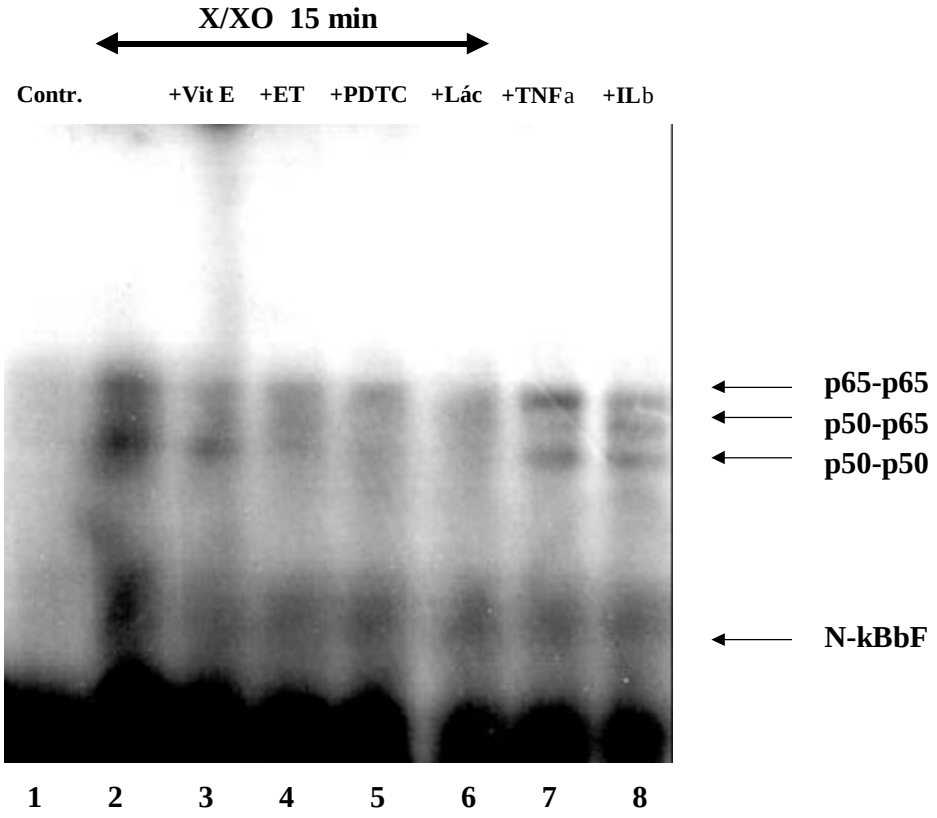


Fig. 38. Treatment with X/XO (pre) mediated an electrophoretic mobility shift which was blocked by antioxidants and lactacystine. *Primary hippocampal cultures of postnatal rats (day 10) were incubated for 15 min with X/XO (pre). The radical-scavengers vitamin E (10 µM) (Vit E) and 2-OH-estradiol (ET) were added simultaneously with the preconditioning agents and removed together with X/XO (pre). PDTC (1 µM), the proteasome–inhibitor lactacystine (0.1 µM) (Lac) and also TNFa and ILb which were used as positive controls, remained in the culture medium until the cells were harvested. Four h later, nuclear extracts were prepared and analyzed for specific DNA-binding activity by EMSA. The autoradiogram shows a representative gel-mobility shift assay with untreated controls (lane 1), X/XO (pre)-treated- (lanes 2-5), TNFa- (lane7) or IL1b-treated (lane 8) nuclear probes The lower band probably represents a recently defined neuronal kappa B-binding factor (N-kBbF) .*

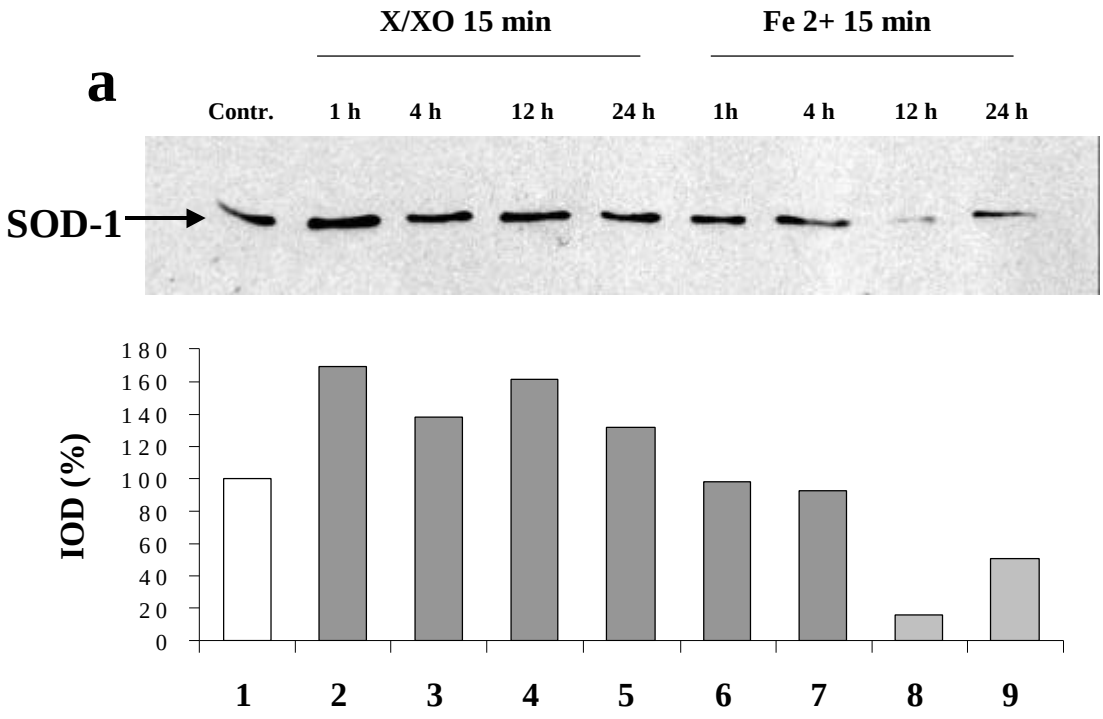
3.3.4 The neuroprotective function of NF-kB is executed by enhanced expression of mitochondrial superoxide dismutase (SOD-2)

The current experiments have demonstrated the importance of NF-kB for the mediation of neuroprotection by moderate ROS-treatment. As activated NF-kB promotes the transcription

of several genes, the involved proteins which executed the observed neuroprotection remained to be clarified. As superoxide dismutase is known to be under the transcriptional control of NF-kB the current thesis further explored the influence of moderate ROS-stimulation on the expression of SOD-1 and SOD-2 and investigated if this effect was NF-kB-dependent.

3.3.4.1 Time-dependent effects of X/XO (pre) or Fe^{2+} on the expression of SOD-1 and SOD-2.

The expression of SOD-1 and SOD-2 after mild stimulation of hippocampal cultures with X/XO (pre) or Fe^{2+} for 15 min was monitored after 1h, 4h, 12, and 24 h. Levels of SOD-1 protein were found to be unaffected by ROS stimulation (Fig. 39a) whereas a marked upregulation of SOD-2 was observed (Fig. 39b). Enhanced expression of SOD-2 was detectable 4 h to 24 h after ROS-treatment.



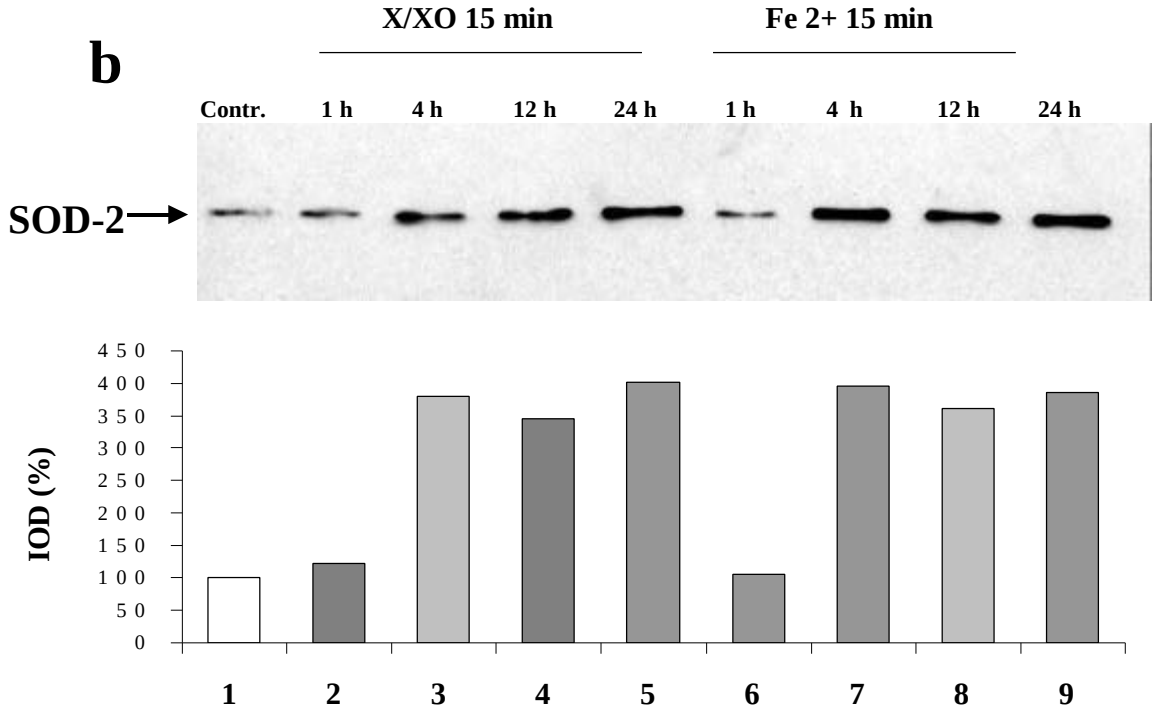


Fig. 39. Time-dependency of X/XO (pre) or Fe^2-regulated expression of SOD-1 and SOD-2. *Primary hippocampal cultures of postnatal rats (day 10) were incubated for 15 min with 100 µM Fe^{2+} or with X/XO (pre). To determine the time-dependency of SOD-1 and SOD-2-expression the cells were collected in lysis buffer after 1 h, 4 h, 12 h and 24 h. SOD-1 (a) and SOD-2 (b) were determined by western blot analysis. Semi-quantitative analysis was performed by determination of the integrated optical density (IOD) using scion image software to support the visual impression. Controls were arbitrarily expressed as 100%.*

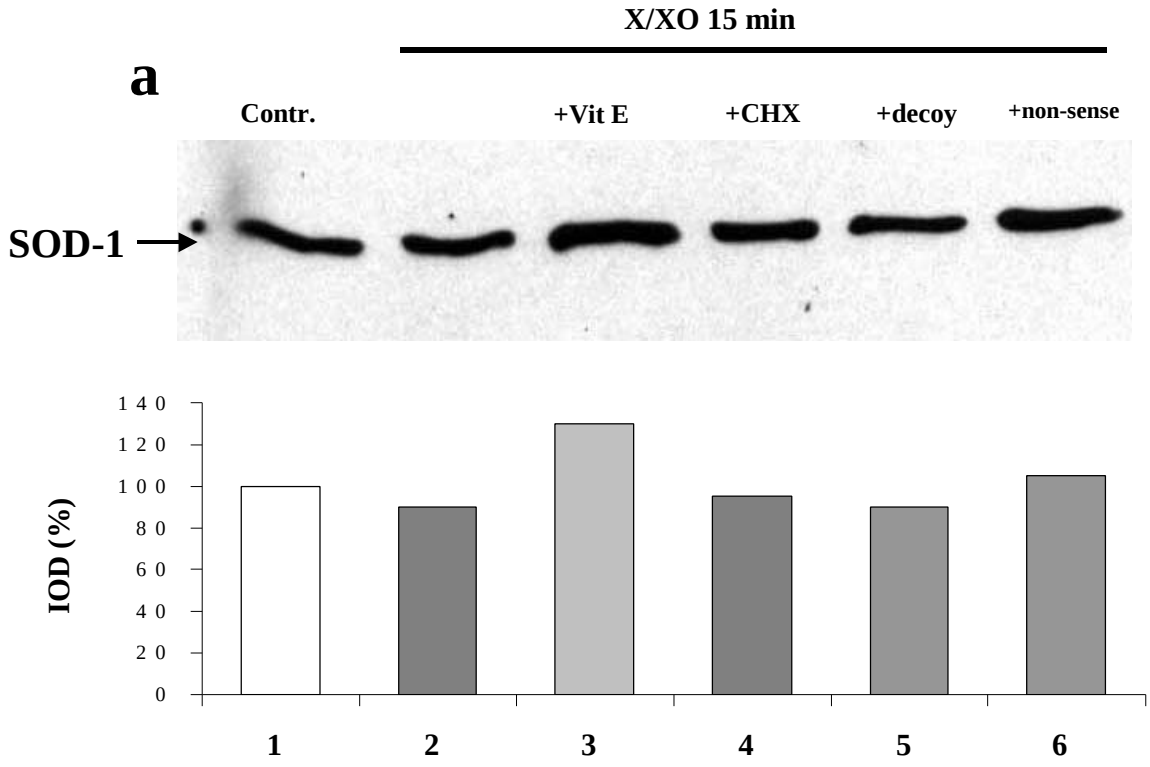
3.3.4.2 The X/XO (pre)-induced upregulation of SOD-2 was blocked by cycloheximide and by decoy

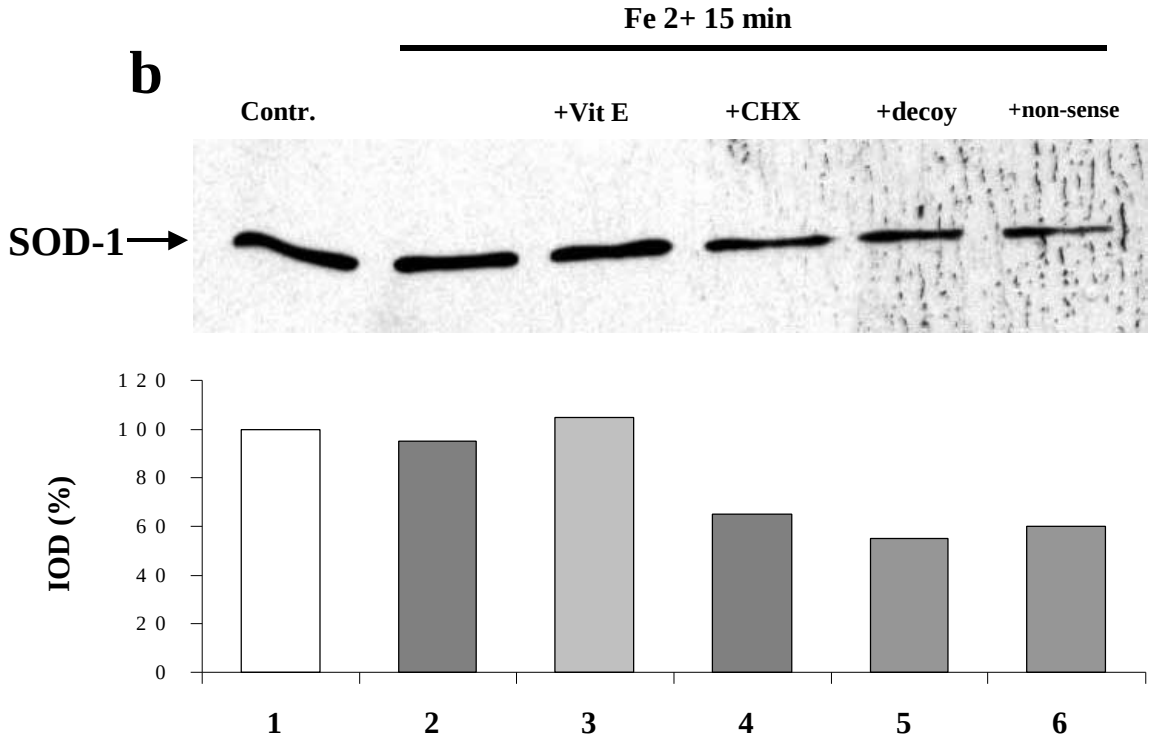
Although the time-course-experiments for SOD-1 and SOD-2 revealed that SOD-1 was rather unaffected by ROS-stimulation, the results were confirmed by another experiment where the influence of the antioxidant vitamin E, the protein-synthesis inhibitor cycloheximide and the specific NF-kB inhibitor decoy on ROS-mediated SOD-1 expression was investigated. Again, there were no remarkable changes in the expression of SOD-1 after X/XO (pre) or Fe^{2+}-stimulation in the absence or presence of the above mentioned inhibitors. Thus, an

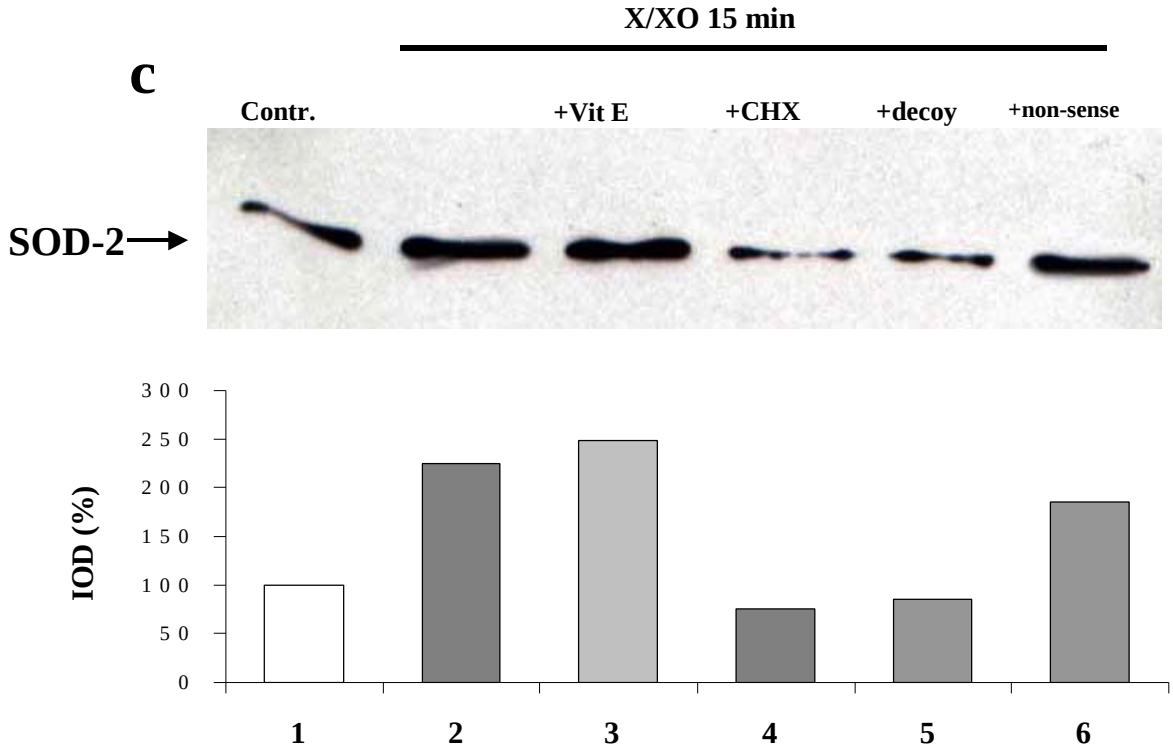
involvement of SOD-1 in the observed neuroprotection by mild ROS-stimulation seems unlikely (Figs. 40a and 40b).

Conversely, the X/XO (pre)-induced upregulation of SOD-2 could be blocked by cycloheximide and by decoy (Figs. 40c and 40d) indicating that ROS caused an NF-kB-mediated transcription with subsequent protein synthesis of SOD-2. Of interest, vitamin E did not block the X/XO (pre)- or Fe^{2+}-mediated SOD-2 upregulation. Further, the Fe^{2+}-induced enhanced SOD-2 expression was not abolished by decoy.

However, these results have to be interpreted carefully as the influence of ROS-stimulation on SOD-2 expression is still under investigation.




b
Fe 2+ 15 min
Contr.
+Vit E
+CHX
+decoy
+non-sense
SOD-1
IOD (%)
120
100
80
60
40
20
0
1
2
3
4
5
6


X/XO 15 min
c
Contr.
+Vit E
+CHX
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+non-sense
SOD-2
IOD (%)
300
250
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50
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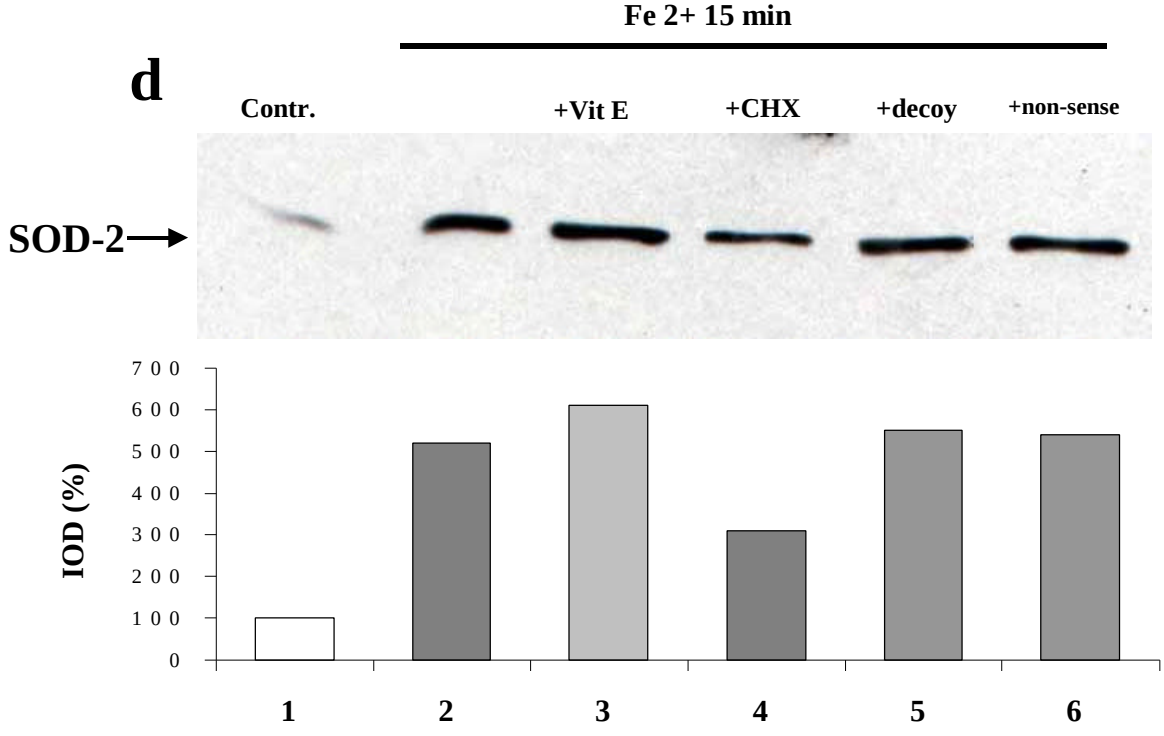

Fe 2+ 15 min
d
Contr.
+Vit E
+CHX
+decoy
+non-sense
SOD-2
IOD (%)
700
600
500
400
300
200
100
0
1
2
3
4
5
6

Fig. 40. Influence of vitamin E, cycloheximide and decoy on the X/XO (pre) or Fe^2-mediated alterations of SOD-1 and SOD-2 expression. *Primary hippocampal cultures of postnatal rats (day 10) were incubated for 15 min with X/XO (pre) or with 100 µM Fe^{2+}. The radical-scavenger vitamin E (10 µM) (Vit E) was added simultaneously with the preconditioning agents and removed together with Fe^{2+} and X/XO (pre). Cycloheximide (CHX) (0.1 µM), decoy (5 µM) and nonsense (5 µM) remained in the culture medium until the cells were harvested. To determine the time dependency of SOD-1 and SOD-2-expression the cells were collected in lysis buffer after 4 h and SOD-1 (a + b) and SOD-2 (c + d) were determined by western blot analysis. Semi-quantitative analysis was performed by determination of the integrated optical density (IOD) using scion image software to support the visual impression. Controls were arbitrarily expressed as 100%.*

4 Discussion

4.1 Reactive oxygen species as mediators of neuronal damage

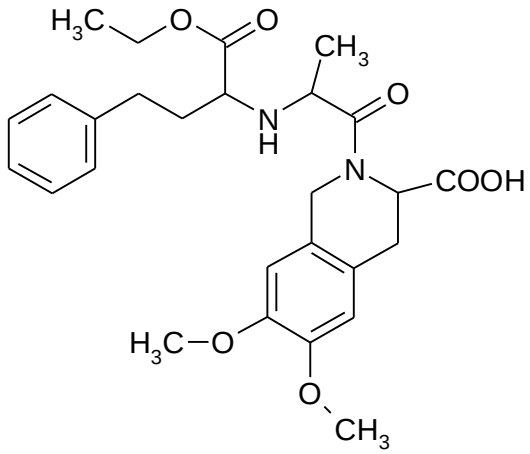
4.1.1 Discussion of drug effects

The excess generation of reactive oxygen species (ROS) was widely described to be a key event in several neurodegenerative diseases such as AD, PD and ALS as well as in stroke. Thus, ROS were considered as important molecular targets for chemical interventions by several drugs. Also the current work investigated the effect of ROS-over-stimulation by different damaging agents on the survival of neuronal cells. Further, drugs with radical scavenging properties which are still promising candidates for the treatment of neurodegenerative diseases were examined in different models of neurodegeneration.

4.1.1.1 Effect of ACE-inhibitors

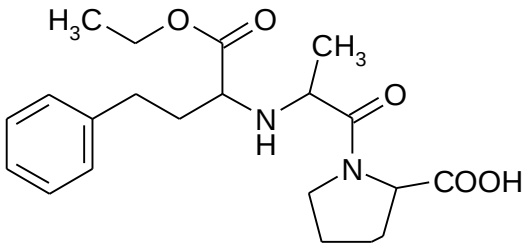
The ACE-inhibitors enalapril and moexipril that were investigated in the current study are established drugs for the therapy of hypertension and chronic heart failure. The antihypertensive mechanism includes reduced formation of angiotensin II which is a potent vasoconstrictor and activator of aldosterone secretion. Further, the endogenous degradation of vasodilatory bradykinins is reduced which might contribute to the antihypertensive properties (Brunner et al. 1988; Schoenberger 1988).

Several studies have described beneficial properties of ACE-inhibitors especially concerning cardio- and nephroprotection ((Megarry et al. 1997; Megarry et al. 1997). In contrast to the heart and the kidney little is known about the effect of ACE-inhibitors on brain function. Since the existence of an intracerebral renin-angiotensin system has been characterized, increasing studies tried to investigate the effect of ACE-inhibition on cerebral pathways.



Moexipril

MW: 499 g/M



Enalapril

MW: 376 g/M

Fig. 41. Chemical structures of enalapril and moexipril

Recently, neuroprotective properties have also been described. In the current study, it was demonstrated that the ACE-inhibitors enalapril and moexipril were protective against glutamate-, staurosporine- or $Fe^{2+/3+}$-induced damage in cultured neurons although the neurotoxic mechanisms of the damaging agents were partially different. Over-activation of glutamate receptors was known to entail excitotoxic cell damage via various processes including Ca^{2+} overload, activation of proteases, phospholipases and endonucleases (Tymianski et al. 1993; Prehn and Krieglstein 1996). Ferrous ions could trigger DNA strand breaks (Hartwig and Schlepegrell 1995). The mycotoxin staurosporine has been shown to induce apoptosis activating a cell death program common to nearly all cells and the pro-apoptotic effect of staurosporine involved activation of the sphingomyelin-ceramide pathway with an onset of caspase cascade (Bertrand et al. 1994; Wiesner and Dawson 1996; Krohn et al. 1998). Besides these different mechanisms, glutamate-, staurosporine- and $Fe^{2+/3+}$-induced elevation of intracellular ROS is a common mechanism of these agents which seems to play an important role in ongoing cell death (Lafon-Cazal et al. 1993b; Prehn et al. 1997).

ROS containing only a single electron in the outer electron orbital, cause high chemical reactivity capable of damaging lipids, proteins and nucleic acids (Barber and Harris, 1994). In addition, an increased release of excitatory amino acids was observed in the presence of oxygen free radicals indicating that ROS reinforce the deleterious cascade of excitotoxic damage (Pellegrini-Giampietro et al. 1988). Several studies revealed that antioxidants like tocopherol, ascorbic acid and glutathione as well as the support of enzymatic defense mechanisms against oxidative damage promoted cellular survival in different in vitro and in vivo models (Liu et al. 1989; Clemens and Panetta 1994; Stoyanovsky et al. 1998). The present study showed that enalapril and moexipril were equally potent free radical scavengers inhibiting staurosporine-, glutamate- and $Fe^{2+/3+}$-induced ROS generation. Both ACE-inhibitors were also able to improve viability of chick embryonic neurons when simultaneously given to the culture medium with the damaging agents L-glutamate or $Fe^{2+/3+}$. In addition, enalapril and moexipril significantly reduced staurosporine-induced neuronal apoptosis. Because the antioxidant activities of the ACE-inhibitors paralleled their neuroprotective capacities, the radical scavenging properties of the ACE-inhibitors enalapril and moexipril seemed to represent the key event in promoting neuronal survival. This is in accordance with other authors who observed protective effects of ACE-inhibitors in myocardial cells due to their ability to exert radical scavenging activities (Anderson et al. 1996; Satoh and Matsui 1997).

The antioxidant properties of ACE-inhibitors could be caused by a direct chemical interaction of ACE-inhibitors and ROS. But it is conceivable that other mechanisms contribute to the observed protection against ROS-induced neuronal damage. Especially, intracellular antioxidant pathways may play a role. It has been described that captopril and enalapril were able to enhance antioxidant defenses by upregulation of superoxide dismutase or glutathione peroxidase in different mouse tissues (de Cavanagh et al. 1997). Thus, the mechanism of ROS-scavenging might include the promotion of intracellular pathways. On the other hand, the observed antioxidative effects of ACE-inhibitors in the current study appeared already after 1 h and were dose-dependent. Further the maximal ROS-scavenging potency against glutamate-, staurosporine- or iron-mediated elevation of ROS-levels was observed at 100 µM enalapril or moexipril supporting the involvement of direct ROS-scavenging.

To clarify whether the neuroprotective properties of the ACE-inibitors enalapril and moexipril obtained in vitro were also relevant in vivo, these drugs were investigated in a mouse model of permanent focal cerebral ischemia by co-workers of the institute (V. Junker and M. Kouklei). This revealed that 1 h pretreatment with 0.3 mg/kg moexipril as well as 0.03 mg/kg enalapril could reduce the infarct volume caused by middle cerebral artery occlusion (data not shown). Neither lower nor higher doses were effective indicating a narrow therapeutic window for ACE-inhibitor treatment (Ravati et al. 1999).

ACE-inhibitors are well known to reduce blood pressure, and although long-term blood pressure control is thought to reduce the incidence of stroke, acute blood pressure reduction after stroke is an event which worsens neurological outcome (Kelley 1996; Lees and Dyker 1996). Therefore, we presumed that doses of enalapril and moexipril higher than 0.3 mg/kg which did not protect mice against cerebral ischemia could decrease mean arterial blood pressure.

To address this hypothesis and to confirm the protective effects observed in the mouse model moexipril was also tested in a rat model of focal cerebral ischemia. Here also the physiological variables such as mean arterial blood pressure, pCO_2, pO_2, pH and glucose levels were monitored. Again, moexipril was able to reduce the brain infarct volume after ischemia caused by middle cerebral artery occlusion. Interestingly, only the dose of 0.01 mg/kg which did not affect blood pressure levels was effective whereas 0.1 mg/kg moexipril that significantly decreased mean arterial blood pressure of normotensive rats when measured 30 and 45 min after drug administration failed to exert protection against ischemic damage. Therefore, in order to gain acute protection against stroke it seems to be important to achieve a plasma concentration of the ACE-inhibitor which does not cause hypotension.

Many pathophysiological events responsible for neuronal degeneration like activation of excitatory processes with subsequent accumulation of intracellular Ca^{2+} are triggered by cerebral ischemia (Choi 1988). These events initiate a cascade with deleterious consequences, the mechanism of which are not yet completely understood. However, the formation of ROS seems to represent a final pathway of ischemic neuronal damage (Siesjo et al. 1989; Christensen et al. 1994). Especially in the penumbra zone of the developing infarct after middle cerebral artery occlusion a persisting blood flow with concomitant oxygen supply is maintained (Ginsberg and Pulsinelli 1994). In the penumbra region free radicals were suggested to be the major mediator of increased neuronal cell death. It was shown that radical scavengers protected penumbral brain tissue and reduced cerebral damage after ischemia

(Wolz and Krieglstein 1996). Thus, also in vivo the radical scavenging properties of the ACE-inhibitors enalapril and moexipril which were clearly demonstrated in vitro could be an important mechanism of the observed cerebroprotective effect.

However, in our study the effective concentrations of the ACE-inhibitors used in vitro were relatively high as 1 µM was necessary to achieve ROS scavenging as well as neuroprotection. In contrast, the presumable plasma concentration of the ACE-inhibitors in vivo following administration of the effective dose (0.01 mg/kg) in the rat model was expected to be lower. As we have no data about the concentration of enalapril and moexipril within brain tissue after intraperitoneal administration it is difficult to compare the doses used in vivo with in vitro concentrations. Nevertheless, the fact that we did not measure the formation of ROS in vivo and the differences in the effective doses of the ACE-inhibitors in vivo and in vitro generates some open questions concerning the participation of free-radical scavenging in the apparent neuroprotective effect in rodents.

Recently, even the fluorescence microscopy methods that we used in vitro to monitor the formation of ROS was partially criticized concerning reliability of the obtained results. Especially publications from Rota et al. which simultaneously appeared in the "Journal of Biological Chemistry" and in "Free Radicals in Biology and Medicine", two high standard journals, gained a lot of attention (Rota et al. 1999; Marchesi et al. 1999). The authors described that measurements of ROS with the frequently used dye 2´-7´-dihydrodichlorofluorescin-diacetate (DCF-DA) are problematic because the dye exhibited auto-oxidative properties in the presence of horse-radish peroxidase. As determined by electron spin resonance spectroscopy in a cell-free system the deacetylation of the dye by esterases caused the chemical or enzymatic generation of H_2O_2. Peroxidase and the self-generated H_2O_2 further oxidised DCF to the semiquinone free radical $DCF^{-\cdot}$ which caused secondary superoxide anion radical generation. Therefore, the authors suggested to measure ROS with the more reliable electron resonance spectroscopy and to be careful with the use of the dye DCF-DA. However, in our study dihydrorhodamine 123 (DHR 123) was used to monitor the formation of ROS. So far no reports appeared that described such strong auto-oxidative capacities for DHR 123. Furthermore, as all cells were treated equally with the dye, a possible auto-oxidative function of DHR 123 would have resulted in a constantly increased basal fluorescence level in all cultures. As only relative fluorescence alterations and no absolute ROS concentrations were determined in our experiments, such a systematic auto-oxidation could hardly influence the reliability of the used fluorescence microscopy. This

view is supported by the fact that the authors in the mentioned studies confessed that the auto-oxidative effect of DCF-DA on the formation of ROS was rather low as compared to the exogenous stimulation with H_2O_2 (Rota et al. 1999).

Apart from such problems the intracellular measurement which requires the application of oxidation sensitive dyes offers the advantage of sub-cellular localization of oxidative processes. For instance, the current investigation revealed that most of the observed fluorescence was probably located in mitochondria which could be identified by a dot-like intracellular fluorescence pattern. Nevertheless, the recent findings strongly suggest that fluorescence microscopy ROS-measurements have to be interpreted cautiously and can only be used for relative comparisons of fluorescence intensities.

In vivo, the formation of ROS or the influence of ACE-inhibitors on ROS generation was not monitored. Thus, it cannot be excluded that other mechanisms of action could also contribute to the observed protective capacities of ACE-inhibitors. Trandolapril and quinapril, for instance, have been demonstrated to protect spontaneously hypertensive rats from stroke by inhibiting fibrinoid necrosis (Richer et al. 1994; Vacher et al. 1993). Furthermore, the role of ACE-inhibitor-induced increase in endogenous bradykinin concentration was considered to be involved in the protection of guinea pig heart by ramipril (Massoudy et al. 1994).

The question arises whether inhibition of the cerebral renin angiotensin system is involved in ACE-inhibitor-mediated neuronal protection. Recently, some studies have focused on the effect of angiotensin II on cellular viability. There is evidence that exogenous angiotensin II precedes deleterious events within several types of cells. For example, angiotensin II was shown to induce apoptosis in cardiac myocytes and human endothelial cells, and different mechanisms like activation of caspases and p53 protein as well as elevation of intracellular Ca^{2+} levels and inactivation of the antiapoptotic protein Bcl-2 have been discussed (Dimmeler et al. 1997; Horiuchi et al. 1997; Leri et al. 1998).

However, it seems unlikely that in the present in vitro studies the ACE-inhibitor mediated suppression of angiotensin II formation is involved in neuroprotection, because even if angiotensin II was secreted by the neurons under damaging conditions it would have been rapidly diluted in the culture medium and thus not reach concentrations high enough to induce neuronal damage. It has been shown in vivo that angiotensin II is a potent cerebral vasoconstrictor of smooth muscle cells directly and indirectly by elevating the release of catecholamines and excitatory neurotransmitters from sympathetic neurons (Dzau 1988). Angiotensin II may impair cerebral blood flow during ischemia and therefore trigger ischemic

damage (Haas et al. 1985). In contrast, there is evidence that angiotensin II may also have protective effects and recent data showed that angiotensin II promoted the regeneration of retinal neurons (Lucius et al. 1998). Moreover, an increase in blood pressure and collateral blood flow has been suggested to mediate the protective effect of angiotensin II in a model of focal cerebral ischemia in gerbils (Kaliszewski et al. 1988). It is assumable that although ACE-inhibition probably occurred, neither of the above mentioned mechanisms related to angiotensin II is a dominant factor in our in vivo models because we showed acute effects while in previous studies a prolonged pre-treatment was examined which was necessary for a constant reduction of angiotensin II levels in the central nervous system (Stier, Jr. et al. 1989).

However, the functional role of cerebral angiotensin II in ischemic processes remains unclear. Because ACE-inhibitors have been shown to cross the blood brain barrier under ischemic conditions and to inhibit ACE in cerebral brain tissue (Jouquey et al. 1995; Werner et al. 1991), further studies have to clarify whether this inhibition of brain renin angiotensin system might contribute to the ACE-inhibitor-mediated acute protection against stroke in normotensive animals.

Taken together, we conclude that the ACE-inhibitors enalapril and moexipril possess neuroprotective properties most importantly due to their ability to scavenge ROS. Beside blood pressure reduction, the neuroprotective capacities of ACE-inhibitors could be an important additional benefit for the treatment of hypertensive patients with an elevated risk of stroke.

4.1.1.2 Effect of estrogens

Within the mammalian organism estrogens fulfil several important regulatory endocrine functions. The physiological estrogen 17ß-estradiol is broadly used for the therapy of estrogen deficits or other therapeutic indications (see introduction). Recently, estrogens have also been found to mediate various functions within the brain (Behl et al. 1995). Such functional regulation included neurotransmitter receptor expression, excitability of neuronal membranes and the process of synapse formation during development and regeneration (Honjo et al. 1995; Green et al. 1997). Further, a pathological or physiological estrogen deficit appeared to cause neurodegenerative processes. For instance, the fact that elderly women are twice as likely to develop AD, as compared to men leading to a faster cognitive decline and

accelerated neuronal cell death, was explained by the postmenopausal estrogen deficit (Rocca et al. 1991; Simpkins et al. 1994).

In the present study, a protective activity of the estrogens 17ß-estradiol and 2-OH-estradiol against iron-induced neuronal cell death in primary cultures of chick embryonic neurons was demonstrated. In addition, the suppression of mitochondrial ROS-production and the neuroprotective effect of these estrogens was shown to be mediated through their antioxidative properties. These data are consistent with previous studies showing that due to their antioxidative capacities estrogens suppressed iron-induced lipid-peroxidation of liver microsomes (Ruiz-Larrea et al. 1994), reduced oxidative damage in rat cortical synaptosomes (Keller et al. 1997) and preserved vascular relaxation due to decrased oxidation of low-density lipoprotein (Keaney, Jr. et al. 1994).

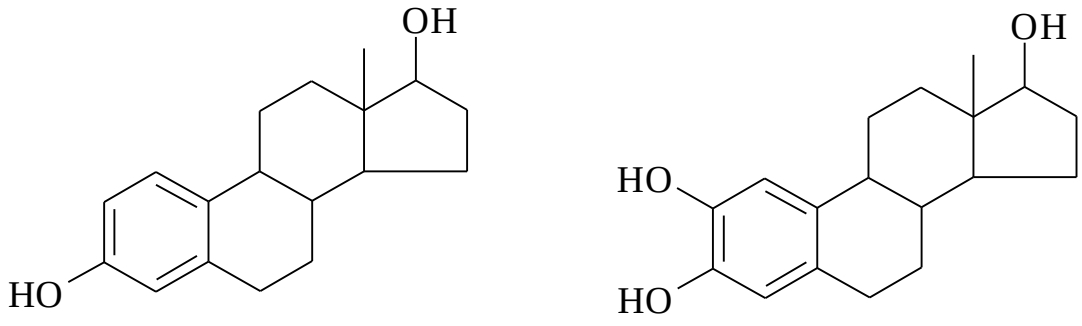
In most of the previous studies micromolar concentrations of the estrogens were used to demonstrate neuroprotection against trophic factor withdrawal and cytotoxic stimuli including Aß, iron, glutamate or hydrogen peroxide (Behl et al. 1997; Goodman et al. 1996;Mattson et al. 1997). The concentrations used in earlier investigations were thus always higher than physiological, nanomolar blood levels. From those in vitro experiments the capacity of estrogens to scavenge cytotoxic free radicals at rather high concentrations was suggested as the underlying mechanism of neuroprotection. By measuring neuronal damage and production of ROS following exposure to iron, the current study demonstrated that 2-OH-estradiol is capable of protecting neurons from oxidative stress already at nanomolar concentrations (10 nM). 17ß-estradiol showed antioxidative effects only if added to the cell culture medium at micromolar concentrations (1-10 µM) which is in line with previous reports. The concentrations of both estrogens required for protection against iron-induced oxidative stress in vitro were in the order of magnitude that were expected to activate their receptors (10-1000 nM).

Thus, the question arose if the observed neuroprotection was at least partially estrogen receptor mediated. These receptors which are expressed in several brain regions, predominantly in the limbic system and in the cortex, are estrogen-responsive transcription factors. Consequently, the synthesis of several important proteins that regulate growth such as NGF, synaptic plasticity and the acetylcholine level in cholinergic neurons is relevant for neuronal functions (Mudd et al. 1998; Honjo et al. 1995). In this line, estrogen receptor-mediated neuroprotection was suggested to include upregulation of neurotrophins such as NGF (Singh et al. 1995) and the NGF high affinity receptor TrkA (Gibbs 1998). 17ß-Estradiol protected human neuroblastoma cells against ß-amyloid toxicity already at nanomolar

concentrations ameliorating both lipid peroxidation and persistent lactate production (Green et al. 1996; Gridley et al. 1997).

Conversely, there is also evidence from literature that neuroprotection by estrogens does not necessarily depend on activation of estrogen receptors because higher concentrations than expected for estrogen receptor stimulation were needed to obtain neuroprotective effects in other studies (Behl et al. 1997; Behl et al. 1995; Goodman et al. 1996). It has been shown in a neuroblastoma cell line that 17-α-estradiol, which does not bind to the estrogen receptor, prevented cell death induced by serum deprivation similar to 17ß-estradiol (Green et al. 1997). Furthermore, 17ß-estradiol prevented iron-induced membrane oxidation independently of estrogen receptor-mediated transcriptional activation in a synaptosome preparation (Keller et al. 1997).

Also in the current study, the estrogen receptor antagonist tamoxifen did not influence either the neuroprotective effect or the antioxidative capacity of the estrogens indicating that the effects were mediated by the inherent antioxidant capacity of the steroids investigated. This hypothesis is supported by the structure-related properties of estrogens (Fig. 42).



17-ß-estradiol 2-OH-estradiol

Fig. 42. Chemical structure of the estrogens 17ß-estradiol and 2-OH-estradiol

Estrogens are steroidal compounds with a phenolic structure. Like vitamin E, they contain a hydroxyl group at a mesomeric ring system and a lipophilic carbohydrate tail. It has been shown that a phenolic hydroxyl-group can donate a hydrogen atom to different ROS and thereby detoxify them (Halliwell and Gutteridge 1988). Concerning 17ß-estradiol, a structure-activity study showed that the hydroxyl group at the C3 position of the steroid A ring confers the neuroprotective antioxidant activity (Behl et al. 1997).

Although neuronal death may be prevented by estrogens through receptor-dependent as well as receptor-independent mechanisms, the present data suggest that estrogen effects were predominantly mediated by a non-genomic, direct, chemical interaction.

To further investigate the relevance of the observed neuroprotection of estrogens in cell cultures another co-worker of the institute (V. Junker) also administered estrogens to mice which were subjected to cerebral ischemia. Four series of ischemia experiments were performed in mice in order to test the protective capacities of 17ß-estradiol and 2-OH-estradiol against brain damage caused by permanent MCA occlusion. Both steroids investigated were able to ameliorate the ischemia-induced brain damage in the model of focal cerebral ischemia in mice (data not shown). 17ß-Estradiol (0.3-3 mg/kg) significantly reduced the infarct area on the mouse brain surface. Doses of 17ß-estradiol lower than 0.3 mg/kg showed no effect. 2-OH-Estradiol reduced the infarct area at dosages of 0.03-30 mg/kg. The lowest effective dosage of 2-OH-estradiol was determined at 0.03 mg/kg, although in one series of experiments this steroid protected brain tissue from ischemic damage already at 0.003 mg/kg (data not shown). Also the mortality of mice which were treated with estrogens was markedly reduced during the entire experiment. In the four series of ischemia experiments performed in this study 8 out of 55 vehicle-treated mice died after MCA occlusion. In contrast, only 4 out of 57 mice and 4 out of 122 animals died in the groups treated with 17ß-estradiol and 2-OH-estradiol, respectively.

There is strong evidence demonstrating that scavenging free radicals exerts protection of brain tissue against damage caused by MCA occlusion (Wolz and Krieglstein 1996). Furthermore, recently published data showed that gender differences in response to focal cerebral ischemia in rats are due to the different estrogen levels in female versus male animals (Alkayed et al. 1998; Zhang et al. 1998). In these studies female rats showed smaller infarct sizes and higher cerebral blood flow than male animals after transient MCA occlusion. Moreover, the loss of female sex steroids in ovarectomized female rats was demonstrated to increase the infarct volume as compared to control female animals (Hurn and Macrae 2000).

Chronically administered 17ß-estradiol abolished the increased susceptibility to ischemic brain damage in ovarectomised rats (Zhang et al. 1998).

The present in vivo experiments have shown that 17ß-estradiol protected brain tissue from ischemic damage in male mice when administration was started 24 h before ischemia. Moreover, 2-OH-estradiol reduced the ischemic brain damage after MCA occlusion already at doses 10 times lower than the 17ß-estradiol doses needed for protection. A similar finding was observed in vitro as far as 2-OH-estradiol reduced intracellular ROS concentration and neuronal cell death at concentrations 100 times lower than that of 17ß-estradiol. These results confirm the view that the structure-related neuroprotective effect of estrogens against oxidative stress in vitro (Behl et al. 1992) also helps to predict cerebroprotective activities in vivo.

In conclusion, the present study demonstrated that neuroprotection against oxidative damage by estrogens was most likely caused by the antioxidant properties of these steroids. The reduced amount of ROS in vitro occurred at concentrations that were relevant for the cerebroprotective activities of the estrogens observed in the mouse model of focal cerebral ischemia. Thus, estrogens and especially the catechol estrogen 2-OH-estradiol which was shown to be more potent than the physiological 17ß-estradiol may prove efficacious as neuroprotectants in the therapy of neurodegenerative diseases and ischemic stroke.

4.2 Reactive oxygen species as mediators of signal transduction and neuroprotection

4.2.1 Neuronal preconditioning by reactive oxygen species – criteria for neuroprotection or neurodegeneration

In the current study, it was demonstrated that a mild and short stimulation of cultured chick neurons with ROS, generated by X/XO (pre) or Fe^{2+}, made them less sensitive to subsequent insults. This effect of pre-treatment which initiated a neuroprotective intracellular pathway was called preconditioning. The fact, that blocking the initial ROS accumulation by antioxidants abolished the neuronal preconditioning, supplies evidence for an important role of ROS signaling for neuronal survival. This preconditioning effect of ROS was similarly observed against glutamate-, staurosporine-, and X/XO (dam)-mediated neuronal damage although the neurotoxic mechanisms of these agents were partially different. Glutamate

mediates its toxicity through various processes including Ca^{2+}-overload, and activation of proteases, phospholipases and endonucleases (Prehn et al. 1997; Tymianski et al. 1993). The mycotoxin staurosporine, widely used for induction of apoptosis, involves PKC inhibition and an activation of the sphingomyelin-ceramide pathway with an onset of the caspase cascade (Wiesner and Dawson 1996; Krohn et al. 1998). In line with previous findings (Lafon-Cazal et al. 1993c; Prehn and Krieglstein 1996), we found a strong enhancement of oxygen radical production by glutamate or staurosporine treatment. As exposure to X/XO (dam) also entailed massive radical formation in our neuronal cultures, elevation of oxygen radicals seemed to represent a common neurotoxic pathway of all the damaging agents used.

On the one hand the current study has revealed that in different models, where excess release of oxygen radicals promoted neurodegeneration, radical scavengers like vitamin E, 17ß-estradiol, 2-OH-estradiol or ACE-inhibitors mediated neuronal survival. Because aberrant ROS formation was shown to be a final pathway of ongoing cell death (Siesjo et al. 1989), the control of cellular ROS release seems to be a promising strategy to prevent neuronal damage.

Conversely, it was also demonstrated that a short and moderate ROS-stimulation of neuronal cultures markedly reduced the glutamate-, X/XO (dam)- or staurosporine-induced burst of oxygen radical formation. The results suggest that such mild ROS-stimulation which entailed resistance to subsequent oxidative death was the common underlying mechanism of the observed neuroprotective effect against different neurotoxic agents.

Such contrary results concerning the effect of ROS leading to either neurodegeneration or neuroprotection, reveals the necessity to obtain further detailed knowledge on how to interfere with the amount of ROS produced in organisms. Thus, the question arose under which conditions ROS promote neuroprotection and when they cause deleterious events leading to neurodegeneration. To investigate this discrepancy of radical action, an incubation-dependency of ROS stimulation by X/XO and Fe^{2+} on neuronal viability was performed.

In a first experimental series a concentration-dependent toxicity of short ROS-stimulation with X/XO or Fe^{2+} concerning neuronal viability was demonstrated. The highest possible subtoxic concentrations (X/XO :10 µM / 0.5 mU x ml^{-1}; Fe^{2+}: 100 µM) were then used to check for time-dependency of the observed preconditioning against glutamate-induced damage. This revealed that ROS incubation with X/XO for 15 min (= X/XO (pre)) significantly reduced neuronal damage by glutamate. The protective effect was still achieved by a 1 h incubation but the efficacy of protection was already lower. ROS-incubation for 4 h

and 8h had no protective effect anymore while 24 h X/XO-treatment aggravated the glutamate-induced neuronal damage. A similar effect was observed when Fe^{2+} was used to stimulate the cells with ROS, showing a maximal reduction of glutamate toxicity at 15 min incubation time. Prolonged exposure to Fe^{2+} caused a severe and time-dependent enhancement of neuronal damage. The turn-round incubation time which revoked neuroprotection into neuronal damage was found between 1h and 4 h ROS stimulation.
Therefore, the effect of exogenous stimulation with ROS-inducing agents is clearly dependent on the concentration of generated ROS and on the duration of exposure. A short and moderate stimulation probably initiates intracellular signal transduction which mediates resistance to oxidative stress. In contrast, exceeding a certain border concentration or incubation time the ROS-mediated direct damage of proteins, lipids and DNA is conceivably overcoming the effect of endogenous protective signaling pathways.

The results have further shown that hydroxyl radicals which are predominantly generated by Fe^{2+}, caused a more severe toxicity at longer exposure times than the X/XO-mediated superoxide anion radicals. This might be explained by the higher reactivity of hydroxyl radicals with intracellular structures leading to an accelerated onset of toxicity (Beckman 1994; Halliwell and Gutteridge 1988). Therefore, also the type of the involved ROS seems to play an important role for the direction of radical functions.

Vitamin E and 2-OH-estradiol were employed as a tool to block the formation of ROS because they had been characterised as effective ROS scavenging compounds in the present study. As already described, the effect of estrogens appeared to be independent of estrogen receptor activation but were rather due to direct chemical interactions requiring a phenolic structural moiety (Moosmann and Behl 1999). Interestingly, both ROS-generators, X/XO (pre) predominantly producing superoxide anion radicals and Fe^{2+} generating hydroxyl radicals were equally effective as preconditioning agents. The most striking difference between both types of stimuli was that the antioxidants vitamin E and 2-OH-estradiol prevented the X/XO (pre)-mediated effects on massive oxygen radical production and neuronal damage, whereas the Fe^{2+}-induced preconditioning could not be blocked by the radical scavengers. Monitoring the ROS-formation kinetics during the 15 min period of preconditioning revealed similar kinetics for X/XO (pre) and Fe^{2+}, but the capacities of the antioxidants to inhibit the initial ROS production were different. While the X/XO (pre)-mediated ROS formation could be immediately reduced, a significant effect of antioxidants on Fe^{2+}-dependent ROS formation could only be measured at 15 min of incubation but not

during the first 10 min. Even when pre-treatment, higher concentrations or other antioxidants were used, the initial Fe^{2+}-induced increase of ROS could not be abolished. This indicates a strong reactivity of the induced Fenton´s reaction by Fe^{2+} probably generating hydroxyl radicals that are highly reactive and immediately oxidize intracellular structures and also the dye DHR 123 during measurement.

Thus, an immediate and continuous radical scavenging seems to be necessary to block ROS-mediated preconditioning by antioxidants. The mild and short 10 min episode of Fe^{2+}-induced ROS formation despite the presence of antioxidants was already sufficient to set a signal initiating intracellular response mechanisms that mediated protection against subsequent insults. This is in line with results from another experimental series where the time window of preconditioning with Fe^{2+} or X/XO (pre) was determined. This revealed that already 10 min of preconditioning mediated significant protection (neuroprotection was maximal at 15 min and disappeared after 1 h).

In the present study, moderate stimulation of neuronal cultures with ROS clearly mediated neuroprotection against different forms of neuronal damage and effective scavenging of ROS during the preconditioning time period abolished this protection. As inhibition of excessive oxygen radical formation under damaging conditions appeared to be the underlying mechanism of such preconditioning, ROS are obviously effective signaling molecules.
Such second messenger functions of ROS makes sense in different ways:

1. Because the half-life of ROS is generally short they are transiently active which means that their signals are only delivered at specific times.
2. As the intracellular concentration of ROS at physiological conditions is rather low and locally bound, their signals can be localized and targeted.
3. ROS can react with antioxidant molecules or enzymes which facilitates easy insulation of the radical signal, thereby minimizing its effect on other pathways.

In line with this, some authors went even further, postulating that too low intracellular levels of ROS are equally deleterious for cellular function and survival as to much oxidative stress (Mattson 1998). The dose-dependency of radical actions on neuronal function, plasticity and death is illustrated in Fig.43.

Conclusively, it seems to be important for the cell to maintain a minimum level of ROS which is sufficient to mediate necessary signal transduction pathways but to stay below the toxic border concentration.

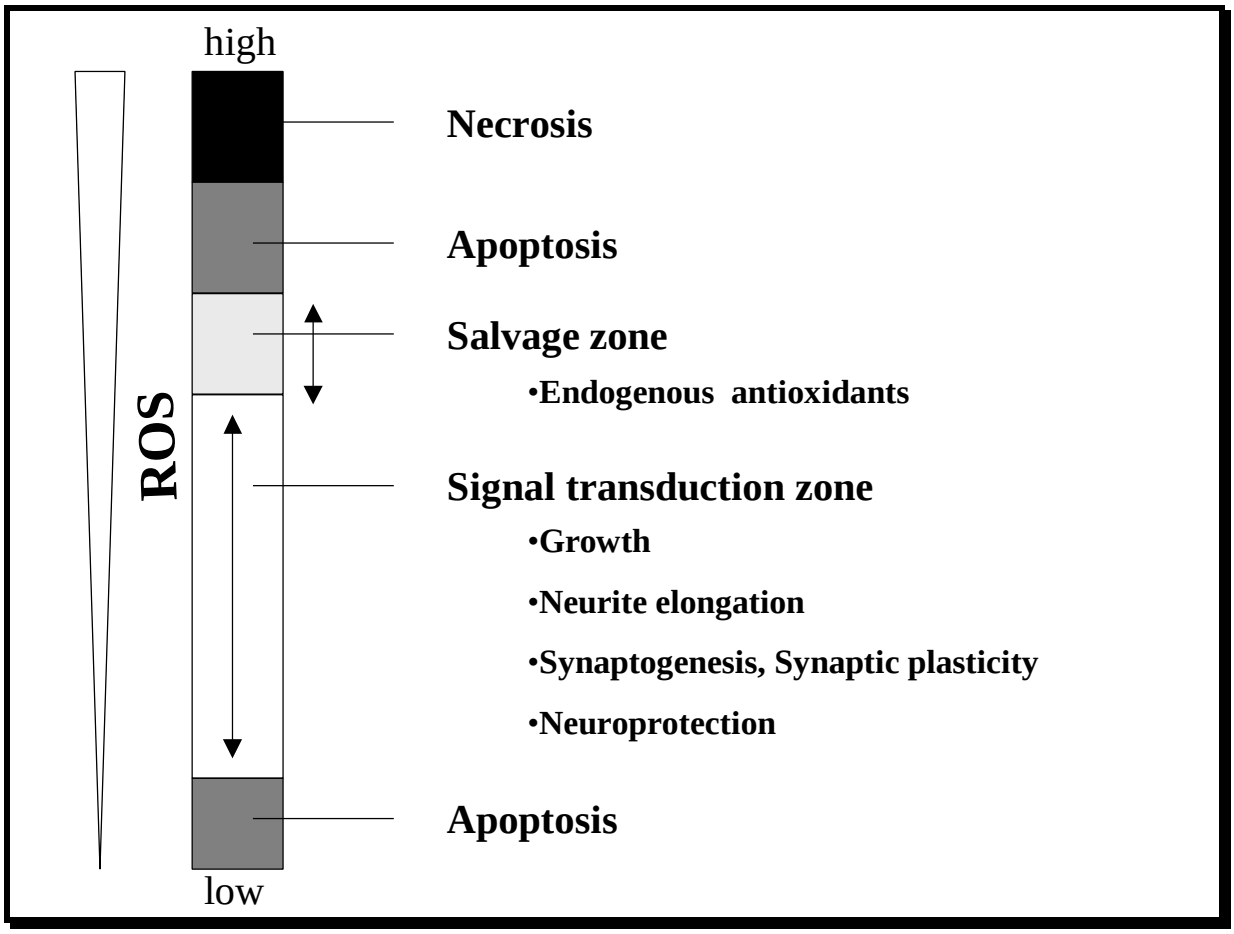


Fig. 43. Role of reactive oxygen species for neuronal function and death (modified from Mattson, 1998)

The induction of mild, sublethal oxidative stress seems to be a beneficial treatment to protect neurons against various insults. However, as the mechanism of the observed ROS-mediated neuroprotection so far remained to be clarified, the model was changed from chick neurons to hippocampal cultures from neonatal rats. While the use of chick neuronal cultures offers some experimental advantages such as appropriate stability and the possibility to investigate neuron-selective pathways, there are also some disadvantages of cultures derived from chick species. For instance, antibodies that were necessary to further investigate intracellular signaling pathways were hardly available. Further, the relevance of the results obtained from experiments with chick neurons has to be interpreted cautiously because such results are far away from clinical models and even from animal models with rodents. Therefore, the model of ROS-mediated preconditioning was established in mixed hippocampal cultures where all further studies were performed.

4.3 Possible mechanisms of reactive oxygen species–mediated neuroprotection and regulation of intracellular signaling

So far, the present study has shown that preconditioning of cultured neurons against subsequent damage was clearly mediated by ROS but the intracellular signaling pathway that could execute such protective role of ROS remained to be clarified.

Generally, the mechanisms of preconditioning or tolerance within the brain which increasingly became object of investigation in recent years are still not fully elucidated. Several enzymes, channels, receptors and modified expression of potentially protective or degenerative proteins have been described to be involved in brain preconditioning.

It is remarkable, that also the mechanism of cyanide-induced preconditioning was investigated in the present study. First investigations by a colleague of the institute (M. Jensen) revealed that short stimulation of cultured neurons with cyanide (0.1 mM; 1h) mediated protection against subsequent severe insults with the same agent (1 mM, 1h). Further, the mechanism of this protection against severe cyanide damage which surprisingly went out to display characteristics of apoptotic cell death, was studied. The results have shown that ROS were not involved as no changes of ROS content were found at any time point after cyanide treatment which is in agreement with prior studies of cyanide in hippocampal cultures (Sengpiel et al. 1998). Cyanide is a potent inhibitor of complex IV of the mitochondrial respiratory chain and therefore, the current study investigated the influence of cyanide-induced preconditioning on mitochondrial functions. Severe cyanide treatment (1 mM, 1h) was found to induce a marked breakdown of mitochondrial membrane potential. Preconditioning with cyanide inhibited the loss of mitochondrial membrane potential which is suggested to contribute to the observed neuroprotection (data not shown). In parallel, moderate cyanide treatment prevented a subsequent loss of the mitochondrial antiapoptotic oncogens Bcl-2 and Bcl-xl further supporting the hypothesis that the mechanism of cyanide preconditioning involved preservation of mitochondrial functions.

Despite the obviously various processes and cascades that are initiated by the onset of preconditioning, the existence of a common downstream regulator collecting the information of intracellular signal transduction has been proposed (Grilli and Memo 1999b).

One interesting candidate could be the transcription factor NF-kB which was found to be activated in the central nervous system by numerous agents, such as the inflammatory cytokines TNFα, IL-1ß, phorbolesters, oxidative stress, UV-light, bacterial and viral proteins,

depolarization, glutamate, opioids, nerve growth factor or Aß (Baldwin, Jr. 1996; O'Neill and Kaltschmidt 1997; Schutze et al. 1992). In the current study the neuroprotective effect of preconditioning with the ROS-stimulating agents X/XO (pre) or Fe^{2+} was accompanied by an activation of NF-kB. We used three different approaches to inhibit NF-kB: the antioxidants vitamin E, 2-OH-estradiol and PDTC, the proteasome inhibitor lactacystine and the oligonucleotide decoy. Apart from scavenging ROS by the used antioxidants which appeared to participate in the inhibition of NF-kB, PDTC has also been described to exert its NF-kB-inhibiting properties independent of antioxidant activity, probably due to direct interaction with the p50 subunit of the dimeric complex (Brennan and O'Neill 1996). Lactacystine blocks the proteolytic complex proteasome which is required to release free cytosolic NF-kB by cleaving the inhibitory subunit I-kB-α upon phosphorylation and ubiquitination while decoy reduces the NF-kB-binding to appropriate gene promotors by competing with nuclear DNA (Kaltschmidt et al. 1997a). In the current experiments each of the mentioned inhibitors blocked the protective effect of preconditioning despite their different mechanisms indicating that this effect was not substance-specific and that the observed neuroprotection evidently involved NF-kB activating pathways. Of interest, it was demonstrated that the Fe^{2+}-mediated preconditioning could be abolished by PDTC but not by vitamin E or 2-OH-estradiol supporting the hypothesis of an additional mechanism of PDTC-mediated NF-kB inhibition.

However, the revealed protective role of NF-kB in our experiments at least partially contrasts the results of former studies. The role of NF-kB as a mediator of either survival or degeneration seems to depend on certain circumstances. One proposed criterion is the activation kinetics of NF-kB which is induced by the stimuli. In the current study, a rather transient activation pattern of NF-kB by the preconditioning agents was found with a maximal activation after 4 h slowly declining to very low activity after 24 h. This finding is in line with previous studies suggesting that transient activation of NF-kB is protective whereas long-term activation entails deleterious events (Schneider et al. 1999). It was shown that NF-kB activation could serve proapoptotic and antiapoptotic functions even within the same cell type depending on different intracellular signaling pathways that mediated the activation (Lin et al. 1999). The functional role of NF-kB seems to rely on further variables including composition of inducible NF-kB complexes, concomitant activation of other transcription factors, intrinsic metabolic and genetic differences between neuronal phenotypes and on the nature and intensity of the activating stimulus (Baichwal and Baeuerle 1997; Grilli and Memo 1997: Lin et al. 1998).

NF-kB-triggered transcriptional activity of neuronal cells results in the expression of proteins with either pro-apoptotic activity such as p53, amyloid precursor protein, bax or interleukin-converting enzyme or with antiapoptotic function such as mitochondrial superoxide dismutase (SOD-2) or bcl-2 (Grilli and Memo 1999b). Especially the exact identification of the genes and proteins that are under transcriptional control of NF-kB will provide further insight into its functional relevance and the possibility for pharmacological intervention.

In the present study it was shown that ROS probably mediated the effect of preconditioning as the suppression of ROS formation abolished the observed neuroprotection (Ravati et al. 2000) but the intermediate mechanisms remained to be elucidated.

While ROS were widely believed to serve only harmful functions, there is increasing evidence that ROS are important intracellular signaling molecules. Low levels of ROS were proposed to modulate the phosphorylation status of several proteins necessary to preserve cellular function and integrity (Finkel 1998a). The authors demonstrated that ROS activated kinases such as mitogen-activated protein kinase (MAPK), c-jun amino terminal kinase (JNK) or extracellular signal-regulated kinases (ERKs) (Guyton et al. 1996; Guyton et al. 1996; Sundaresan et al. 1995; Finkel 1998b). Further, superoxide anion radicals as well as hydrogen peroxide markedly decreased the activity of tyrosine phosphatase 1B (PTP 1B) (Barrett et al. 1999). In line with this, basal protein phosphorylation was decreased and tyrosine phosphatase activity increased by the antioxidants NAC and PBN in primary rat glia (Robinson et al. 1999). Therefore the intracellular balance was proposed to be moved to a higher phosphorylation status and thereby initiating several phosphorylation-dependent intracellular signaling cascades. The exact mechanism of such modulation of kinases and phosphatases by ROS is still poorly understood but recent studies proposed that all the mentioned molecules affected by ROS contain a redox-sensitive cysteine residue in the active site of the enzyme (Barrett et al. 1999).

Also transcription factors were shown to be directly regulated by ROS. Oxidation of free sulfhydryl-groups to disulfide-bonds in the active center of the transcription factor SoxR which leads to an alteration of protein conformation was described to modify the transcripitonal activity of SoxR (Storz and Imlay 1999). An oxidation of sulfur-associated cations located in the active center of some transcription factors was discussed to be involved in ROS-gated activation pathways (Hidalgo et al. 1997). It was further shown that endogenous antioxidants such as glutathione or vitamin E can regenerate the oxidized

molecules (Gaudu et al. 1997). However, the exact mechanism of ROS-mediated NF-kB activation is not known but an indirect influence of ROS by augmenting the activity of the NF-kB activating kinases NIK or IKK or a direct chemical modulation of NF-kB subunits by ROS are probably involved.

Concerning NF-kB activation, besides ceramide, ROS seem to be common second messengers finally executing various NF-kB activating signals (Baldwin, Jr. 1996; O'Neill and Kaltschmidt 1997). The ROS-mediated activation pattern of NF-kB also appeared to be dose-dependent. When cultured neurons were treated with low concentrations of hydrogen-peroxide there was a marked increase in NF-kB activation whereas high doses of inhibited the p65 subunit and perinuclear aggregates were found (Kaltschmidt et al. 1997b). This observation supports the hypothesis that moderate ROS-stimulation induced beneficial pathways which were mediated by NF-kB.

The fact that preconditioning diminished ROS production under damaging conditions suggests that an enhancement of antioxidative enzymatic defense mechanisms, possibly promoted by de novo protein synthesis, could be responsible for the observed effect. Among the potential antioxidative neuroprotective proteins that are under the transcriptional control of NF-kB, mitochondrial manganese superoxide dismutase (SOD-2) plays an important role for the control of intracellular ROS generation. It was shown in vivo that induction of brain tolerance by moderate oxidative stress was associated with an upregulation of SOD-2 (Ohtsuki et al. 1992). Such an adaptive response may require translational activity and it has been demonstrated that a 3-nitropropionic acid- or hyperbaric oxygenation-induced preconditioning against transient cerebral focal ischemia, which also involved the formation of oxygen radicals, was blocked by inhibitors of translation (Schumann et al. 1998).

In the current study, it was demonstrated that stimulation with moderate amounts of ROS by X/XO resulted in an enhanced expression of SOD-2. This upregulation could be blocked by the protein synthesis-inhibitor cycloheximide and by the specific NF-kB-inhibitor decoy indicating that NF-kB-gated transcriptional and translational activation was required for the observed alterations of SOD-2 expression. It was further shown that such preconditioning with modest amounts of ROS prevents the subsequent oxygen radical burst of the damaging agents. Therefore, the intracellular signaling pathway of ROS-mediated neuroprotection becomes clearer. The underlying mechanism of mild ROS stimulation-mediated preconditioning appears to include an activation of NF-kB which entails enhanced

protein synthesis of SOD-2 that in turn serves as an effective neuroprotectant by preventing severe secondary oxygen radical burst (Fig. 44).

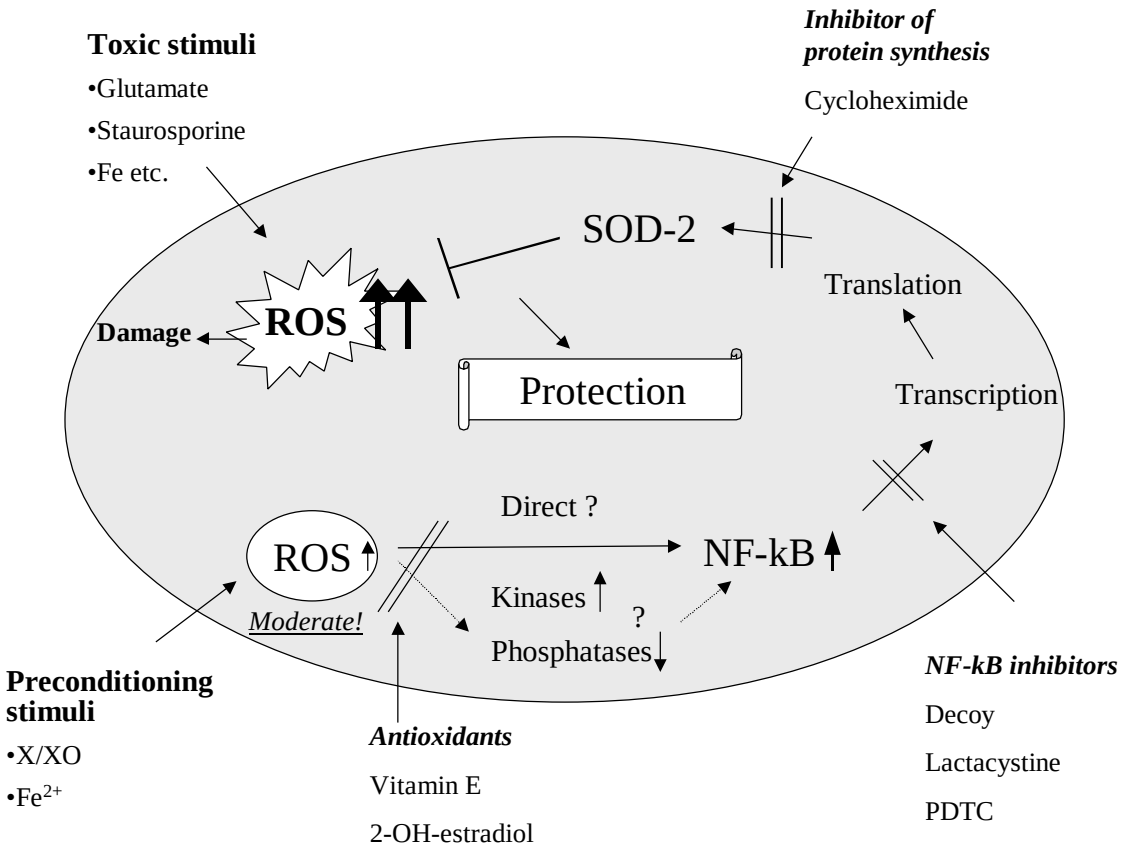


Fig. 44. Proposed mechanism of the observed neuroprotection by moderate stimulation with ROS

The fact that in the current study the Fe^{2+} -mediated preconditioning could not be blocked as effectively by the NF-kB-inhibitors as the X/XO (pre)-induced protection, might be explained by the finding that different kinds of ROS provoke distinct intracellular reactions and cause different functional alterations. Especially concerning NF-kB, different activating properties were found for different ROS. It was shown that superoxide anion radicals which are predominantly generated by the reaction of xanthine and xanthine oxidase were more potent activators of NF-kB than hydroxyl radicals generated by Fe^{2+} (Wang et al. 1999). On the other hand, the incomplete blockade of the observed neuroprotection by the NF-kB-inhibitors suggests that other pathways might also be involved in the ROS-mediated neuroprotection which do not require NF-kB activation.

As the used mixed hippocampal culture system contained neurons (~60%) and astrocytes (~40%) it could not be distinguished if the effect of preconditioning was mediated by neurons or astrocytes. NF-kB immunoreactivity was observed in neurons as well as in astrocytes. Using Aß the same stimulus was shown to cause opposite response of neurons and astrocytes concerning NF-kB activation (Bales et al. 1998). Although it was described that neurons were the dominant cell type within the CNS expressing active NF-kB, other authors also pointed out a pivotal role for NF-kB in neurons and astrocytes. Thus, it was proposed that NF-kB functions as an important cross-talk molecule to deliver information either for neuron-glial-interaction or to mediate retrograde signal transduction from far areas such as axons, dendrites or synapses (Kaltschmidt et al. 1999a; Gisiger 1998). However, in the current study, both astrocytes and neurons were assumed to be involved in the observed stimulation of ROS-gated signal transduction. This was supported by the immunostaining results of the present study showing that nuclear translocation of NF-kB upon ROS-stimulation was observed in both cell types of the used hippocampal culture.

Therapeutic interventions targeting only part of the complex network of mediators that contribute to the progression of neuronal damage after stroke and neurodegenerative diseases mostly produced only subtle protection (Liu et al. 2000). Therefore, preconditioning which initiates several pathways preserving neuronal function and integrity could be a promising strategy to overcome the problems of monocausal treatment. The current study indicates that mild oxidative stress could protect cultured neurons against subsequent damage by activation of NF-kB and subsequent enhancement of SOD-2 expression. Upon preconditioning treatment moderate amounts of ROS evidently function as effective molecules for neuroprotective intracellular signaling pathways.

Especially the activation of NF-kB which seems to represent a point of convergence for several endogenous and exogenous stimuli opens a valuable target for pharmacological interference which could be beneficial for the treatment of neurodegenerative diseases.

5 Summary

Reactive oxygen species (ROS) were often defined as aggressive molecules being broadly involved in numerous pathological situations and diseases. In the periphery, ROS were shown to participate in nearly all degenerative processes including cancer, inflammation, neuralgia, infertility, nephrotoxicity and hepatotoxicity. Concerning the central nervous system ROS play a pivotal role in the pathology of the neurodegenerative diseases AD, PD and ALS as well as in stroke. Thus, for a long time it was common understanding that the generation of ROS only promotes deleterious events and therefore has to be inhibited.

Recently, increasing evidence pointed towards an opposite face of radical action. It was suggested that ROS might also be involved in signal transduction pathways that contribute to cellular function and survival. The current study investigates both possible roles of ROS and tries to interfere with the consequences of damaging and beneficial ROS stimulation for neuronal cells.

First aim of the study was to examine the damaging effects of excess radical formation on neuronal cell cultures and to establish neuroprotection by treatment with drugs that possess potential antioxidant properties.

Initially the ROS-generating damaging models had to be characterized. Glutamate, staurosporine, and iron were used as damaging agents to establish defined necrotic or apoptotic neuronal cell death. The excitotoxin glutamate that leads to a cellular calcium-overload and iron ions that have been shown to intercalate with DNA predominately mediate necrotic cell death whereas the toxicity of the mycotoxin staurosporine which involves a loss of mitochondrial membrane potential and activation of the caspase cascade displays most characteristics of apoptosis. However, the exact pro-apoptotic mechanism of staurosporine still remains to be clarified.

In the present study, it has been demonstrated by fluorescence microscopy measurements that besides such different neurotoxic mechanisms of glutamate, staurosporine and iron, a strong elevation of ROS is a common pathway which is involved in the process of neurodegeneration by these agents. Therefore, the ROS formation kinetics of these agents was further characterized in cultured chick neurons.

The results have shown that the glutamate- and the iron-induced severe ROS generation occurred rapidly after stimulation showing a peak of radical formation already after 15 min and 30 min, respectively which was followed by a gradual decline of intracellular ROS content until 24 h.

In contrast, the staurosporine-induced ROS formation was rather delayed as the maximal level was reached after 4 h and remained constantly elevated until 24 h. This gradual rise of ROS might at least partially explain the pro-apoptotic mechanism of staurosprorine as the cell has enough time to start an active cell death program upon increasing ROS burden, resulting in morphological and biochemical features of apoptosis 4 - 24 h after exposure. Thus, such ROS formation kinetics of staurosporine might contribute to clarify the yet unknown mechanism of staurosporine-induced neuronal apoptosis.

Among the interesting candidates that might be able to interfere with the excess and deleterious formation of ROS in neurons, ACE-inhibitors could play an important role. Previously, it was found that ACE-inhibitors mediate cardio- and nephro-protective properties which could not only be related to their antihypertensive effect as such protection was markedly superior to other drugs that solely reduced blood pressure. Increasing evidence suggested an antioxidative capacity of ACE-inhibitors in peripheral organs. Although recent investigations revealed the surprising existence of an intracerebral renin-angiotensin system, the effect of ACE-inhibitors on brain functions is still poorly investigated. Therefore, the current study explored the influence of ACE-inhibitors on neurodegeneration caused by the mentioned agents.

Enalapril and moexipril, simultaneously applied with the damaging agents, similarly protected against glutamate and Fe-induced necrotic cell death as identified by morphology changes and trypan blue staining. Both ACE-inhibitors also ameliorated staurosporine- and also serum deprivation-induced neuronal apoptosis which was characterized by morphological alterations after Hoechst-staining.

Further studies which investigated the effect of enalapril and moexipril on glutamate-, iron- or staurosporine-induced ROS formation revealed that the underlying mechanism of the observed neuroprotection by ACE-inhibitors was an acute and marked prevention of the deleterious oxygen radical burst.

The relevance of these findings in pure neuronal cultures was confirmed in vivo as enalapril and moexipril significantly reduced the infarct volume after focal cerebral ischemia

by middle cerebral artery occlusion in mice and rats (in vivo experiments were predominantly performed by the co-worker V. Junker).

The question arose whether an inhibition of neuronal angiotensin II formation was involved in the neuroprotective mechanism of the investigated ACE-inhibitors because angiotensin II was discussed to promote neuronal damage within the brain. The influence of ACE-inhibition on cerebral renin-angiotensin system was not directly explored in the current work. Nevertheless, it can be assumed that at least in vitro such potentially possible effects do not fundamentally contribute to the observed neuroprotection because high concentrations of 1 – 100 µM, that were far above relevant plasma concentrations for ACE-inhibition, were required to achieve neuroprotection as well as radical scavenging by enalapril and moexipril. However, the cerebral renin-angiotensin system is an emerging new field in neuroscience and further studies will intensively investigate its functional relevance.

Also estrogens which were examined in the current study were considered as promising drugs that exert additional benefits apart from hormone function. In the current study the physiological 17-ß-estradiol and the synthetic estrogen 2-OH-estradiol significantly reduced neuronal damage caused by iron ions. This neuroprotection was paralleled by strong radical scavenging properties of the estrogens effectively preventing the iron–mediated elevation of ROS.

As low concentrations in a nanomolar range were sufficient to mediate the protective actions of 17ß-estradiol and 2-OH-estradiol a conceivable involvement of estrogen receptors in the observed effects were studied. The unspecific estrogen receptor antagonist tamoxifen neither reversed the neuroprotection nor influenced the antioxidative effect of the applied estrogens. These findings suggest that the mechanism of the beneficial 17ß-estradiol and 2-OH-estradiol treatment were receptor-independent and probably due to direct chemical detoxification of ROS which might be explained by their phenolic structure.

Again, the results from neuronal cultures were confirmed in an in vivo model employing cerebral ischemia in mice by permanent MCA occlusion, which showed that estrogens markedly reduced brain infarct area after stroke.

In both models, in vitro and in vivo, 2-OH-estradiol was the more effective drug concerning neuroprotection or reduction of infarct size as up to 100-fold lower concentrations of 2-OH-estradiol were sufficient to achieve protective effects as compared to the physiological 17-ß-estradiol. This might be explained by the stronger antioxidative capacity

of the biphenolic 2-OH-estradiol as already 0.01 µM of the drug was sufficient to exert radical scavenging whereas 1 µM 17ß-estradiol was required.

Such impressive antioxidative capacity of 2-OH-estradiol at concentrations that can be easily reached after pharmacological application in the human organism supports the view that this drug could be an appropriate candidate for the treatment of neurodegenerative diseases and stroke.

So far, the current study presented evidence that the excess release of ROS after stimulation with different agents promoting necrosis or apoptosis is a common mediator of neurodegeneration. Second, direct and effective ROS-scavenging by drugs such as ACE-inhibitors or estrogens is still a promising strategy to prevent the deleterious cascade of aberrant radical action.

However, as emerging evidence also suggested a role of controlled ROS formation for intracellular signaling, the current study explored such possible opposite function of ROS as mediators of neuroprotection.

First, a model was established to stimulate neuronal cells with defined low amounts of ROS in order to investigate if such moderate ROS treatment caused secondary resistance against neuronal damage. The characterization revealed that simultaneous application of low amounts of xanthine and xanthine oxidase (X/XO pre; 10 µM / 0.5 mU x ml^{-1}) for 15 min which are known to promote predominantly the formation of superoxide anion radicals caused a mild rise of intra-neuronal ROS content. A similar effect could be achieved with 15 min incubation of 100 µM iron ions (Fe^{2+}) prevalently inducing hydroxyl radical generation by the Fenton´s reaction.

Short and moderate ROS-stimulation of cultured chick neurons by X/XO (pre) or by Fe^{2+} for 15 min effectively mediated protection against subsequent damage induced 24 h later by the different oxidative stress-inducing agents glutamate (1 mM; 1h), X/XO (dam) (500 µM / 5 mU x ml^{-1}, 1 h) and staurosporine (200 nM, 24 h). Such preceding treatment that initiates intracellular protective responses was called preconditioning.

The underlying mechanism of the observed neuroprotection appeared to be an induced resistance to subsequent oxidative stress. It could be demonstrated in the present study that such preconditioning prevented the severe oxygen radical burst that occurred in not-preconditioned cultures after stimulation with glutamate, X/XO (dam) or staurosporine.

To confirm that ROS really mediated the observed neuroprotection, the radical scavengers vitamin E (10 µM) and 2-OH-estradiol (1 µM) were simultaneously administered with the preconditioning stimuli. The results have shown that the X/XO (pre)-induced neuroprotection was blocked in the presence of the antioxidants indicating that the moderate formation of ROS was involved in neuroprotection.

However, the Fe^{2+}-mediated protection could not be abolished by the radical scavengers. To investigate this phenomenon, the ROS formation kinetics during the 15 min preconditioning time period was monitored. This revealed that the initial ROS formation stimulated by X/XO (pre) was continuously blocked by the antioxidants vitamin E and 2-OH-estradiol, whereas the Fe^{2+} mediated ROS generation could only be reduced after 15 min but not during the first 10 minutes of incubation. Also pre-incubation or higher doses (of vitamin E and 2-OH-estradiol) and other antioxidants such as n-acetylcysteine, ascorbic acid or PDTC were unable to abolish this initial formation of ROS by Fe^{2+}.

Therefore, an immediate and continuous radical scavenging is indispensable to block the effects of ROS preconditioning and it seemed that already 10 min of mild ROS incubation was sufficient to set an intracellular signal leading to neuroprotection.

As it was shown in the present study that ROS could either confer deleterious or beneficial consequences for neuronal function and survival an interesting point was to investigate in more detail under which conditions the one or the other quality of radical action prevails.

An incubation response study with X/XO (pre) concerning preconditioning against glutamate toxicity revealed that neuroprotection was maximal when the cultures were exposed for 15 min, followed by 24 h recovery. Also a 1 h incubation was still protective while 4 h and 8h had no more preconditioning effect and 24 h even aggravated the glutamate-induced damage. A similar time-window was observed for the Fe^{2+}-mediated preconditioning showing an optimal protection after 15 min incubation but significant toxicity already started at 4 h Fe^{2+}-stimulation. The turn-around time for ROS-stimulation by X/XO (pre) or Fe^{2+} mediating either protection or cell death seemed to be between 1 h and 4 h of moderate ROS incubation in the present study.

Therefore, these results strongly suggest that the consequences of radical action for neuronal survival or neurodegeneration depend on the source, the type, the generated amount and the exposure time of ROS. This view is supported by recent findings of other groups which went even further reporting that too low amounts of ROS exert equally deleterious

neurodegeneration as excessive radical formation and created the term “reductive stress” for this phenomenon.

It was shown that exogenous moderate stimulation of neuronal cells with ROS entailed neuroprotection by promoting neuronal oxidative stress resistance. However, the intracellular mechanisms that were responsible for these effects remained to be clarified. Because a 24 h recovery time was always given to neuronal cells after the mild ROS-stimulation it was imaginable that intracellular signal transduction pathways were involved possibly requiring transcriptional and translational activity.

The transcription factor NF-kB was a promising candidate that could participate in the observed effects of protective ROS-stimulation although very contradictory qualities and functions of this transcription factor were reported in literature. Therefore, the present study examined the role of ROS-gated NF-kB pathways in hippocampal cell cultures of neonatal rats.

It was demonstrated by immunocytochemistry that such moderate ROS stimulation with X/XO (pre) or Fe^{2+} mediated a nuclear translocation of NF-kB which could be blocked by the antioxidant vitamin E indicating that ROS were responsible for the observed NF-kB activation. In addition, the ROS treatment caused a decrease of I-kBα expression as determined by western blotting which indirectly proved the ROS-mediated NF-kB activation. This can be explained by the fact that the inhibitory subunit I-kBα is cleaved by the proteasome upon activation of NF-kB and is therefore not detectable any more.

Antioxidants as well as the NF-kB inhibitor lactacystine blocked the I-kBα degradation by ROS which confirms the hypothesized pathway. Finally, an electrophorectic mobility shift assay proved that NF-kB activation definitely occurred under the conditions of ROS preconditioning. Again, this activating effect of ROS could be reduced by antioxidants and inhibitors of NF-kB.

Further studies revealed that the NF-kB inhibitors PDTC, lactacystine and the specific NF-kB-binding oligonucleotide “decoy” abolished the neuroprotective effect of ROS-mediated preconditioning against staurosporine-induced neuronal apoptosis.

Taken together, the results clearly indicate that the neuroprotective effect of ROS-stimulation is mediated by an activation of the transcription factor NF-kB.

It was now shown that moderate ROS stimulation induced neuroprotection against subsequent neuronal damage and the intracellular signal transduction involved an activation of NF-kB. However, the final executioner of the observed resistance against oxidative stress still had to be investigated.

Therefore, the current study examined the effect of ROS stimulation on the expression of the antioxidative enzymes superoxide dismutases 1 and 2. This revealed that the cytosolic Cu/Zn-SOD (SOD-1) was not affected by ROS treatment. In contrast, the expression of mitochondrial Mn-SOD (SOD-2) was markedly enhanced by a 15 min stimulation with X/XO (pre) after 4 h, 8h and 24 h. This up-regulation of SOD-2 could be blocked by PDTC, cycloheximide and by the specific NF-kB-binding oligonucleotide decoy.

Although the results concerning SOD-2 regulation have to be interpreted carefully as they are still under investigation at the moment, it seems that the promotion of endogenous antioxidative enzymatic defenses is the underlying mechanism of the NF-kB-gated neuroprotection by ROS.

Therefore, it may be suggested form the results of the current thesis that the broad and uncontrolled intake of radical scavengers such as vitamins C or E might induce a lack of physiologically relevant “good oxygen radicals” and therefore promote a kind of “reductive stress” which should be avoided.

Taken together, the current study reveals that ROS can exert ambivalent functions in neuronal cells. Abundantly released ROS no doubt contribute to neurodegenerative processes and effective radical scavenging is still a promising strategy to interfere with ROS-mediated neurodegeneration.

However, the current study also enlightened a new and therefore “radical” view of oxygen radicals: When ROS are produced at moderate conditions they initiate important intracellular signaling pathways which can mediate resistance to several potential threats for neuronal survival and seem to be indispensable for neuronal function and integrity.

6 Zusammenfassung

Sauerstoffradikale, die auch als reaktive Sauerstoffspezies (ROS) bezeichnet werden, wurden in der Vergangenheit als Mediatoren verschiedenster pathologischer Phänomene und Erkrankungen identifiziert. In zahlreichen Studien konnte gezeigt werden, daß ROS außerhalb des zentralen Nervensystems an der Pathogenese von Krebs, Entzündungen, Infertilität, Leber- und Nierenschäden sowie Neuralgien beteiligt sind. Im Gehirn spielen sie offenbar eine Rolle bei Entwicklung und Fortschreiten neurodegenerativer Erkrankungen wie Morbus Alzheimer, Morbus Parkinson, Amyotropher Lateralsklerose sowie im Schlaganfallgeschehen.

Neuerdings mehrten sich jedoch Hinweise darauf, daß es offensichtlich auch ein „Kehrseite der Medaille“ bezüglich der Funktion von ROS gibt. Es wurde diskutiert, daß ROS auch an der Vermittlung intrazellulärer Signalkaskaden beteiligt sind und somit eine wichtige Rolle für neuronale Funktionsvorgänge übernehmen könnten.
Die vorliegende Arbeit untersucht beide mögliche Funktionen von Sauerstoffradikalen und versucht die Diskrepanz der ROS-vermittelten Wirkungen bezüglich Degeneration oder Protektion aufzuklären.

Erstes Ziel der Arbeit war die Untersuchung der degenerativen ROS-induzierten Wirkungen an neuronalen Zellkulturen. Daher wurden Modelle zur oxidativen Schädigung etabliert und charakterisiert, sowie Substanzen mit potentiell antioxidativen und damit neuroprotektiven Eigenschaften untersucht.

Hierzu wurden Glutamat, Eisenionen und Staurosporin als schädigende Agenzien eingesetzt, um einen kontrolliert nekrotischen sowie apoptotischen Neuronalschaden zu erzeugen. Glutamat, welches vorwiegend über Öffnung von Calciumkanälen zu einem intrazellulären Calciumoverload führt, sowie Eisenionen, von denen bekannt ist, DNA-Strangbrüche zu verursachen, führten hauptsächlich zu einem nekrotischen Zellschaden, was durch morphologische Untersuchungen nach Trypanblaufärbung gesehen wurde. Das Mykotoxin Staurosporin hingegen verursachte eine relativ selektive Apoptose, die durch morphologische Untersuchungen mit dem Kernfarbstoff Höchst 33258 charakterisiert wurde. Obwohl in Studien gezeigt werden konnte, daß Staurosporin einen Zusammenbruch des mitochondrialen Membranpotentials sowie die Aktivierung der Caspasen-Kaskade bewirken

kann, konnte der genaue Mechanismus der proapoptotischen Wirkung bis heute nicht genau geklärt werden.

In der vorliegenden Arbeit wurde durch fluoreszenzmikroskopische Untersuchungen deutlich, daß neben den verschiedenen neurotoxischen Wirkmechanismen der genannten schädigenden Agenzien, eine starke Freisetzung von Sauerstoffradikalen einen gemeinsamen Mechanismus darstellt, der wesentlich zu der beschriebenen Neurodegeneration beiträgt. Daher wurde die ROS-Bildungskinetik der genannten Substanzen näher untersucht.
Es zeigte sich, daß der durch Glutamat und Eisenionen bedingte starke Anstieg der intrazellulären ROS-Konzentration unmittelbar nach Stimulation (nach 15 – 30 min) maximal war und dann allmählich bis hin zum letzten gemessenen Zeitpunkt abfiel. Im Gegensatz dazu trat nach Staurosporinstimulation der Anstieg der ROS verzögert auf, erreichte erst nach 4 h ein Maximum und blieb relativ konstant bis 24 h.

Die hier gezeigte ROS-Bildungskinetik von Staurosporin könnte zumindest teilweise zur Aufklärung des unbekannten proapoptotischen Wirkmechanismus beitragen, da der Zelle, im Gegensatz zu der unvermittelt brachial einsetzenden ROS-Wirkung von Glutamat oder Eisenionen, hier genügend Zeit bleibt, ein aktives Zelltodprogramm zu starten. Das Zeitfenster der ROS-Bildung würde dabei auch zu den morphologischen Befunden passen, bei denen erste Anzeichen von Apoptose durch Staurosporin nach 4 h auftraten.

Unter den Substanzen mit potentiell antioxidativen Eigenschaften, welche die unkontrolliert starke Freisetzung von ROS unterbinden könnten, sind ACE-Hemmer interessante Kandidaten. Vor kurzem wurde gezeigt, daß ACE-Hemmer kardio- und nephroprotektive Eigenschaften besitzen deren Wirkung deutlich über das Maß einer reinen Blutdrucksenkung hinausgehen. An peripheren Organen mehrten sich die Hinweise darauf, daß sauerstoffradikalfangende Eigenschaften an den protektiven Effekten beteiligt sein könnten. Obwohl neuere, überraschende Befunde für die Existenz eines intrazerebralen Renin-Angiotensin-Systems sprechen, wurden die Wirkungen von ACE-Hemmer auf neuronale Funktionsprozesse bislang wenig erforscht. Daher wurde in der vorliegenden Arbeit der Einfluß der ACE-Hemmer Enalapril und Moexipril auf die beschriebenen oxidativen neurodegenerativen Prozesse nach Stimulation mit den schädigenden Agenzien näher untersucht.

Anhand von quantitativen morphologischen Untersuchungen mit Trypanblau und Hoechst 33258 konnte gezeigt werden, daß die Applikation von Enalapril und Moexipril die

neuronalen Zellen sowohl vor einem glutamat- oder eisenionenbedingten nekrotischen, als auch vor einen durch Staurosporin hervorgerufenen apoptotischen Zellschaden schützte.
Weitere Studien zur Beeinflussung der Sauerstoffradikalbildung nach Glutamat-, Eisen- oder Staurosporininkubation ergaben, daß der durch diese Agenzien hervorgerufene starke ROS-Anstieg wirkungsvoll mit Enalapril oder Moexipril unterbunden werden konnte. Der Mechanismus der Neuroprotektion beinhaltet also eine bedeutende antioxidative Kapazität der ACE-Hemmer.

Die Relevanz dieser in vitro Befunde wurde auch in vivo bestätigt, da Enalapril und Moexipril das Infarktvolumen nach fokaler zerebraler Ischämie durch Okklusion der mittleren Zerebralarterie (MCA) von Mäusen und Ratten signifikant reduzierten (die in vivo Arbeiten wurden von den Kollegen des Arbeitskreises, V. Junker und M. Kouklei durchgeführt).
Es stellte sich jedoch die Frage, ob eine Inhibition der Freisetzung von neuronalem Angiotensin II, von welchem neurodegenerative Funktionen beschrieben wurden, an der protektiven Wirkung der ACE-Hemmer beteiligt gewesen sein könnte. Dies ist anhand der vorliegenden Befunde nicht auszuschließen, da keine dahingehenden weiteren Versuche gemacht wurden, erscheint jedoch zumindest in vitro eher unwahrscheinlich, da sehr hohe Konzentrationen von 1 µM – 100 µM benötigt wurden, um eine signifikante Protektion oder das Abfangen von ROS zu erzielen. Die hier verwendeten Konzentrationen liegen also deutlich über dem für eine effektive ACE-Inhibition benötigten Dosen (ca. 1-10 nM). Dennoch stellt die Entdeckung des Renin-Angiotensin-Systems im Gehirn einen bedeutenden Forschungsansatz für die Neurowissenschaft dar, der in nächster Zeit sicher noch weitergehend charakterisiert wird.

Auch Estrogene, die in der vorliegenden Arbeit untersucht wurden, sind vielversprechende Substanzen, die abgesehen von der Hormonfuktionen noch zusätzliche protektive Effekte aufweisen.
In der vorliegenden Arbeit konnte gezeigt werden, daß das physiologische 17ß-Estradiol sowie das synthetische Estrogenderivat 2-OH-Estradol einen eiseninduzierten Neuronalschaden deutlich reduzieren konnten. Der neuroprotektive Effekt war mit einer ausgeprägten Radikalfängereigenschaft der Estrogene gepaart. Da sehr niedrige Konzentrationen, die sich teilweise im nanomolaren Bereich bewegten, für die Neuroprotektion ausreichten, wurde eine mögliche Beteiligung von Estrogenrezeptoren an den beobachteten Effekten untersucht. Da der unspezifische Estrogenrezeptorantagonist Tamoxifen jedoch weder die estrogenvermittelte Neuroprotektion noch die antioxidativen

Eigenschaften blockieren konnte, wurde ein rezeptorunabhängiger Mechanismus angenommen. Dieser begründet sich wahrscheinlich mit einer direkten chemischen Detoxifikation von Sauerstoffradikalen mittels Oxidation der phenolischen Estrogenstruktur zum Chinon.

Auch bei den Estrogenen wurde die Relevanz der Befunde an in vivo Modellen abgesichert. Hier zeigte sich, daß bereits niedrige Dosen von 17ß-Estradiol und 2-OH-Estradiol die Infarktfläche nach permanenter fokaler Zerebralischämie durch MCA-Okklusion signifikant reduzierten.

Sowohl in Zellkultur als auch am Tiermodell war 2-OH-Estradiol, verglichen mit 17ß-Estradiol die effektivere Substanz, da eine Neuroprotektion oder eine Reduktion des Infarktes bereits in 100-fach niedrigeren Dosen erzielt werden konnte. Dies könnte auf einer stärkeren antioxidativen Eigenschaft des biphenolischen 2-OH-Estradiol beruhen, da in der vorliegenden Arbeit gezeigt werden konnte, daß bereits 0,01 µM 2-OH-Estradiol signifikant den intraneuronalen ROS-Gehalt verminderte, während mindestens 1 µM des physiologischen Estrogens notwendig waren.

Eine solch beeindruckende antioxidative Potenz von 2-OH-Estradiol in Konzentrationen, die nach oraler Applikation erreicht werden könnten, gibt Anlaß zu der Hoffnung, daß diese Substanz möglicherweise ein probater Kandidat für die Behandlung neurodegenerativer Erkrankungen und des Schlaganfalls sein könnte.

Soweit konnte in der vorliegenden Arbeit gezeigt werden, daß die exzessive Freisetzung von Sauerstoffradikalen nach Stimulation mit verschiedenen nekrose-oder apoptoseerzeugenden Noxen einen gemeinsamen Mediator der Neurodegeneration darstellt. Daher stellt auf der einen Seite die Applikation von Substanzen mit antioxidativen Eigenschaften, wie ACE-Hemmern oder Estrogenen nach wie vor eine effektive Strategie zur Verminderung deliziöser oxidativer Zellschäden dar.

Andererseits jedoch gab es in jüngster Zeit vermehrt Befunde, die für eine Beteiligung von Sauerstoffradikalen an wichtigen intrazellulären Signaltransduktionskaskaden sprechen. Daher beschäftigt sich die vorliegende Arbeit im folgenden mit der Erforschung dieser möglichen gegensätzlichen Funktion von ROS als Mediatoren neuroprotektiver Prozesse.
Dazu wurde zunächst ein Modell etabliert und charakterisiert, in dem neuronale Zellen einer definierten niedrigen Menge an ROS stimuliert werden konnten. Ziel der Untersuchung war es dabei festzustellen, ob eine solche moderate Stimulation mit Sauerstoffradikalen zu einer sekundären Resistenz gegen einen Neuronalschaden führen kann.

Die Charakterisierung des Modells ergab, daß die simultane Applikation von Xanthin und Xanthinoxidase (X/XO prä; 10 µM / 0,5 mU x ml^{-1}) für 15 Minuten von der bekannt ist, prävalent Superoxidanionradikale zu generieren, eine geeignete Kombination zur milden Steigerung des intrazellulären ROS-Gehalts darstellte. Ein ähnlich moderater ROS-Anstieg konnte mit 100 µM Eisenionen für 15 min erzielt werden, die im Zuge der intrazellulär ablaufenden Fenton-Reaktion hauptsächlich an der Entstehung von Hydroxylradikalen beteiligt sind.

Im folgenden konnte demonstriert werden, daß die eben beschriebene kurze und milde Stimulation mit X/XO (prä) oder Eisenionen für 15 min nach einer Erholungszeit von 24 h neuronale Zellen vor oxidativem Schaden durch Glutamat (1 mM; 1h), X/XO (in hohen Konzentrationen = X/XO (dam); 500 µM / 5 mU x ml^{-1}) oder Staurosporin (200 nM; 24 h) schützte.

Diese Form der Stimulation mit der subtoxischen Menge eines Agens, welches eine protektive intrazelluläre Antwort initiiert, die gegen eine späteren Schaden schützt, wird auch als Präkonditionierung bezeichnet.

Der zugrundeliegende Mechanismus der hier gezeigten neuronalen Präkonditionierung scheint eine induzierte Resistenz gegen nachfolgenden oxidativen Streß zu beinhalten. Diesbezüglich konnte in der vorliegenden Arbeit demonstriert werden, daß eine solche Präkonditionierung den durch die schädigenden Agenzien Glutamat-, X/XO (dam)- oder Staurosprorin-bedingten starken Anstieg der ROS-Bildung unterdrücken konnte.

Um die direkte Beteiligung von ROS an der beobachteten Neuroprotekton abzusichern, wurden die Sauerstoffradikalfänger Vitamin E (10 µM) und 2-OH-Estradiol (1 µM) gleichzeitig mit den präkonditionierenden Stimuli appliziert. Es zeigte sich, daß die X/XO (prä)-induzierte Neuroprotektion in Gegenwart der Antioxidanzien geblockt wurde, was auf eine klare Beteiligung von ROS an der protektven Wirkung hindeutet.

Die eiseninduzierte Protektion konnte hingegen nicht mit Antioxidatien verhindert werden. Zur Untersuchung dieses Phänomens wurde die Sauerstoffradikalbildungskinteik innerhalb des fünfzehnminütigen Präkonditionierens gemessen. Es zeigte sich, daß die initiale X/XO (prä)-vermittelte ROS-Bildung wirkungsvoll und kontinuierlich während der gesamten Zeit des Präkonditionierens durch Vitamin E und 2-OH-Estradiol unterdrückt werden konnte. Im Gegensatz dazu jedoch war die eiseninduzierte Generation von ROS während der ersten 10 min nicht blockierbar, auch nicht durch höhere Antioxidanzienkonzentrationen oder durch

Vorbehandlung. Sogar andere Antioxidanzien wie N-Acetylcystein oder PDTC, die zu diesem Zweck getestet wurden, waren diesbezüglich unwirksam.

Daher ist ein unmittelbares und kontinuierliches Radikalfangen zur Blockade der ROS-vermittelten Neuroprotektion unabdingbar und es kann geschlossen werden, daß 10 min der milden Sauerstoffradikalstimulation ausreichend waren, ein intrazelluläres Startsignal zu setzen, welches zu einer entsprechenden Neuroprotektion führte.

Da in der vorliegenden Arbeit demonstriert wurde, daß ROS sowohl degenerative als auch protektive Wege anstoßen können, wurde die interessante Fragestellung aufgeworfen, unter welchen Bedingungen jeweils die eine oder die andere Eigenschaft von Sauerstoffradikalen zum Tragen kommt.
Untersuchungen der Arbeit zeigten diesbezüglich, daß die Protektion mit X/XO (prä) gegen eine Glutamatschädigung nach der beschriebenen 15 min Inkubation bereits maximal war. Eine 1 h Stimulation war ebenfalls noch protektiv, 4 h und 8 h hatten keinen Effekt während 24 h die Toxizität von Glutamat potenzierte. Ein ähnliches Zeitfenster konnte für das eisenvermittelte Präkonditionieren gezeigt werden, jedoch mit dem Unterschied, daß die Toxizität bereits nach 4 h einsetzte.

Die Konsequenzen der ROS-Einwirkung für neuronale Zellen hängen also in erster Linie von der Art, der Menge und der Inkubationsdauer der Sauerstoffradikale ab. Die Ermittlung der optimalen Bedingungen zur protektiven Stimulation mit ROS scheint also ein kritischer „Drahtseilakt“ zwischen einer noch nicht zur Stimulation ausreichenden zu schwachen ROS-Einwirkung und einer bereits zu hohen, toxischen Dosis zu sein. Diese Ansicht wird noch deutlicher durch neuere Studien, die eine zu niedrige Menge an intrazellulären ROS als ebenso deliziös ansehen, wie eine exzessive Radikalbildung und dieses Phänomen mit dem Begriff „reduktiver Streß“ umschrieben.

Es konnte also soweit gezeigt werden, daß die exogene, moderate Stimulation neuronaler Zellen mit Sauerstoffradikalen eine Resistenz der Neurone gegen oxidativen Streß vermittelte, was sich in Neuroprotektion gegen schädigende Agenzien widerspiegelte. Die genauen intrazellulären Mechanismen, die durch die ROS-Stimulation angestoßen wurden, bedurften jedoch noch der Klärung.

Da den Zellen immer ein Zeitabstand von 24 Stunden nach Stimulation mit der moderaten Menge an ROS gegeben wurde, war anzunehmen, daß intrazelluläre

Signaltransduktionswege an der Neuroprotektion beteiligt sind, die möglicherweise auch Transkription und Translation umfassen.
Der Transkriptiosfaktor NF-kB könnte dabei eine wesentliche Rolle bei der Vermittlung ROS-induzierter Neuroprotektion spielen, da es in der Literatur Hinweise gibt, daß seine Aktivierung möglicherweise über oxidative Prozesse gesteuert wird. Andererseits jedoch sind die bislang beschriebenen NF-kB-Funktionen in neuronalen Zellen äußerst konträr. Die vorliegende Arbeit untersuchte daher den Zusammenhang zwischen der ROS-vermittelten Neuroprotektion und einer NF-kB-Aktivierung in hippocampalen Zellkulturen neonataler Ratten.

Immuzytochemische Untersuchungen mittels Laserscanningmikroskopie zeigten, daß eine moderate ROS-Stimulation durch X/XO (prä) oder Eisenionen eine Translokation von NF-kB aus dem Zytosol in den Zellkern vermittelte. Die durch X/XO (prä) verursachte NF-kB-Translokation konnte mit dem Antioxidans Vitamin E verhindert werden, was auf eine direkte Beteiligung von Sauerstoffradikalen an der NF-kB Aktivierung hindeutet. Des weiteren konnte die ROS-vermittelte NF-kB Aktivierung auch mittels I-kB Westernblots und EMSA bestätigt werden, da auch hier Antioxidatien den Effekt blockten. Darüber hinaus verhinderten auch die NF-kB-Inhibitoren PDTC und Laktazystin die ROS-vermittelte NF-kB Aktivierung, was den Befund insgesamt abrundet.

Weitere Studien zur Hemmbarkeit der Neuroprotektion zeigten, daß sowohl die antioxidativen NF-kB-Inhibitoren Vitamin E, 2-OH-Estradiol und PDTC, der Proteasominhibitor Laktazystin, als auch das spezifische NF-kB-bindende Oligonukletid „Decoy“ die ROS-vermittelte Protektion gegen die staurosporininduzierte Apoptose aufheben konnten.

Zusammenfassend kann man feststellen, daß die Resultate klar für eine wesentliche Beteiligung des Transkriptionsfaktors NF-kB an der ROS-vermittelten Neuroprotektion sprechen.

Dennoch war noch der letztlich ausführende Faktor der durch milde ROS-Behandlung und nachfolgende NF-kB-gesteuerte Transkription erzeugten neuronalen Resistenz gegen oxidativen Streß aufzuklären.

Es wurde daher noch der Effekt von X/XO (prä) und Eisenionen auf die Expression der antioxidativen endogenen Enzyme Superoxiddismutase I und II (SOD I und II) untersucht. Zunächst zeigte sich, daß die zytosolische SOD-1 nicht durch die ROS-Stimulation beeinflußt wurde. Im Gegensatz dazu war nach 15 min Behandlung mit X/XO

(prä) eine markant gesteigerte Expression der mitochondrialen SOD II festzustellen. Diese Hochregulation konnte mit PDTC, Cycloheximid und „Decoy“ verhindert werden, was für eine ROS- und NF-kB-Abhängigkeit der verstärkten SOD-II Expression spricht.

Obwohl die Ergebnisse bezüglich der SOD-Regulation vorsichtig interpretiert werden müssen, da sie noch Gegenstand der derzeitigen Untersuchungen sind, scheint eine Verstärkung endogener ROS-Detoxifizierungskapazitäten der letztlich zugrundeliegende Mechanismus der ROS-induzierten Neuroprotektion zu sein.

Die vorliegende Studie setzt daher auch ein Warnsignal bezüglich der weitverbreiteten Angewohnheit einer unkontrollierten, überdosierten „prophylaktischen“ Einnahme von Antioxidantien wie Vitamin C und Vitamin E. Eine Blockade der physiologischen Signalübertägerfunktion von Sauerstoffradikalen in Form von „reduktivem Streß“ könnte dabei unabsehbare Folgen für den menschlichen Organismus haben.

Resümierend läßt sich feststellen, daß Sauerstoffradikale ambivalente Funktionen in neuronalen Zellen erfüllen können.

Die unter pathologischen Bedingungen auftretende, unkontrolliert exzessive Bildung von Sauerstoffradikalen trägt zweifelsohne zu der Progression neurodegenerativer Erkrankungen bei. Daher ist auch in solchen Situationen die Applikation effektiver und gut verträglicher Radikalfänger, wie z.B. ACE-Hemmer oder Estrogene, nach wie vor eine vielversprechende Behandlungsstrategie.

Die vorliegende Arbeit zeigt jedoch auch ein anderes Gesicht von Sauerstoffradikalen und präsentiert daher einen neuen Denk- und Forschungsansatz. Wenn ROS in moderaten Mengen auf neuronale Zellen einwirken, erfüllen sie durch das Anstoßen von Signaltransduktionswegen wichtige Funktionen für die Integrität, Plastizität und Streßresistenz von Neuronen.

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