# Neuronal death and survival
# - targeting glutamate-induced cell death mechanisms

by

Gro Haarklou Mathisen


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Department of Pharmaceutical Biosciences

School of Pharmacy

Faculty of Mathematics and Natural Sciences

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Gro Haarklou Mathisen

## Abbreviations

| | |
|---|---|
| AA | - arachidonic acid |
| AIF | - apoptosis inducing factor |
| AMPA | - α-amino-3-hydroxy-5-methylisoxazole-4-propionate |
| APAF-1 | - apoptotic protease-activating factor 1 |
| APF | - 2-[6-(4'-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid |
| Bad | -Bcl-associated death promoter |
| Bax | - Bcl-2-associated X-protein |
| Bcl-2 | - B-cell lymphoma-2 |
| CGN | - cerebellar granule neurons |
| Cyt c | - cytochrome c |
| Cys | - cysteine |
| DHR | - dihydrorhodamine 123 |
| GCS | - γ-glutamyl-cysteine synthetase |
| Glu | - glutamate |
| Gly | - glycine |
| GSH | - reduced form of glutathione |
| GSSG | - oxidized form of glutathione |
| HE | - dihydroethidium |
| $H_2O_2$ | - hydrogen peroxide |
| HPF | - 2-[6-(4'-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid |
| MT Red | - MitoTracker Red CM-$H_2$XROS |
| NGF | - nerve growth factor |
| NGFI-B | - NGF-inducible clone B |
| NMDA | - N-methyl-D-aspartate |
| MOMP | - mitochondrial outer membrane permeabilization |
| $^{\cdot}NO$ | - nitric oxide radical |
| $^{\cdot}NO_2$ | - nitrogen dioxide radical |
| Nor1 | - neuron derived orphan receptor-1 |
| NOS | - nitric oxide synthase |
| Nurr1 | - Nur-related factor-1 |

| | |
|---|---|
| $O_2^{\cdot -}$ | - superoxide anion radical |
| $^{\cdot}OH$ | - hydroxyl radical |
| $ONOO^-$ | - peroxynitrite |
| PARP-1 | - poly(ADP-ribose) polymerase |
| $PLA_2$ | - phospholipase $A_2$ |
| RNS | - reactive nitrogen species |
| ROS | - reactive oxygen species |
| RXR | - retinoid X receptor |
| $sPLA_2$-IIA | - secretory phospholipase $A_2$ type IIA |

## List of papers

I. Secretory $PLA_2$-IIA and ROS generation in peripheral mitochondria are critical for neuronal death (2007). **Gro H. Mathisen**, Inger H. Thorkildsen, Ragnhild E. Paulsen. Brain Research 1153; 43-51.

II. Preconditioning with estradiol abolishes its neuroprotection in cerebellar neurons (2007). Åsa B. Fallgren[1], **Gro H. Mathisen[1]**, Jan Mæhlen, Rune Blomhoff, Ragnhild E. Paulsen. Biochemical and biophysical research communications 352; 966-972.
[1] These authors contributed equally to this work.

III. Chicken cerebellar granule neurons rapidly develop excitotoxicity in culture (2006). Chris M. Jacobs[1], Petra Aden[1], **Gro H. Mathisen[1]**, Erica Khuong, Mona Gaarder, Else M. Løberg, Jon Lømo, Jan Mæhlen, Ragnhild E. Paulsen. Journal of Neuroscience Methods 156; 129-135.
[1] These authors contributed equally to this work.

IV. NGFI-B induction in glutamate stimulated neurons maintains its death pathway and allows late protection by 9-*cis* retinoic acid (2008). **Gro H. Mathisen[1]**, Åsa B. Fallgren[1], Bjørn O. Strøm, Beata U. Mohebi, Ragnhild E. Paulsen. Submitted.
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## Aims of the present study

The excitatory neurotransmitter glutamate may act as an excitotoxin when glutamate receptors are overstimulated, e.g. during stroke. This results in increased intracellular calcium, a situation that can be mimicked by treatment with calcium ionophore. The rise in intracellular calcium may activate phopholipases capable of breaking down the cell membrane and liberating arachidonic acid. Reactive oxygen species are generated from calcium-activated enzymes. NGFI-B has been shown to play a key role in regulating apoptosis by translocating to the mitochondria, and glutamate is capable to induce this translocation. The focus of this work has been on the above mentioned factors, with emphasis on cell death and protection. More specific, the aims have been to:

1) Investigate the role of ROS generation in glutamate-induced cell death; with emphasis on identifying toxic ROS, and pinpoint the location of this ROS and the enzymes involved in the ROS generation (papers I, II, III, and IV).
2) Investigate if different neuroprotector molecules show different long-term efficiency as neuroprotectors against glutamate-induced toxicity due to interference with endogenous production of glutathione, the main endogenous antioxidant (paper II).
3) Establish chicken neurons as a supplementary model to study cell death mechanisms involved in excitotoxicity (paper III).
4) Investigate the role of NGFI-B in glutamate-, calcium ionophore-, and arachidonic acid-induced cell death in rat and chicken neurons (paper IV).

# 1. Introduction

## 1.1 Cell death types

Apoptosis is an essential mechanism for the selective elimination of cells during development, homeostasis of tissues with cell turnover, and removal of aging and abnormal cells (Steller 1995). Morphological changes that occur during apoptosis includes cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation (Kerr et al. 1972). Apoptosis may affect scattered individual cells and the apoptotic bodies formed are phagocytosed by macrophages and adjacent cells (Leist and Nicotera 1998). The apoptotic process depends on the ability of the dying cell to produce ATP. Therefore, it has been regarded as an active form of cell death. Apoptosis is also called programmed cell death (the cell commits suicide through a built-in program) (McConkey and Orrenius 1995), however, programmed cell death generally denotes all forms of cell death mediated by a cell death program and is not linked to a specific morphology (Leist and Nicotera 1998). Dead cells may also be characterized by other morphological changes. Cells characterized by swelling of organelles, breakdown of cellular membranes and cell disintegration are said to be necrotic (Kerr et al. 1972; Wyllie 1997). Whereas apoptotic cells are phagocytosed by macrophages and adjacent cells, necrotic cells lyse and may therefore cause an inflammatory reaction (Leist and Nicotera 1998). Necrosis may be induced experimentally by impairing the ability to produce ATP; therefore, necrosis has been considered an uncontrolled form of cell death. Evidence suggests that cell death with necrotic morphological characteristics also can occur as a programmed event (Jagtap and Szabo 2005). Thus, cell death is not only apoptosis or necrosis. Cell death has been divided into four different groups (Leist and Jaattela 2001):

*"Apoptosis* is defined by stereotypic morphological changes, especially evident in the nucleus where the chromatin condenses to compact and apparently simple geometric (globular, crescent-shaped) figures… In its most classic form, apoptosis is observed almost exclusively when caspases, in particular caspase-3, are activated…

*Apoptosis-like PCD* is used here to describe forms of PCD with chromatin condensation that is less compact/complete than in apoptosis (geometrically more complex and lumpier shapes), and with the display of phagocytosis-recognition molecules before lysis of the plasma membrane. Any degree and combination of other apoptotic features can be found. Most published forms of 'caspase-independent apoptosis' fall into this class…
*Necrosis-like PCD* is used here to define PCD in the absence of chromatin condensation, or at best with chromatin clustering to speckles. Varying degrees of other apoptosis-like features — including externalization of phosphatidylserine — might occur before the lysis. Necrotic PCD usually involves specialized caspase-independent signaling pathways…
*Accidental necrosis/cell lysis* is the conceptual counterpart to PCD, as is prevented only by removal of the stimulus. It occurs after exposure to high concentrations of detergents, oxidants, ionophores or high intensities of pathologic insult. Necrosis is often associated with cellular OEDEMA (organelle swelling)… The necrotic tissue morphology is, in large part, due to postmortem events (occurring after lysis of the plasma membrane)."

Autophagy, or cellular self-digestion, is a process where parts of the cytoplasm and intracellular organelles are sequestered within characteristic double- or multi-membrane autophagic vacuoles (named autophagosomes) which are delivered to lysosomes for degradation. Autophagy is cytoprotective in response to most forms of cellular stress; however, autophagy can lead to cell death, possibly through activation of apoptosis or as a result of the degradation of large amount of cytoplasm contents [reviewed in (Maiuri et al. 2007; Scott et al. 2007)]. Thus, autophagy represents an additional form of cell death.

### 1.2 Molecular mechanisms of cell death

Apoptosis triggers include overactivation of glutamate receptors (Ankarcrona et al. 1995), increased oxidative stress (Mattson 1998; Sastry and Rao 2000), and deprivation of neurotrophic factors (Cheng and Mattson 1991). Most apoptotic pathways converge on

a restricted number of effectors (Sastry and Rao 2000). Important groups of apoptosis-related proteins include:

- the caspase (cysteine aspartate proteases) family (Thornberry and Lazebnik 1998)
- AIF (apoptosis inducing factor) (Susin et al. 1999)
- smac/DIABLO (Verhagen et al. 2000)
- cytochrome c (Liu et al. 1996)
- the Bcl-2 (B-cell lymphoma-2) protein family (Chao and Korsmeyer 1998)

Caspases are central to apoptosis of many cell types (Chan and Mattson 1999). Caspases require processing at specific cleavage sites to generate the active enzyme (Stennicke and Salvesen 1999), and the first caspase to be activated (initiator caspase) triggers downstream caspases (effector caspases) giving rise to a cascade of caspase activation. AIF translocates from the mitochondrial intermembrane space to the nucleus where it binds to DNA and induces chromatin condensation and DNA fragmentation and therefore induces caspase-independent apoptosis (Daugas et al. 2000). Smac/DIABLO inhibits a family of proteins that function as inhibitors of apoptosis (IAPs) (Du et al. 2000; Verhagen et al. 2000) by inhibiting caspase activation (Liston et al. 1996; Deveraux et al. 1997), and it is released from the mitochondria together with cytochrome c. Cytosolic cytochrome c is one of the components of the apoptosome (caspase 9-activating complexes) (Hengartner 2000). The Bcl-2 family of proteins includes both pro- apoptotic [e.g. Bax (Bcl-2-assoociated X-protein) and Bad (Bcl-associated death promoter)] and anti-apoptotic (Bcl-2 and Bcl-$x_L$) members (Pellegrini and Strasser 1999) which regulates mitochondrial permeability.

Two major apoptosis pathways can be differentiated by the relative timing of caspase activation and mitochondrial release of cytochrome c. In the first (the mitochondrial pathway/the intrinsic pathway), cytochrome c is released from the mitochondrial intermembrane space prior to caspase activation. In the second (the extrinsic pathway), effector caspases are activated prior to mitochondrial alterations by activation of death receptors (Lossi and Gambino 2008). The mitochondrial pathway includes up-regulation and/or translocation of proapoptotic proteins, e.g. Bax and p53, to the mitochondria, the release of cytochrome c and/or AIF from the mitochondria through permeability transition pores (PTP) formed in the mitochondrial membrane, and

activation of caspase 9 which activates effector caspase 3, resulting in cleavage of numerous protein substrates. The anti-apoptotic Bcl-2 protein prevents the release of cytochrome c from the mitochondria, and Bcl-$x_L$ also inhibits activation of caspase 9 by interacting with APAF-1. The extrinsic pathway includes ligands activating death receptors in the cell membrane which recruits and activates caspase 8, which then activates caspase 3. Caspase 3 plays a major role in executing the apoptotic cell death process. Apoptosis in most mammalian cells proceeds via the mitochondrial/intrinsic pathway (figure 1) (Green and Kroemer 2005), and the mitochondrial outer membrane permeabilization (MOMP) is characteristic (Green and Kroemer 2004). Two classes of mechanisms for MOMP have been described; one involving the inner membrane and the other only involving the outer membrane. The first class of mechanism involves the opening of a pore in the inner membrane, and most models of this pore postulates a role for the adenosine nucleotide transporter (ANT) in the inner membrane and the voltage dependent anion channel (VDAC) in the outer membrane (Green and Kroemer 2004). The second class of mechanism for MOMP appears to be mediated by members of the Bcl-2 family of apoptosis-regulating proteins acting directly on the outer mitochondrial membrane (Green and Kroemer 2004). Both mechanisms involve release of proteins located to the mitochondrial intermembrane space (IMS).

### 1.3 Excitotoxicity

Excitotoxicity refers to the ability of glutamate and structurally related excitatory amino acids to destroy neurons (Olney 1986), and this pathological condition may occur in acute neurodegenerative conditions such as e.g. stroke and trauma, as well as in Alzheimer's disease and motor system disorders (Choi 1988; Mattson 2000). During stroke, neurons die of hypoxia following blood vessel occlusion or hemorrhage. Most of the neuronal death following a stroke occurs over a period of days following the initial insult and reflects excitotoxicity elicited by glutamate, the major excitatory neurotransmitter in the brain (Olney et al. 1973; Hara and Snyder 2007). Glutamate is present in millimolar tissue concentrations in the brain; however, glutamate is cleared from the synaptic and

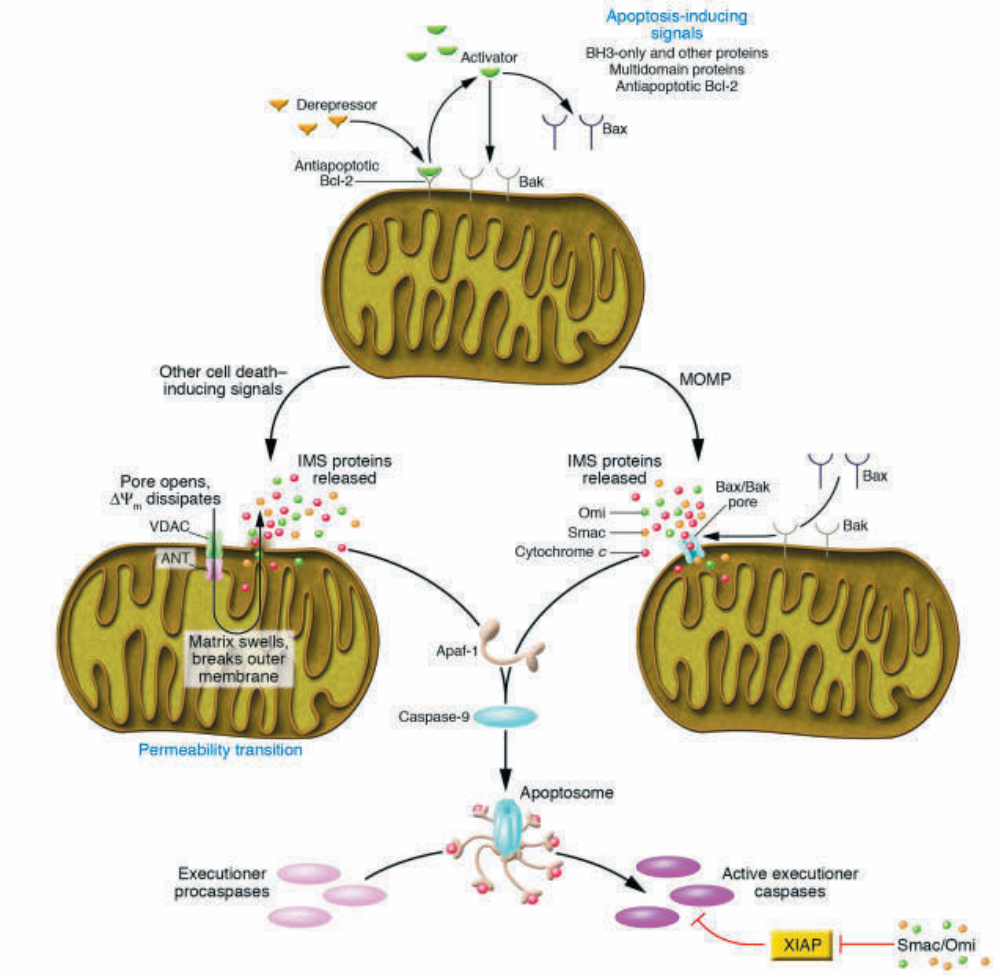


**Figure 1**. "**Checkpoints for apoptosis in the mitochondrial pathway.** Most mammalian cell death proceeds via the mitochondrial pathway, as illustrated. Stimuli for the induction of apoptosis predominantly act by engaging proapoptotic members of the Bcl-2 family, which work to cause MOMP, and this is countered by the antiapoptotic Bcl-2 family members. Other cell death stimuli can cause MOMP by the induction of a mitochondrial permeability transition. In either case, release of proteins from the intermembrane space triggers the activation of caspases via the formation of an Apaf-1 apoptosome, which recruits and activates caspase-9. This, in turn, cleaves and activates the executioner caspases. The activation of caspase-3, -7, and -9 is antagonized by XIAP, which in turn can be inhibited by Smac, Omi, and other proteins released upon MOMP. .. ANT, adenosine nuclear transporter; VDAC, voltage-dependent anion channel; IMS, intermembrane space; $\Delta\Psi_m$, mitochondrial transmembrane potential; MOMP, mitochondrial outer membrane permeabilization". *Figure and legend are from Green, D.R., and Kroemer, G. (2005). The journal of clinical investigation 115: 2610-17.* XIAP = X-chromosome-linked inhibitor of apoptosis, one of the IAPs.

extracellular environment by glutamate transporters leaving micromolar extracellular concentrations (Rothstein 1996). During ischemia there is an increase in the extracellular glutamate concentration, and this is largely due to reversed operation of neuronal glutamate transporters (Rossi et al. 2000). Abnormally intense exposure to glutamate can be lethal to neurons both *in vivo* and *in vitro* (Olney 1986). Many factors, including increased intracellular calcium concentration [a result of activation of the N-methyl –D-aspartate (NMDA) subtype of glutamate receptors (Choi 1988; Tymianski et al. 1993)], activation of caspases, Bcl-2, activation of NOS and $PLA_2$, and activation of poly(ADP-ribose) polymerase (PARP-1) (genetically knocking out PARP-1 protects neurons from excitotoxic death) (Yu et al. 2002), have been reported to contribute to the development of glutamate-induced excitotoxicity (Dawson et al. 1991; Frandsen and Schousboe 1993; Martinou et al. 1994; Dugan et al. 1995; Reynolds and Hastings 1995; Ciani et al. 1996; Tenneti et al. 1998; Mandir et al. 2000) (paper I). Effects observed at the mitochondria include mitochondrial calcium overload (Peng et al. 1998), mitochondrial depolarization (White and Reynolds 1996), opening of mitochondrial permeability transition pore (PTP) (Alano et al. 2002), and release of AIF and cytochrome c (Wang et al. 2004). The mitochondria possess an enormous capacity to sequester $Ca^{2+}$ driven by the 180 mV mitochondrial membrane potential ($\Delta_{\psi m}$) (Budd and Nicholls 1996). Mitochondrial $Ca^{2+}$ uptake is required for NMDA-induced ROS generation, and ROS generation during the early stage of acute glutamate excitotoxicity is mainly localized to the mitochondria (Duan et al. 2007). Depolarizing mitochondria, and thereby eliminating the driving force for mitochondrial $Ca^{2+}$ uptake (Stout et al. 1998), protects neurons from excitotoxic death. In mitochondria of cortical neurons treated with high levels of glutamate (100 μM for 15 min), significant $Ca^{2+}$ injury to the oxidative phosphorylation is detected prior to any commitment to cell death (Kushnareva et al. 2005). Glutamate causes little acute elevation in cytoplasmic free $Ca^{2+}$ or total cellular $Ca^{2+}$ content when the mitochondrial $Ca^{2+}$ accumulation is inhibited, and it is not able to induce acute excitotoxicity in cells with previously depolarized mitochondria (Budd and Nicholls 1996). Duan et al. have demonstrated that mitochondrial ROS generation serve as a signal to activate PARP-1, thus linking mitochondrial $Ca^{2+}$ uptake and PARP-1 activation (figure 2).

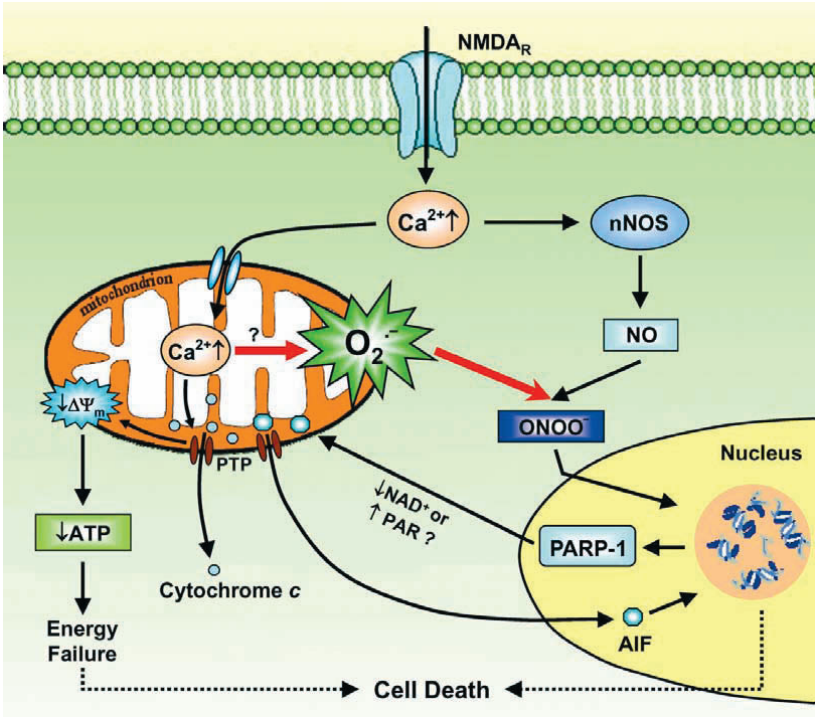


**Figure 2. "Diagram of the excitotoxic cascade showing mitochondrial ROS generation as the link between mitochondrial $Ca^{2+}$ uptake and PARP-1 activation in excitotoxicity.** Under pathological conditions, the massively activated NMDA receptors cause a strong increase in the cytosolic $Ca^{2+}$ level, which is then taken up by mitochondria. The mitochondrial $Ca^{2+}$ overload leads to a loss of $\Delta\Psi_m$ and an explosion of $O_2^{\cdot-}$ generation, probably through the opening of PTP and the subsequent release of cytochrome *c*. The elevated cytosolic $Ca^{2+}$ also activates nNOS and increases NO production. $ONOO^-$ is formed from the reaction of $O_2^{\cdot-}$ with NO, and then diffuses into the nucleus to cause DNA damage. In response to this DNA damage, PARP-1 is activated, which results in excessive production of PAR polymers and depletion of $NAD^+$. The activation of PARP-1 further induces AIF translocation from the mitochondria to the nucleus, which causes DNA fragmentation. The mechanism for the PARP-1 activation-dependent AIF translocation is still not fully understood, both the depletion of $NAD^+$ and the accumulation of PAR polymer could be the hypothetic signal. Finally, the profound DNA damage in combination with the energy failure caused by mitochondrial dysfunction and $NAD^+$ depletion leads to cell death. $NAD^+$, nicotinamide adenine dinucleotide". *Figure and legend are from Duan Y., Gross R. A., and Sheu S. S. (2007). Journal of Physiology 585: 741-58.*

To be able to interfere with the cell death process initiated by excitotoxicity, it is important that mechanisms essential for the mediation of the cell death is fully characterized. The localization of toxicity mediating ROS to peripheral mitochondria

(paper I), the role of secretory $PLA_2$-IIA (paper I), and the role of NGFI-B in neuronal excitotoxic cell death (paper IV) are novel findings.

### 1.4 Glutamate and glutamate receptors

Glutamate is the major excitatory neurotransmitter in the mammalian nervous system. The postsynaptic effects of glutamate are mediated by two subfamilies of glutamate receptors, the ionotropic receptors and the metabotropic receptors (Ozawa et al. 1998). The ionotropic receptors may be pharmacologically divided into NMDA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainate (KA) receptors. The NMDA receptors are composed of assemblies of four subunits derived from three distinct gene families (NR1, NR2A-D and NR3A-B). The stoichiometry of NMDARs has not been established definitely, but the consensus is that NMDA receptors are tetramers that most often incorporate two NR1 and two NR2 subunits of the same or different subtypes (Dingledine et al. 1999; Paoletti and Neyton 2007). The extracellular portion of NR1 and NR2 consists of two different types of domains, N-terminal domains and agonist binding domains (figure 3). Glycine and D-serine are both agonists for NR1 (Johnson and Ascher 1987; Panatier et al. 2006). Glutamate and NMDA are agonists for NR2. To activate the receptor both glutamate and glycine must be bound to their respective agonist domains. The NMDA receptors are ion channels permeable to $Ca^{2+}$, $Na^{+}$ and $K^{+}$ in a voltage-dependent manner. Under basal conditions $Mg^{2+}$ binds inside the ionic channels and prevents ionic flow even when the receptor is activated. When the membrane is depolarized, the affinity of $Mg^{2+}$ for its binding site is reduced, allowing ionic flow through the channel if the receptor is activated by ligand. $Zn^{2+}$ is a ligand for the modulatory domain of NR2, and binding of $Zn^{2+}$ elicits a voltage-independent block (Peters et al. 1987; Westbrook and Mayer 1987). Due to disruption of the energy metabolism (the driving force for the $Na^{+}$ pump that maintains the resting membrane potential) during neurotoxic insults, neurons are depolarized relieving the $Mg^{2+}$ block of the NMDA receptor, and the reversed operation of neuronal glutamate transporters results

in increased extracellular glutamate. Glutamate-induced overactivation of NMDA receptors then results in increased intracellular calcium, activating cell death processes.

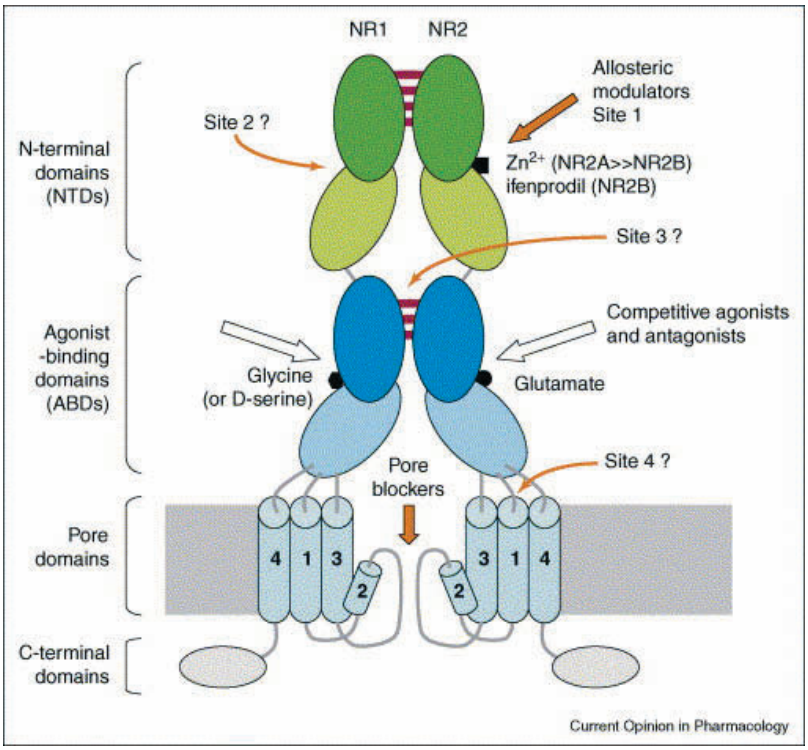


**Figure 3.** "**Potential sites for ligand binding at NMDA receptors.** Most NMDAR are believed to assemble as tetramers, associating two NR1 and two NR2 subunits in a 'dimer of dimers' quaternary architecture. For clarity, only one of the two NR1/NR2 heterodimers is shown… The NR2 ABD binds glutamate, whereas the NR1 ABD binds the co-agonist glycine (or D-serine). White arrows indicate binding sites for competitive agonists and antagonists. Thick orange arrows indicate sites known to bind allosteric modulators such as endogenous zinc (NR2A and NR2B NTDs) or ifenprodil-like compounds (NR2B NTDs), both acting as non-competitive antagonists. The ion-channel domain also forms binding sites for pore blockers such as endogenous $Mg^{2+}$ and MK-801…, acting as uncompetitive antagonists. Thin orange arrows indicate putative modulatory sites, which can bind either positive or negative allosteric modulators. The only known NMDAR antagonists that display strong subunit selectivity are the NR2 NTD ligands $Zn^{2+}$, which selectively inhibits NR2A-containing receptors at nanomolar concentrations, and ifenprodil-like compounds, which selectively inhibit NR2B-containing receptors". *Figure and legend are from Pierre Paoletti P. and Neyton J. (2007). Current Opinion in Pharmacology 47: 39-47.*

### 1.5 Reactive oxygen and nitrogen species

A free radical is defined as any molecular species capable of independent existence that contains one or more unpaired electrons (Halliwell and Gutteridge 1999). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are collective terms, ROS includes both oxygen radicals and some non-radicals that are oxidizing agents and/or easily converted into radicals (examples are peroxynitrite and hydrogen peroxide), and RNS includes nitrogen radicals and non-radicals (Halliwell 2006). The toxicity of ROS/RNS is directly related to their reactivity. In healthy aerobic organisms, production of ROS and RNS are approximately balanced by antioxidant defense systems. Oxidative stress, situations of serious imbalance between production of ROS/RNS and the antioxidant defense, may result in oxidative damage of molecules (Halliwell and Gutteridge 1999).

If a single electron is supplied to oxygen the superoxide anion radical ($O_2^{\cdot -}$) is formed. The two-electron reduction product of oxygen is hydrogen peroxide ($H_2O_2$), and the four-electron product is water.

$$O_2 \xrightarrow{\text{one-electron reduction}} O_2^{\cdot -}$$

$$O_2 \xrightarrow{\text{two-electron reduction (plus 2H+)}} H_2O_2$$

$$O_2 \xrightarrow{\text{four-electron reduction (plus 4H+)}} 2H_2O$$

The hydroxyl radical ($^{\cdot}OH$) is generated from decomposition of hydrogen peroxide, and this process is accelerated in the presence of iron or copper in a reaction called the Fenton reaction (Halliwell and Gutteridge 1999).

Fenton reaction: $H_2O_2 + Fe^{2+} / Cu^{+} \rightarrow {}^{\cdot}OH + {}^{-}OH + Fe^{3+} / Cu^{2+}$

Nitric oxide ($^{\cdot}NO$) reacts very fast with superoxide anion radical to form peroxynitrite ($ONOO^{-}$), a non-radical product.

$${}^{\cdot}NO + O_2^{\cdot -} \rightarrow ONOO^{-}$$

Peroxynitrite rapidly protonates to peroxynitrous acid (ONOOH), a very reactive non-radical, at a physiological pH. Peroxynitrous acid can cause damage by undergoing homolytic fission to hydroxyl radical and nitrogen dioxide.

$$ONOOH \rightarrow {}^{\cdot}NO_2 + {}^{\cdot}OH$$

Superoxide anion radical and hydrogen peroxide are highly selective in their reactions with biological molecules, whereas hydroxyl radical attacks everything around it (Halliwell 2006).

In cerebellar granule neurons it has been shown that the calcium-activated enzymes NOS and $PLA_2$ (including secretory $PLA_2$ type IIA) are involved in glutamate-induced generation of toxicity-mediating ROS (Ciani et al. 1996) (Paper I). $PLA_2$ releases arachidonic acid which yields superoxide anion radical through its subsequent metabolism by lipoxygenases and cyclo-oxygenases, and NOS generates nitric oxide.

Oxidative stress may cause damage to e.g. DNA, lipid membranes, and proteins. In DNA ROS may cause modification of DNA-bases, single and double strand DNA breaks, loss of purines, damage to deoxyribose sugars, DNA-protein cross-linkage, and damage to the DNA repair system (Kohen and Nyska 2002). Lipid peroxidation has been implicated in modification of membrane structure, and modification of DNA and proteins (Porter et al. 1995). ROS-induced damage on proteins includes damage to specific amino acid residues, changes in structure, and fragmentation (Halliwell and Gutteridge 1999).

Reactive oxygen species generated during apoptosis have been recognized as mediators of intracellular apoptotic signaling cascades (Greenlund et al. 1995; Cai and Jones 1998; Esteve et al. 1999; Valencia and Moran 2001). Some intracellular sources of ROS and their interaction with the apoptotic pathway, e.g. release of cytochrome c and activation of caspase 3 are shown in figure 4.

### 1.6 Antioxidant defenses

From the biological aspect, an antioxidant is defined as a compound which, in low concentrations, after reaction with a free radical, is relatively stable either in a radical or

non-radical form, and does not trigger successive radical reactions during which other new free radicals and oxidized substrates would be produced (Bergendi et al. 1999).

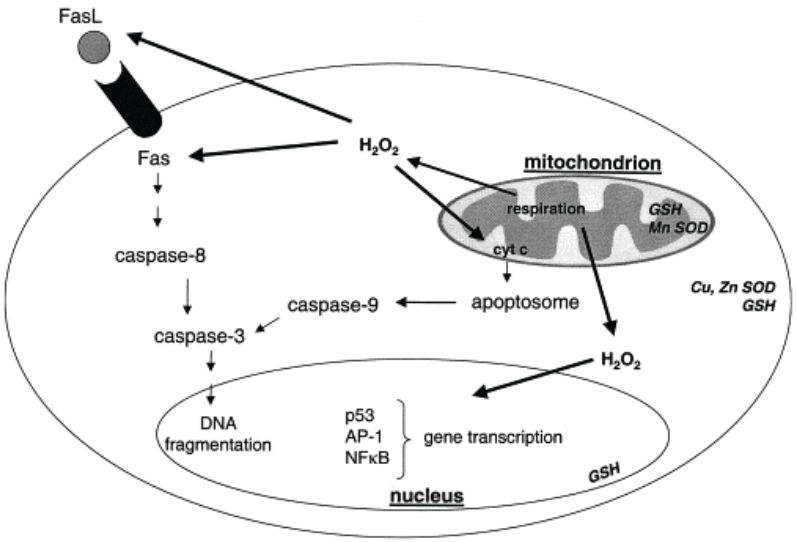


**Figure 4.** "**Intracellular sources of ROS and their interaction with the apoptotic pathway**. Mitochondria are the most notorious producers of ROS ($O_2^{\bullet-}$, $H_2O_2$). One model proposed for $H_2O_2$ induction of apoptosis is upregulation of the Fas-FasL system, leading to activation of caspase-8 and downstream caspases... $H_2O_2$ can cause the release of cytochrome c from mitochondria into the cytosol. In the cytosol, cytochrome c binding to Apaf-1 is a critical step in formation of the apoptosome. The apoptosome complex activates caspase-9, which then activates caspase-3. $H_2O_2$ may also activate nuclear transcription factors, like NFκB, AP-1, and p53, which may upregulate death proteins or produce inhibitors of survival proteins. Cellular defenses against ROS include GSH, which is synthesized in the cytosol but is transported into nuclei and mitochondria. The SOD enzymes are also important antioxidants. Mn SOD is localized to mitochondria while Cu, Zn SOD exists in the cytosol". *Figure and legend are from Chandra J., Samali A., Orrenius S. (2000)**. Free Radical Biology and Medicine 29: 323-333.***

### 1.6.1 Endogenous antioxidant defense and protection

All parts of the nervous system contain superoxide dismutase (SOD), an enzyme that catalyzes dismutation of superoxide to hydrogen peroxide and molecular oxygen.

$$2\ O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Animals have MnSOD in the mitochondrial matrix and CuZnSOD in the mitochondrial intermembrane space and in the rest of the cell (Liochev and Fridovich 2005).
To remove hydrogen peroxide, the most important enzymes in the brain are glutathione peroxidases (GPx) (Brigelius-Flohe 1999) and peroxiredoxins (Rhee et al. 2005), whereas catalases are not very important in the brain. Peroxidases use $H_2O_2$ to oxidize a substrate. For GPx the substrate is glutathione (GSH), which may exist in a reduced form (GSH) or an oxidized from (GSSG). GPx can also reduce other peroxides (Brigelius-Flohe 1999).

$$H_2O_2 + 2\ GSH \rightarrow 2GSSG + 2\ H_2O$$

GSH is present in all mammalian cells at millimolar intracellular concentrations (1-11 mM) (Meister and Anderson 1983) and is an important part of the antioxidant defense of cells. In addition to protect cells against oxidative stress as a cofactor for enzymes (e.g. GPx), glutathione may scavenge hydroxyl radical and singlet oxygen directly, and it may regenerate vitamin C and vitamin E back to their active forms (Masella et al. 2005; Valko et al. 2007). Glutathione is a tripeptide that consists of glutamate, cysteine and glycine. GSH is synthesized in the cytosol of all mammalian cells via two ATP-requiring enzymatic steps: the formation of γ-glutamylcysteine from glutamate and cysteine, and formation of GSH from γ-glutamylcysteine and glycine. The first and rate-limiting step is catalyzed by γ-glutamyl-cysteine synthetase (GCS), and the second step is catalyzed by glutathione synthetase (Anderson 1998). The GCS expression level is regulated by 3 different redox-sensitive response elements (Lu 1999), and may therefore be regulated/influenced by antioxidant stroke therapy (paper II).

### 1.6.2 Exogenous antioxidant protection

Several dietary constituents, including vitamin C and vitamin E, have been suggested to exert antioxidant effects in vivo. At physiologically pH the favored form of vitamin C (ascorbic acid) is ascorbate (Beyer 1994). Ascorbate has been shown to have a multiplicity of antioxidant properties, including scavenging of superoxide and hydroxyl radical (Beyer 1994). In this process a much less reactive ascorbyl radical is generated. Vitamin E is a nutritional term; it does not refer to a particular chemical structure. Eight naturally occurring substances with vitamin E activity have been found (d-α-, d-β-, d-γ-,

d-δ-tocopherols and -α-, d-β-, d-γ-, d-δ-tocotrienols) (Traber 1994). The most effective form in animals is α-tocopherol, and the term α-tocopherol and vitamin E are now used almost interchangeably. Vitamin E reacts at an almost diffusion-controlled rate with the hydroxyl radical, but slowly with superoxide. Vitamin E is fat-soluble and resident in membranes and in lipoproteins (Traber 1994). Vitamin E scavenges peroxyl radicals faster than these radicals can react with adjacent fatty acid side-chains or with membrane proteins, and is probably the most important inhibitor of the free-radical chain reaction of lipid peroxidation in animals (Halliwell and Gutteridge 1999). During this process α-tocopherol is converted to the radical α-tocopheryl, and it has been shown, e.g. in cultured cells, that ascorbate can reduce α-tocopheryl back to α-tocopherol (Beyer 1994). Thus, the two are often used together in cell culture systems. Dietary sources of antioxidants have been associated with lower stroke risk, however, antioxidant supplementation has not reduced stroke risk in clinical trials (Ding and Mozaffarian 2006).

### 1.7 The NGFI-B subfamily of nuclear receptors

NGFI-B (nerve-growth-factor-induced clone B) was originally identified because of its rapid induction by nerve growth factor (NGF) in rat PC12 (pheocromocytoma) cells (Milbrandt 1988). The mouse NGFI-B homologue is Nur77 (Hazel et al. 1988), and the human homologue is TR3 (Chang et al. 1989). NGFI-B, Nurr1 (Nur-related factor 1) and Nor1 (neuron derived orphan receptor-1) constitute the NR4A subfamily of nuclear receptors (Maruyama et al. 1998). As other nuclear receptors, NGFI-B consists of three major domains; a variable amino-terminal region, a highly conserved DNA binding domain (DBD), and a relatively well conserved carboxyl-terminal domain (Evans 1988; Milbrandt 1988). DNA binding is mediated by two zinc fingers (folded loops of protein stabilized by $Zn^{2+}$ ions) located in the DBD. The ligand binding domain is localized to the carboxyl-terminal domain. NGFI-B has been classed as an orphan receptor because it does not have any known ligand (O'Malley and Conneely 1992; Mangelsdorf and Evans 1995), and the ligand binding domains of NGFI-B do not contain a pocket large enough

to support the binding of a small molecule (Baker et al. 2003; Wang et al. 2003). Evidence supports that NGFI-B does not require ligand binding for physiological function. Therefore, to allow control of NGFI-B *in vivo*, other mechanisms such as protein expression, nuclear accumulation, and post-translational modifications are likely to exist (Wingate and Arthur 2006). The transcription of *NGFI-B* is controlled by external stimuli, and it has been shown to be up-regulated by glutamate-induced activation of NMDA receptors and increased intracellular calcium (Bading et al. 1995). We show that buffer (activation of voltage dependent calcium channels), glutamate and calcium ionophore induced *NGFI-B* expression (paper IV).

NGFI-B has been implicated in the regulation of cell survival and apoptosis. During apoptosis, NGFI-B translocates to the mitochondria to initiate the apoptotic process (Li et al. 2000) as a NGFI-B/RXR heterodimer (Cao et al. 2004). Bcl-2, localized in the outer membrane of the mitochondria, functions as a receptor for NGFI-B, and NGFI-B converts Bcl-2 from a protector to an apoptosis inducer and cytochrome c is released (Li et al. 2000; Lin et al. 2004). 9-*cis* retinoic acid (9cRA), a high affinity ligand for RXRs (Heyman et al. 1992), suppresses a nuclear export sequence (NES) activity present in the carboxyl terminus of RXRα by inducing RXRα homodimerization or altering RXRα/NGFI-B heterodimerization (Cao et al. 2004). Consequently, RXR ligands were shown to inhibit mitochondrial targeting of RXRα/NGFI-B heterodimers as well as their ability to induce apoptosis (Cao et al. 2004). Similar results were obtained in paper IV. It has been shown that overexpression of a dominant negative Nur77 protein or inhibition of *Nur77* expression by antisense Nur77 inhibited apoptosis, whereas constitutive expression of *Nur77* resulted in massive apoptosis (Liu et al. 1994; Woronicz et al. 1994; Woronicz et al. 1995; Uemura and Chang 1998; Li et al. 2000). In paper IV we report that in neurons treated with glutamate or calcium ionophore new synthesis of NGFI-B is important for maintenance of the death mechanism, and that this allows late protection by 9cRA.

## 2. Discussion of methods

### 2.1 Primary cultures of cerebellar granule neurons from rat, chicken, and transgenic mouse

In primary cultures the cells are more similar to cells in their natural environment *in vivo* compared to cell lines. Primary cultures of cerebellar granule cells have the advantages of a cell line without the drawbacks of transformed tumor cells (Vaudry et al. 2003). It has been reported that this neuronal model is well suited to identify factors that control neuronal differentiation and apoptosis (Vaudry et al. 2003), although excitotoxicity seldom occurs in the human cerebellum. It has been described that rat cerebellar granule neurons are 95% pure neuronal cultures (Gallo et al. 1987; Ciani and Paulsen 1995), and that they express glutamate receptors (Gallo et al. 1987) and NGFI-B (Jacobs et al. 2004). The cultures were used to investigate mechanisms involved in cell death induced by glutamate, calcium-ionophore, or arachidonic acid, including ROS generation and the effect of scavengers and enzyme inhibitors, the effect of glutamate and different antioxidant treatment on the level of glutathione, and expression and localization of NGFI-B. The chicken neurons were used as a supplement to the rat neurons. Advantages with chicken neurons are that there is no need for animal facilities, and that they can be cultivated at a physiological potassium level (paper III). Also, the transfection efficiency is higher than in rat neurons, which makes it possible to measure luciferase generated from reporter genes (paper IV). Mouse cerebellar granule neurons were made from transgenic mice with a luciferase reporter coupled to the GCS promoter (the rate-limiting enzyme in the glutathione synthesis). The cultivation of these neurons made it possible to investigate the effect of both glutamate and different antioxidant treatments on the transcription of GCS, and thus determine if changes in the level of reduced glutathione were due to an increased oxidation of GSH or reduced synthesis of glutathione (paper II).

## 2.2 Cell death

### 2.2.1 Models

Cell death mechanisms following three different stimuli that increase the intracellular calcium concentration have been investigated. The cell death stimuli included treatment with i) glutamate (100 μM for 15 minutes), ii) calcium ionophore (0.1 μM directly in the medium), and iii) arachidonic acid (10 μM directly in the medium). Overstimulation of glutamate receptors may induce cell death, e.g. following a stroke, and it results in excessive calcium influx and disturbance of the postsynaptic calcium homeostasis (Choi 1992). This is reported to induce both apoptotic and necrotic cell death depending on the dosage and time of exposure (Ankarcrona et al. 1995). It has been reported that a low concentration of calcium ionophore (0.1 μM) induces apoptosis whereas 10-fold higher concentration induces caspase 3 independent cell death in cerebellar granule neurons (Takadera and Ohyashiki 1997; Slagsvold et al. 2003). Arachidonic acid is released during traumatic brain injury, ischemia, or convulsion (Katsuki and Okuda 1995), and has been suggested to be involved in ischemia-induced cell death (Dhillon et al. 1997). Arachidonic acid induces increased intracellular $Ca^{2+}$ and $Na^{+}$, and the mitochondrial $Na^{+}$ overload is important for caspase 3-dependent apoptosis (Fang et al. 2008).

### 2.2.2 Cell death measurement

Cell death was measured by trypan blue exclusion assay. Intact cell membranes are impermeable for trypan blue, whereas cells with ruptured membranes are stained blue. Blue neurons were scored as dead, whereas non-stained neurons were scored as live. Since the cultures contains other cells than cerebellar granule neurons, this method was used to be able to visually verify that only granule neurons were scored as live or dead during the evaluation of cell death. We have also tested the MTT assay (shows the mitochondrial activity) to be able to evaluate the viability of the neurons at an earlier time point. However, these results did not correlate with the results from the trypan blue exclusion assay, probably due to the influence of the mitochondria in the astrocytes. Although the number of astrocytes in the cultures was very low compared to the number of granule neurons, they have a larger cytosolic volume, and contain more mitochondria

(as observed with the microscope). Therefore, the MTT assay was unsuitable for the detection of viability of the cerebellar granule neurons. Cell death may also be measured by other methods, e.g. flow cytometri. However, this method can not be used on these neurons since they are adherent, and can not be trypsinized.

## 2.3 ROS

While ROS predominantly are implicated in causing cell damage, they also play a major physiological role in several aspects of intracellular signaling and regulation (Droge 2002). Thus, it is important to be able pinpoint the role of different ROS in the cell when ROS generated in response to different stimuli is characterized, and in doing so, which processes a specific scavenger may interfere with. The ideal chemical ROS probe would be highly reactive at low concentrations, specific, sensitive, nontoxic, well-characterized chemically, and easy to load into cells/organelles without unwanted diffusion, excretion, or metabolism (Wardman 2007).

### 2.3.1 ROS detection

Several probes have been developed to detect the generation of ROS in living cells. Some probes are easier to oxidize than others, and these probes may be used as more general ROS detectors than probes that only may be oxidized by a few ROS (Setsukinai et al. 2003). To detect reactive species two different probes were used, DHR (dihydrorhodamine 123) and Mt Red (MitoTracker Red CM-$H_2$XROS), and this made it possible to characterize the localization of buffer- and glutamate-induced ROS. DHR has been reported to be a sensitive and efficient trap for peroxynitrite and may therefore be used to detect peroxynitrite generation (Kooy et al. 1994). Oxidation of DHR results in the formation of the fluorescent product rhodamine 123, which preferentially accumulates in mitochondria according to the trans-mitochondrial potential although DHR can be oxidized in different compartments throughout the cell (Pias et al. 2003). MT Red is oxidized inside the mitochondria and can therefore be used to investigate mitochondrial ROS generation. The absolute increase in the neuronal ROS level induced by buffer or glutamate treatment was similar. The oxidation of DHR and the cell death

were reduced by inhibition of enzymes generating nitric oxide or superoxide (the combination product is peroxynitrite) in glutamate treated neurons (paper I). Buffer induced oxidation of DHR was also reduced by inhibition of NOS and $PLA_2$. Buffer induced the ROS level in somal mitochondria whereas glutamate induced the ROS level in peripheral mitochondria. This indicates that both buffer and glutamate induces the production of peroxynitrite in the neurons, but that the localization is different.

### 2.3.2 ROS scavenging

The effect of α-estradiol, β-estradiol, or vitamin C combined with vitamin E, as scavengers for glutamate-induced ROS, were evaluated (paper II). These scavengers all reduced glutamate-induced cell death, showing that ROS is involved in cell death.
Five different probes usable for detection of ROS were also used as scavengers: DHR, MT Red, HE (dihydroethidium), HPF (2-[6-(4'-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid), and APF (2-[6-(4'-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid). The specificity of DHR and MT Red is described in 2.3.1. HE is frequently used as a probe for superoxide, and the oxidation of HE is reported to be selective for superoxide in rat hippocampal pyramidal neurons in culture and in brain slices (Bindokas et al. 1996). HPF is reported to detect hydroxyl radical and peroxynitrite, whereas APF detects hydroxyl radical, peroxynitrite and hypochlorite (Setsukinai et al. 2003). Since oxidation of the probe reduces the ROS level, ROS probes may be used as scavengers. We showed that DHR, HPF and APF all scavenged glutamate-induced toxic ROS, whereas HE did not scavenge the toxic ROS (paper I). Thus, it is likely that glutamate induces the generation of peroxynitrite, and that this peroxynitrite is toxic to the neurons.

## 2.4 Pharmacological inhibitors

MK 801 [(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate] is a non-competitive NMDA receptor antagonist (Wong et al. 1986). It blocked cell death, and completely reduced glutamate-induced ROS generation and expression of NGFI-B (paper 1 and IV). MK 801 (10 μM) has also been reported to be an antagonist on

the nicotinic acetylcholine receptor (Galligan and North 1990). However, since the protection mediated by MK 801 (1 μM) in cerebellar granule neurons is similar as the protection mediated by a competitive NMDA receptor antagonist (CGP 39551), it is likely that the observed effects of MK 801 is due to interference with the NMDA receptor (Jacobs et al. 2006). Verapamil is an inhibitor of voltage-dependent calcium channels (Ferrari 1997), and it significantly reduced buffer-induced ROS generation and expression of NGFI-B (paper I and IV), showing that other channels than the NMDA receptor contribute to the increased ROS and NGFI-B mRNA observed. It has been reported that addition of verapamil to cultured cerebellar granule neurons before, during, and after glutamate treatment reduced glutamate-induced cell death (Pizzi et al. 1991).

Activated $PLA_2$ may generate superoxide anion radical, and their activity can be inhibited by OBAA (a general inhibitor of $PLA_2$). Secretory $PLA_2$-IIA inhibitor 1 has been shown to selectively inhibit $sPLA_2$ type IIA (Church et al. 2001). The specificity of this inhibitor can be evaluated by measuring the activity of $sPLA_2$-IIA or by immunostaining (active and inactive $sPLA_2$-IIA has different locations). However, there was no specific assay for the detection of the $sPLA_2$-IIA activity available at the time when paper I was prepared. The synthesis of nitric oxide was inhibited by nitro-arginine (inhibitor of nNOS and iNOS). Inhibition of $PLA_2$ and NOS significantly reduced glutamate-induced cell death. It also reduced glutamate-induced ROS generation, but only OBAA and nitro-arginine reduced buffer-induced ROS generation (paper I). Nitric oxide and superoxide combine to generate peroxynitrite, and this process is only limited by the diffusion rate (Murphy et al. 1998). Peroxynitrite induces the generation of 3-nitrotyrosine on proteins. It is likely that peroxynitrite is generated in glutamate treated neurons since the level of 3-nitrotyrosine was significantly increased (paper I).

Vitamin C combined with vitamin E are ROS scavengers (section1.6.2), and they reduced glutamate-induced ROS generation and cell death, but had no effect on the expression of NGFI-B or the transcription of GCS (paper I, II, and IV). Both α-estradiol and β-estradiol may scavenge ROS and therefore protect the neurons against glutamate-induced toxicity (paper II), however, it did not affect the expression of *NGFI-B* (paper IV). Preincubation of the neurons with estradiol for 24 h before glutamate treatment abolished the acute protection (paper II). Thus, although vitamins and estradiol scavenge

ROS and reduce neuronal death, they may have different effects on other ROS regulated processes.

Caspase 3 activation was detected in chicken neurons using Ac-DEVD-amc, a fluorometric caspase 3 substrate. The caspase 3 activity was significantly increased in glutamate-treated neurons, and the addition of a caspase 3 inhibitor significantly reduced the cell death. This is consistent with previous results in rat cerebellar granule neurons (Slagsvold et al. 2003).

Addition of 9cRA, a high-affinity RXR ligand, reduced cell death induced by glutamate and calcium ionophore both when 9cRA was added before and when it was added 1 or 2 hrs after treatment. 9cRA arrests RXR in the nucleus (Cao et al. 2004) and therefore inhibits translocation of the NGFI-B/RXR heterodimer to the nucleus. Since RXR may dimerize with other transcription factors, addition of 9cRA may also have an effect on these processes (Szanto et al. 2004).

In total, this supports that critical actors in glutamate-induced neuronal death includes activation of NMDA receptors which increase intracellular calcium resulting in ROS generation by NOS and $PLA_2$, and activation of caspase 3 and NGFI-B.

### 2.5 Expression

Models used in this thesis to express reporter genes or to overexpress proteins, includes transgenic mice with firefly luciferase coupled to the GCS promoter, chicken cerebellar granule neurons transfected with an experimental reporter containing 8 NGFI-B response elements in front of a basal prolactin promoter driving the expression of the firefly luciferase reporter gene (NBRE-luc), and rat cerebellar granule neurons transfected with NGFI-Bgfp. In transiently transfected cells, the number of cells containing the transfected plasmid depends on the transfection efficiency, and the cells only express the gene for a limited period. The transfection procedure may also have an effect on the cells and may affect the results. Therefore, Renilla luciferase was used as an internal control for the transfection efficiency. NGFI-Bgfp was overexpressed and this may have an effect on the localization of the protein. However, the localization of NGFI-Bgfp in untreated neurons

was similar to the localization of wild type NGFI-B shown by immunostaining (Jacobs et al. 2004). It has also been shown by immunostaining that wild type NGFI-B translocates to the mitochondria in glutamate treated neurons (Jacobs et al. 2004). The localization of the wild type protein was not shown by immunostaining in paper IV. This is because it was not longer possible to find an antibody lot that could be used in immunostaining (the efficiency varies between different lots).

In transgenic animals, the reporter gene is expressed in all cells. This eliminates the problem with delivery of the plasmid to the cells. However, making transgenic animals is expensive and demands more resources than transient transfection. The transgenic animals may also be used to image *in vivo* bioluminescence. This would make it possible to evaluate if the estradiol preconditioning is a general phenomenon in the brain. The preconditioning phenomenon correlated with the amount of GSH detected by a glutathione assay. This assay can be used to quantify the amount of GSH (the reduced form of glutathione), but not GSSG (the oxidized form of glutathione) or tissue-linked glutathione.

## 3. Discussion of the results

The results obtained in the present thesis are discussed in 3.1-3.4, and are illustrated in figure 5.

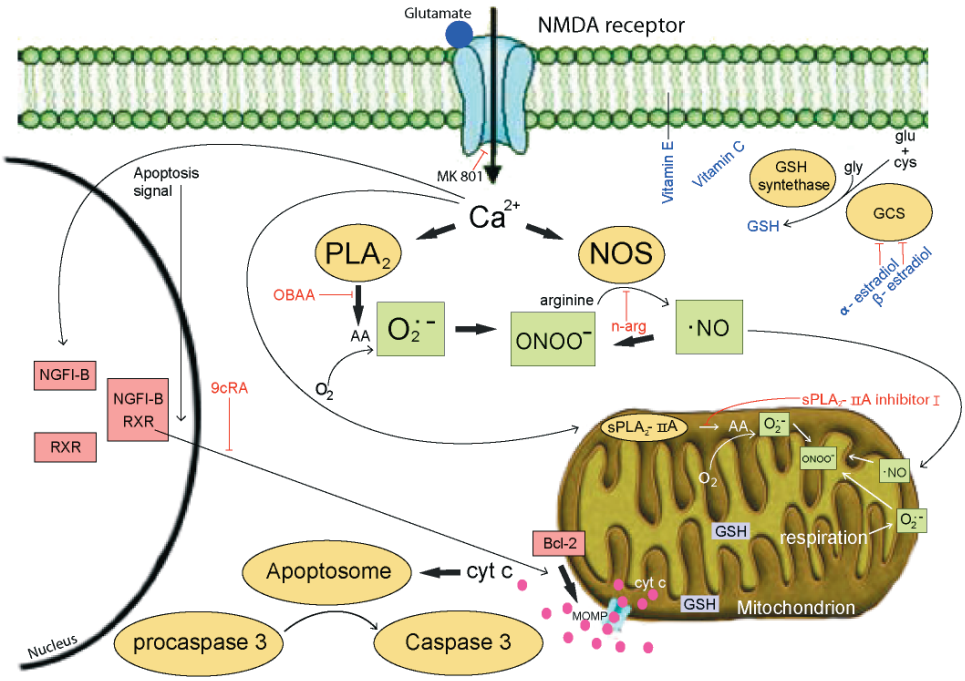


**Figure 5. Pathways involved in glutamate-induced cell death and survival.** Activation of the NMDA receptor results in increased intracellular $Ca^{2+}$. MK 801 is a non-competitive NMDA receptor antagonist. $PLA_2$, sPLA$_2$-IIA and NOS are $Ca^{2+}$ -dependent enzymes, generating $O_2^{\cdot-}$ or $^{\cdot}NO$, respectively, and the combination product is $ONOO^-$. OBAA ($PLA_2$ inhibitor), sPLA2-IIA inhibitor I, and n-arg (NOS inhibitor) reduce ROS generation. GSH, an important part of the cellular antioxidant defense, is synthesized via two steps catalyzed by GCS and GSH synthetase, respectively, and GSH is also located inside the mitochondria. Estradiol reduces the synthesis of GCS and therefore the level of GSH. Vitamin E targets to membranes whereas estradiol exerts its function inside cells. $Ca^{2+}$ induces the expression of NGFI-B. In response to an apoptosis signal (unknown signal) NGFI-B/RXR translocates from the nucleus to the mitochondria and converts Bcl-2 from an apoptosis protector to an apoptosis inducer releasing cyt c through MOMP. Cytosolic cyt c is required for the initiation of the apoptosome and the activation of caspase 3. 9cRA arrests RXR in the nucleus preventing the translocation of NGFI-B/RXR out of the nucleus. Abbreviations are shown on page 2 and 3. The mitochondrion is taken from figure 1and the membrane is taken from figure 2.

### 3.1 Glutamate-induced ROS

In paper I we show that glutamate induces ROS generation in peripheral mitochondria, whereas buffer treatment induces ROS generation in somal mitochondria. However, only increased ROS in the peripheral mitochondria were involved in the mediation of the toxicity. This may be due to differences in the mitochondrial populations. It is well known that the mitochondrial population is heterogeneous with regard to its enzymatic activities, chemical makeup, and sedimentation characteristics (Kuff and Schneider 1954). For instance, it has been shown in serotonergic neurons that mitochondria in the cell body contains monoamine oxidase type B, whereas mitochondria in axon terminals lacks this enzyme (Arai et al. 2002). Comparing brain and liver mitochondria isolated from 12-month-old rats showed a difference in the susceptibility to oxidative damage due to a difference in the antioxidant mechanisms (Santos et al. 2001). It has been found that the susceptibility to oxidative stress depends on mitochondrial contents of both antioxidants and cytochromes, and a study of three mitochondrial fractions from rat liver showed that the heavy fraction containing more mature mitochondria, was characterized by the lowest antioxidant level, the highest cytochrome content, and the lowest capacity to oppose an oxidative challenge (Di Meo et al. 1996; Venditti et al. 2002). Mitochondria are synthesized in the cell bodies of neurons and are then transported down the axon (Hollenbeck 1996). Analysis of mitochondrial potential during transport in neurons derived from the dorsal root ganglia of chicken embryos has shown that ~90% of mitochondria with high potential move towards the growth cone and ~80% of mitochondria with low potential move towards the cell body (Miller and Sheetz 2004). Thus, somal and peripheral mitochondria represent different mitochondrial populations. It is not known if NGFI-B preferentially interacts with any of these.

Both DHR and HE were used to detect glutamate-induced ROS generation (paper I), and the results showed that the level of oxidation was similar, however, only ROS detected by DHR were involved in the mediation of toxicity. DHR and HE were also used to detect ROS generation induced by an other cell death stimuli, potassium and serum deprivation (Miller and Johnson 1996), in the neurons (results not shown). The ROS generation in potassium and serum deprived neurons were approximately similar as

in glutamate treated neurons, however, only ROS detected by HE was involved in the mediation of toxicity. This shows that these two probes detect different ROS, and that they may be used as scavengers for specific ROS. To obtain increased selectivity of ROS probes they may be linked to a specific location in the cell, e.g. by targeting antioxidants to mitochondria (Murphy and Smith 2007). Such probes were not commercially available when paper I was made.

### 3.2 Long-term effect of antioxidants

It has been reported that estrogen treatment can protect against a wide range of neurotoxic insults, including free radical generation, excitotoxicity, and ischemia (Dubal et al. 1998; Green and Simpkins 2000; Behl 2002). Pretreatment for 15 minutes with β-estradiol or its non-estrogenic stereoisomer α-estradiol, or vitamin C combined with vitamin E, reduced neuronal death in glutamate treated neurons (paper II). Since β-estradiol and α-estradiol offered similar protection, it is likely that the protection is due to a non-genomic mechanism. Several potential mechanisms have been reported to be involved in the neuroprotective effect of estrogens, including its antioxidant properties (Kelly and Wagner 1999). In the presence of β-estradiol, α-estradiol, or vitamin C combined with vitamin E, the oxidation of DHR in glutamate treated neurons was reduced, showing that they reduce the ROS level in the cells, and supporting that the protection is due to an antioxidant mechanism. Preconditioning with estradiol for 24 h before glutamate treatment reduced its subsequent acute neuroprotection, whereas the neuroprotection by vitamin C combined with vitamin E offered full neuroprotection following a similar preconditioning period. These results show that combined treatment with vitamin C and vitamin E offers more effective long-term neuroprotection than estrogens. The reduced protection by long-term treatment with estradiol may be due to an effect on the synthesis of glutathione, the main endogenous antioxidant. In a PC12 model where the preconditioning phenomenon is mimicked the effect of estradiol on the GCS promoter is abolished when estradiol is linked to the membrane by a fatty acid tail (eicosaestradiol) (unpublished results by master student G.R. Øverby). Thus, the different

effects of vitamins versus estradiol on the GCS transcription may be due to the localization of the antioxidants; vitamin E targets to membranes (Monroe et al. 2005) whereas estradiol exerts its function inside cells. ROS that is involved in the mediation of toxicity must be different than ROS responsible for down-regulation of the GCS promoter.

### 3.3 Preconditioning

Preconditioning is the phenomenon that pretreatment of cells, tissues or organisms with a stimulus promote a transient response altering the result of a subsequent treatment. Preconditioning is described in different cell types, and the results of preconditioning may be e.g. increased or reduced toxicity of the subsequent treatment. In paper II we show that sustained (24 h) preconditioning with estradiol reduced the synthesis of the rate-limiting enzyme in glutathione synthesis resulting in a lower level of GSH, and the neurons were therefore more sensitive to excitotoxicity. This is a simple preconditioning mechanism. On the other hand, preconditioning may also be regulated by complex mechanisms. It is well established that preconditioning of cardiac and brain tissue with sublethal insults prepares cells to better withstand subsequent injury, e.g. ischemia (Chen and Simon 1997; Dekker 1998; Ferdinandy et al. 1998). A brief exposure to ischemia/reperfusion before sustained ischemia enhances the ability of the heart to withstand a subsequent ischemic insult (Ferdinandy et al. 2007). Similar, neurons may be protected against ischemia by sublethal ischemic insults via oxygen and glucose deprivation, and cultured rat cerebellar granule neurons may be protected against the excitotoxic effects of glutamate by subtoxic agonist-mediated stimulation of NMDA receptors (Marini and Paul 1992). The genomic expression pattern in response to ischemia is unique in a preconditioned animal, and differs considerably from the pattern activated by ischemia in a non-preconditioned animal (Stenzel-Poore et al. 2003). Preconditioning has been reported to e.g. induce the synthesis of the antiapoptotic proteins Bcl-2 and Bcl-$x_L$ (Shimizu et al. 2001; Wu et al. 2003). In general, preconditioning agents/conditions appear to act by inflicting sublethal stresses on neurons

that cause a responsive upregulation of intrinsic neuroprotective survival mechanisms (Chen and Simon 1997).

### 3.4 NGFI-B and cell death

NGFI-B has been recognized to be involved in apoptosis in different cell types. In several cancer cells NGFI-B is overexpressed (Zhang 2007), and apoptosis-inducing cancer therapy by AHPN has been reported to result in the translocation of NGFI-B/RXR to the mitochondria, initiating the apoptotic process (Zhang et al. 2002). It has also been reported that NGFI-B may be involved in neuronal cell death (paper IV) (Jacobs et al. 2004). NGFI-B translocates as a heterodimer with RXR to the mitochondria where it induces apoptosis by converting Bcl-2 from an antiapoptotic protein to a proapoptotic protein which induces cytochrome c release (Li et al. 2000), and the release of cytochrome c is critical for the activation of caspase 3 (Hengartner 2000). The protection induced by 9cRA (arresting NGFI-B/RXR in the nucleus) reduces cell death, but to a lesser degree than inhibition of caspase 3 (paper III) (Slagsvold et al. 2003). This indicates that other mechanisms than mitochondrial translocation of NGFI-B/RXR also is involved in the release of cytochrome c. We have shown that treatment with glutamate or calcium ionophore increased the expression of NGFI-B and the NGFI-B protein level (by transfecting chicken cerebellar granule neurons with NBRE-luc). To directly measure the protein level of NGFI-B, NGFI-B Western analysis should be done. However, since there was no antibody lot that could be used for Western available, this was not done. To further investigate the translocation of NGFI-B to the mitochondria, NGFI-B Western should be done on mitochondrial fractions. It would also be interesting to investigate if the glutamate induced ROS generation is involved in the mitochondrial translocation of NGFI-B. Therapy preventing the translocation of NGFI-B/RXR to the mitochondria may be reducing neuronal death. Thus, cancer therapy and neurodegeneration therapy may have the opposite effect on the localization of NGFI-B. This implicates that the side effect of such therapies must be thoroughly evaluated.

## 4. Concluding remarks

- Overactivation of glutamate receptors induces ROS, which is critical for neuronal death, due to secretory $PLA_2$-IIA associated with peripheral mitochondria. Buffer treatment induced generation of non-toxic ROS predominantly in somal mitochondria, whereas glutamate treatment led to ROS production predominantly in peripheral mitochondria. Inhibition of secretory $PLA_2$-IIA in glutamate treated neurons blocked the cell death and reduced ROS generation in peripheral mitochondria, whereas it did not reduce ROS production in buffer treated cells.

- Preincubation of cultured cerebellar granule neurons with α-estradiol, β-estradiol, vitamin C combined with vitamin E, or progesterone, for 15 min before glutamate treatment, reduced the neuronal death. Preconditioning with α-estradiol or β-estradiol for 24 h before glutamate treatment strongly reduced its subsequent acute neuroprotection, whereas vitamin C combined with vitamin E, or progesterone, offered full neuroprotection following a similar preconditioning period. The reduction was accompanied by an inhibition of the γ-glutamylcysteine synthetase promoter and a reduced level of GSH when preconditioning was combined with subsequent glutamate treatment.

- Cerebellar granule neurons from chicken may be used as a method to study excitotoxicity. These neurons respond to glutamate excitotoxicity similar to rat neurons (shown by ROS production and caspase 3 activation), they may be grown in a physiologically potassium concentration (5 mM), and have a larger transfection efficiency.

- Neuronal death is induced in neurons treated with glutamate, calcium ionophore, or arachidonic, and they trigger translocation of NGFI-B out of the nucleus. The *NGFI-B* expression is induced by glutamate and calcium ionophore, and this induction is important for maintenance of the death mechanism. NGFI-B

translocates out of the nucleus as a heterodimer with RXR, and RXR is arrested in the nucleus in the presence of the RXR ligand 9-*cis* retinoic acid. Therefore, the need for new production of NGFI-B to maintain the cell death pathway allows late protection by 9-*cis* retinoic acid from glutamate- and calcium ionophore-induced death.

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