Delayed Cell Death after Traumatic Brain Injury

Role of Reactive Oxygen Species

BY

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ABSTRACT

Clausen, F. 2004. Delayed Cell Death after Traumatic Brain Injury. Role of Reactive Oxygen Species. Acta Universitatis Upsaliensis. *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1359. 75 pp. Uppsala ISBN 91-554-5990-0

Traumatic brain injury (TBI) is a leading cause of death and disability. TBI survivors often suffer from severe disturbances of cognition, memory and emotions. Improving the treatment is of great importance, but as of yet no specific neuroprotective treatment has been found. After TBI there are changes in ion homeostasis and protein regulation, causing generation of reactive oxygen species (ROS). Overproduction of ROS can lead to damage in cell membranes, proteins and DNA and secondary cell death. In the present thesis experimental TBI in rats were used to study the effects of the ROS scavengers α-phenyl-N-tert-butyl-nitrone (PBN) and 2-sulfophenyl-N-tert-butyl-nitrone (S-PBN) on morphology, function, intracellular signalling and apoptosis.

Posttreatment with PBN and S-PBN resulted in attenuation of tissue loss after TBI and S-PBN improved cognitive function evaluated in the Morris water maze (MWM). Pretreatment with PBN protected hippocampal morphology, which correlated to better MWM-performance after TBI.

To detect ROS-generation in vivo, a method using 4-hydroxybenzoic acid (4-HBA) microdialysis in the injured cortex was refined. 4-HBA reacts with ROS to form 3,4-DHBA, which can be quantified using HPLC, revealing that ROS-formation was increased for 90 minutes after TBI. It was possible to attenuate the formation significantly with PBN and S-PBN treatment.

The activation of extracellular signal-regulated kinase (ERK) is generally considered beneficial for cell survival. However, persistent ERK activation was found in the injured cortex after TBI, coinciding with apoptosis-like cell death 24 h after injury. Pretreatment with the MEK-inhibitor U0126 or S-PBN significantly decreased ERK activation and reduced apoptosis-like cell death. Posttreatment with U0126 or S-PBN showed robust protection of cortical tissue.

To conclude: ROS-mediated mechanisms play an important role in secondary cell death following TBI. The observed effects of ROS in intracellular signalling may be important for defining new targets for neuroprotective intervention.

Keywords: Traumatic brain injury, Reactive oxygen species, Fluid percussion injury, Controlled cortical impact, Weight drop injury, Extracellular signal-regulated kinase, apoptosis, free radical scavenging, morphology, functional outcome

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Articles included

This thesis is based on the following articles, which will be referred to by their Roman numerals. Reprints were made with the permission of the publishers;

I   Clausen F and Hillered L (2004)
        Intracranial Pressure Changes During Fluid Percussion, Controlled Cortical Impact and Weight Drop Injury in Rats
        Submitted

II   Marklund N, Clausen F, McIntosh TK and Hillered L (2001)
        Free Radical Scavenger Posttreatment Improves Functional and Morphological Outcome after Fluid Percussion Injury in the Rat
        Journal of Neurotrauma 18:8:821-32

III   Marklund N, Clausen F, Lewander T and Hillered L (2001)
        Monitoring of Reactive Oxygen Species Production after Traumatic Brain Injury in Rats with Microdialysis and the 4-Hydroxybenzoic Acid Trapping Method
        Journal of Neurotrauma 18:11:1217-27

IV   Marklund N, Lewander T, Clausen F and Hillered L (2001)
        Effects of the Nitrone Radical Scavengers PBN and S-PBN on In Vivo Trapping of Reactive Oxygen Species after Traumatic Brain Injury in Rats
        Journal of Cerebral Blood Flow and Metabolism 21:11:1259-67

V   Clausen F, Lewén A, Marklund N, McArthur D, Olsson Y and Hillered L (2004)
        Correlation of Hippocampal Morphology and Morris Water Maze Performance Following Graded Cortical Contusion Injury in Rats
        Submitted

VI   Clausen F, Lundqvist H, Ekmark S, Lewén A, Ebendal T and Hillered L (2004)
        Oxygen Free Radical Dependent Activation of Extracellular Signal-regulated Kinase (ERK) Mediates Apoptosis-like Cell Death after Traumatic Brain Injury

        Submitted

ABBREVIATIONS USED IN THE THESIS

ADP Adenosine diphosphate

ATP Adenosine triphosphate

BBB Blood brain barrier

CCC Controlled cortical contusion, also called WDI

CCI Controlled cortical impact

CNS Central nervous system

DAI Diffuse axonal injury

DAB Diamino benzidine

3, 4-DHBA 3,4-dihydroxybenzoic acid

EAA Excitatory amino acids

ECF Extracellular fluid

ECM Extracellular matrix

EGF Epidermal growth factor

ERK Extracellular signal-regulated kinase

FPI Fluid percussion injury

GFAP Glial fibrillary acidic protein

2-HBA 2-hydroxybenzoic acid; salicylate

4-HBA 4-hydroxybenzoic acid

HDG Hilus of the dentate gyrus

HPLC High performance liquid chromatography

ICP Intracranial pressure

i.p. Intraperitoneal

i.v. Intravenous

JNK c-jun N-terminal kinase

MAP2 Microtubule associated protein 2

MAPK Mitogen activated protein kinase

MEK MAP kinase kinase

MD Microdialysis

NADP Nicotinamide dinucleotide Phosphate

NGF Nerve growth factor

NO Nitric oxide

NOS Nitric oxide synthase

NtBHA N-t-butyl hydroxylamine

PARP Poly (ADP-ribose) polymerase

PBN α-phenyl-N-tert-butylnitrone

PCD Programmed cell death

PTP Protein tyrosine phosphatase

ROS Reactive oxygen species

SAH Subarachnoidal hemorrhage

SOD Superoxide dismutase

S-PBN 2-sulfophenyl-N-tert-butyl-nitrone

TBI Traumatic Brain Injury

TUNEL Terminal deoxynucleotide Transferase (TdT) mediated dUTP nick end labeling

WDI Weight drop injury

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BACKGROUND

General

Traumatic brain injury (TBI) is a global medical problem that leads to death, disability and mental deficits. In young adults TBI is most often the result of motor vehicle accidents, and in the elderly TBI is most often the result of falls (205). Incidentally, in the Pacific islands, falling coconuts have been reported as a culprit of TBI (223). TBI is often complicated by other factors, such as fractures, injury to other organs or intoxication. In developed is the major cause of mortality and lifelong disability for people under the age of 45 (146). Survivors suffer long periods of rehabilitation, neurological deficits, memory impairment decreased quality of life and often behavioural changes (91, 166). Behavioural changes consists of depression, disinhibition, anxiety, aggressive behaviour, decreased motivation and personality changes (109, 239), causing much distress for the patient's family (65, 199). Although most of these symptoms relate to patients subjected to moderate to severe TBI, mildly injured persons also show post traumatic changes. Mild TBI is usually without or with minimal loss of consciousness and typical sequelae are headache, dizziness, irritability, insomnia, and concentration and memory difficulty (32).

In Scandinavia, the incidence of hospitalisation after TBI have been reported to between 200-550 / 100 000 (10, 261), which translates into around 18 000 - 50000 cases per year in Sweden. In the USA there are over 500 000 cases per year, and 10 % are fatal (3) and 20 % of the cases result in permanent disabilities (311). The socioeconomic effects of TBI are substantial; the hospitalisation and rehabilitation in the USA due to severe cases has been estimated to cost 10 billion dollars (228), out of which 3 billion dollars are spent on initial care (210).

Presently, the best way to protect the brain is to prevent the incident or decrease the severity of the insult. Because motor vehicle accidents are a major cause of TBI, car manufacturers have added numerous safety features in an attempt to lower the frequency of TBI as the result of vehicle accidents (3, 205, 233). An important element in preventing brain injuries is the helmet and the introduction of helmet laws for motor cycles and mopeds have successfully reduced the number of head traumas (275). The use of bicycle helmets has been proposed to reduce the risk of a severe brain injury with 60% after a collision or fall (63).

Pathophysiology of TBI

The severity of the initial injury is closely connected to the final outcome, in terms of physical and neurological function. The anatomic damage caused at the time of injury results in mechanical disruption of brain parenchyma, contusion formation and direct membrane damage to cells and blood vessels. There is also primary axotomy and stretching of axonal tracts, causing diffuse axonal injury (DAI) (36, 250).

There are also molecular and cellular injury mechanisms associated with the acute phase, such as disturbed cellular energy metabolism, free radical generation, glutamate excitotoxicity, and calcium and sodium influx into the cell (230-232, 238) leading to subsequent phenomena, for instance cellular swelling, damage to mitochondria, protein and DNA changes, and eventually cell death. These events are referred to as secondary injury mechanisms.

Secondary injury can be caused by pathophysiological factors – such as raised intracranial pressure (ICP) as the result of the formation of haematoma or oedema, hypotension, hypoxia, seizures, and hyperthermia (34, 88, 147, 255). These events can be treated neurosurgically and with neurointensive care. In the last 20 years neurointensive care units have been introduced throughout the Western world leading to a significant increase in favourable outcome (78, 83, 324). For instance, the mortality has decreased from 41 to 7 % for patients admitted to hospital with neurointensive care after severe TBI (83). Secondary cellular and molecular events include mitochondrial injury, protein changes, DNA-damage, inflammatory response, and delayed cell death. Cell death has been shown to continue for at least a year in both humans and rats (287, 327).

Experimental animal models of TBI have been developed to study injury mechanisms and to examine possible neuroprotective effects of pharmacological substances or critical care paradigms. The models have been designed to be highly reproducible to facilitate quantification of tissue, cellular and cell organelle effects with a minimum of intra- and inter-laboratory variations. Animal research has discovered several potential drug targets although as of yet no drug has made it through the clinical trials and entered clinical practice. However, intensive care measures such as monitoring the intracranial pressure, recording the neurochemical situation with microdialysis (MD) and treatment with hypothermia have become clinical standard (59, 134).

INTRODUCTION

Animal models of TBI

To simulate the human injury in rodents a number of different models have been developed. One category of models produces a cortical contusion and others are designed to accelerate and decelerate the brain producing a more diffuse injury. However, no model can completely simulate all the facets of human brain injury and the mechanisms behind the human pathology (Table 1).

	CCC	CCI	FPI	CHI
Cortical contusion	+	+	+	-
Subarachnoid haemorrhage	+	+	+	+
Subdural haematoma	+	+	+	+
Intraparenchymal haematoma	+	+	+	+
Hippocampal cell loss	+	+	+	-
Axonal injury	+	+	+	+
Oedema	+	+	+	+
Altered cerebral blood flow	+	+	+	+
Ischemia	+	+	-	-
Altered metabolism	+	+	+	+
Blood-brain barrier dysfunction	+	+	+	+
Inflammation	+	+	+	-

Table 1. Morphological and cerebrovascular responses observed after experimental TBI with the most common contemporary rodent models, adapted from Kline and Dixon, Headtrauma 2001 (159).

Feeney et al.(87) introduced a model for cortical contusion injury in the rat, the weight drop injury model (WDI), also called controlled cortical contusion (CCC). Briefly, a piston in the end of a tube is hit by a free falling weight within the tube and delivers a compression to the exposed brain. The severity of the injury is determined by the length of the piston, which controls the depth of the compression, which determines the severity of the injury. The deeper the compression, the more severe the injury.

Controlled cortical impact (CCI) is another injury model based on cortical compression. In rats it was originally characterised by Dixon et al. (79). The injury device uses an electronically controlled pneumatic piston to produce the trauma. Similarly to CCC the depth of the compression can be controlled, but in CCI the speed of the piston can be varied. Higher piston velocity results in a more forceful impact because more energy is delivered to the brain.

The lateral (parasagittal) fluid percussion injury (FPI) model (212) uses a transient fluid pressure pulse delivered to the brain surface to produce the injury. FPI is graded by manipulating release angle of the hammer that hits the piston in the cylinder of the FPI-device, thereby varying the pressure of the fluid pulse. If the pressure pulse is strong enough FPI produces a cortical cavity but also diffuse axonal injury (DAI) and bilateral hippocampal damage. The physiological reactions to FPI resemble those seen in humans more than CCI. Directly after the trauma mean arterial pressure rises and after the initial rise hypotension is registered. One com-

plicating factor with FPI is the possibility of brain stem injury at the severe setting, which leads to a higher degree of mortality than in the compression models.

Another frequently used injury model is the closed head injury (CHI) technique, which produces TBI by inducing an impact-acceleration injury (229). The most common model works by placing the anaesthetised rat on a styrofoam bed and hit it on the head with a weight falling down a guide rail, accelerating the head into the foam. This can be done with (94, 198) or without (276) protection of the rat skull. This produces a trauma that is very similar to that seen in humans, with diffuse brain injury and especially diffuse axonal injury (DAI). Experiments without protecting the skull bone are prone to great heterogeneity, which of course is the case in human TBI, but in the experimental setting it translates into larger groups of animals in the study.

Morphological outcome

The methods used to analyse brain injury depend on the aim of the study. A common way of assessing the changes after any insult to the brain is to prepare tissue sections by freezing the specimen and cut it at sub zero conditions; this is called cryo sectioning. Another way is to remove the water of the brain using an alcohol gradient and embedding it in paraffin before sectioning. Changes in the anatomy (cavity formation or viable tissue) can be studied using histochemistry on sections of around 40 µm thickness. For immunohistochemistry sections of between 5-10 µm are used to facilitate the penetration of the antibodies.

Immunohistochemical staining that uses antibodies to identify antigens is a widely used technique for examining changes in proteins, and in this thesis several proteins have been studied with respect to TBI: microtubule-associated protein 2 (MAP2); neuronal-specific nuclear protein (Neu-N); glial fibrillary acidic protein (GFAP); vimentin, OX-42; cleaved caspase-3 and phosphorylated extracellular signal-related kinase 1/2 (p-ERK). To detect cell death transferase (TdT) mediated dUTP nick end labelling, commonly called TUNEL-staining, was used.

MAP2 acts as a microtubule stabiliser in dendrites and the perisoma, and is useful to visualise surviving tissue after trauma it is possible to use MAP2 stained sections to more closely study changes to the dendritic tree, such as in the hippocampus. Neu-N stains the nuclei of neurones and is particularly well suited for use in double immunofluorescense stainings to determine what cell type is positive for the second protein of interest.

GFAP is a structural protein found in reactive astrocytes, and after TBI they are mostly found in areas of astrogliosis. Vimentin is another glial structural protein, but is normally only expressed in immature astrocytes during development. After TBI however, transiently vimentin positive cells can be found in the injured cortex, with a possible link to cell proliferation (305). OX-42 is a marker for microglia and binds to CD11b, a membrane receptor (115).Like Neu-N it is suited for double stainings. Cleaved caspase-3 and p-ERK are described in more detail below.

Functional outcome

To evaluate the effects of different treatments, it is important to do motor and cognitive tests to make sure that neuroprotection not only attenuates tissue loss but also preserves function (64). One could argue that studying functional outcome in rodents is not comparable to humans or non-human primates, but this depends on how well the tests are modified to reflect the same function regardless of phylogenetical differences to the cytoarchitecture of the brain (49, 73).

In study 1 and 2, this has primarily been done using the Morris water maze (MWM) (221, 222), described in detail under materials and methods. Briefly, the rats are to navigate in a water tank using visual cues to find a hidden (1 cm below the surface) platform. This tests the ability to recognize the cues and remember were the platform is hidden. The MWM was first used in experimental TBI by Smith et al. after lateral fluid percussion injury in rats (289), and have since been used by other TBI laboratories (68, 135, 252).

One of the most critical cerebral structures to learning and spatial memory is the hippocampus. Therefore the trauma is often placed over the parietal cortex to also injure the hippocampus. Morphological damage to the hippocampus has been demonstrated following FPI (92, 131, 188), CCI (97) and CCC (176, 182). According to one study (66) one side of the hippocampus is enough to support the animal in learning the MWM task and according to Warburton et al.(322) there has to be a bilateral injury to elicit spatial deficits. Only some experimental TBI studies have shown a bilateral hippocampus injury (132, 329).

To study neurological deficits after trauma, a scoring system has been devised (22, 212). It is a composite score were forelimb flexion and resistance to lateral push is scored using a graded scale where 0 indicates normal, 1 indicates abnormal, but not marked deficit and 2 indicates marked paresis. The inclined plane is used to test the ability of the animal to cling on to the surface of a gradually steeper angle. The angle at which the rat slips gives an indication of the neurological status. Other motor function tests include beam walking, where the rodent's ability to traverse a narrow beam with upright pegs is evaluated, beam balance, where the number of foot faults is counted; rotarod (rotate-a-rodent) test where a rotating cylinder with increasing speed is used to produce a fall and the time it takes for the rodent to fall is measured. Out of these three, the rotarod test has been rated as the most accurate way to score motor function (123).

Translating basic research on neuroprotection into the clinical setting

Over the last several decades numerous treatment concepts and pharmacological substances have shown beneficial results in animal models and the most promising treatments have entered clinical trials. Unfortunately, none of these studies

have shown significant enough results in humans to become a clinical reality. Most substances have been targeted against a single mechanism. To date however, no "magic bullet" has been approved for pharmacological treatment after TBI. Often the phase II trials have indicated favourable effects, but the subsequent phase III trial have failed to show efficacy (41). As of yet, only Nimodipine, a calcium channel antagonist, have been shown to positively affect outcome, although only in aneurismal subarachnoidal haemorrhage (SAH) (124). In a workshop arranged by the National Institute of Neurological Disorders and Stroke in USA this fact was discussed and the proceedings published as a comprehensive review article by Narayan et al. (225). The different clinical trials discussed therein are presented in Table 2. The failed clinical trials seem to be the result of both limitations of the experimental research and the design flaws of the clinical trials.

Animal models like CCC, CCI, and FPI are designed to study mechanistic details, but none of them represents the complexity and heterogeneity of clinical TBI (251). There is probably a need to test a substance in more than one trauma model to conclude that a mechanism is properly targeted in the trial. Most animal studies are

Substance	Mechanism	Side effects	Clinical trial	Results
Selfotel (Ciba Geigy)	Competitive glutamate antagonist	Psychomimetic/ psycoactive effects in volunteers	Phase III	Excess mortality in stroke
Cerestat (Cambridge Neuroscience)	Non-competitive glutamate antagonist		Phase III	Large intervariablility in participating clinics, small pharmaceutical company unable to finance proper preclinical studies
CP 101-606 (Pfizer)	Second generation NMDA-receptor antagonist, subtype receptor specific		Phase IIb	Not yet reported
D-Cpp-ene (Sandoz)	Glutamate antagonist	Longer time to come off ventilation, longer to leave ICU	Phase III	Treatment resulted in slightly worse outcome than placebo
Steroids (dexamethasone, trimcinalone	Proposed to decrease oedema, radical scavenging		Phase III	No clear benefits of treatment
Tirilazad (Upjohn)	Free radical scavenging		Phase III	Poor patient selection, intervariability between participating clinics
PEG-SOD	Free radical scavenging, ihibition of lipid peroxidation		Phase III	Improvements in outcome, but not enough to reach significance
IGF-1/Growth hormone (Genentech)	Treat negative nitrogen balance, catabolism	Increased susceptibility to infection	Phase II	Stopped for safety reasons by Genentech, no positive effects on neuropsychology
Nimodipine (Bayer)	Calcium channel blocker		Phase III	Positive results in patients with spontaneous SAH
Bradycor (Smithkline-Beecham)	Bradykinin antagonist, reduction of ICP		Phase III	Improvement in outcome, albeit not statistically significant, trend towards reducion in ICP. Study stoppedd by the company after adverse effects in a rat study, which later turned out to be due to poor laboratory technique, not the substance
Dexanabinol (Pharmos)	Non-competitive NMDA-recpetor antagonist, radical scavenger		Phase IIb	Non-significant decrease in mortality, fever and hypotension, significant decreasein ICP
SNX-111	Calcium channel blocker	Hypotension	Phase II	Increased mortality after treatment

**Table 2.** Clinical trials of pharmacological treatments after TBI.

performed on relatively young, male and inbred rodents, which does not correspond to age, sex, and genetic differences in patients (297). One consistent problem with the animal models is that the design seldom includes secondary insults, although some studies were made in the nineties (306). Over the last couple of years the number of studies including secondary insults such as hypoxia, hypotension, and hyperthermia, which all exacerbates the injury, has increased (20, 37, 207).

Clinical trials have been criticised for having too insensitive outcome measures compared to animal studies (41). Expanded and standardised scales have been proposed to lower the variability and enhance the sensitivity (310, 328). There is also a discrepancy between dosages of the substance. In preclinical studies the doses are calculated from body weight, whereas in clinical trials the doses are uniformly administered to the patients (41). Furthermore, most clinical studies use a therapeutic window of over four hours for the same substances that were administered less than an hour after trauma in the preceding animal studies (225). Microdialysis studies could further refine the evaluation of a drug's ability to enter the brain parenchyma in relevant doses (8), though not all drugs have to penetrate the BBB to be effective.

Secondary injury mechanisms

Experimental and clinical research has identified a multitude of potentially harmful events after TBI. The injury machinery that starts with the impact to the head continues for months and years after the incident (287, 327). Progressive neurodegeneration leads to a gradual decrease in cortical grey matter and expansion of the cerebral ventricles, with neuropsychological effects as a result (30, 31). Behind this phenomenon there are several secondary injury mechanisms, starting immediately after the initial impact with a widespread depolarisation and alteration of brain electrolytes (Figure 1) (84). Potassium efflux and calcium and sodium influx to the intracellular compartment disturbs the ion homeostasis (152, 230, 232).

The intracellular accumulation of Ca^{2+}-ions is a pivotal event in TBI because it causes mitochondrial damage, increases ROS-formation, activates proteins and changes the gene expression (211, 336). The calcium influx has been implicated to sustain the extended depolarisation after glutamate induced neurotoxicity, possibly by altering membrane proteins (183). Increased calcium in axons leads to calpain activation with subsequent proteolysis of neurofilament sidechains and over time to secondary axotomy (209).

Another important event in the acute phase after TBI is the release of excitatory amino acids (EAA) from pre-synaptic vesicles into the extracellular space. Among the EAA, glutamate is particularly harmful if it is released in excess of what the surrounding astrocytes can absorb using ATP-dependent glutamate transporters (125, 352). If the EAA is released at pathophysiological levels it starts a process called

Figure 1.
Biochemical events involved neuronal damage and cell death.
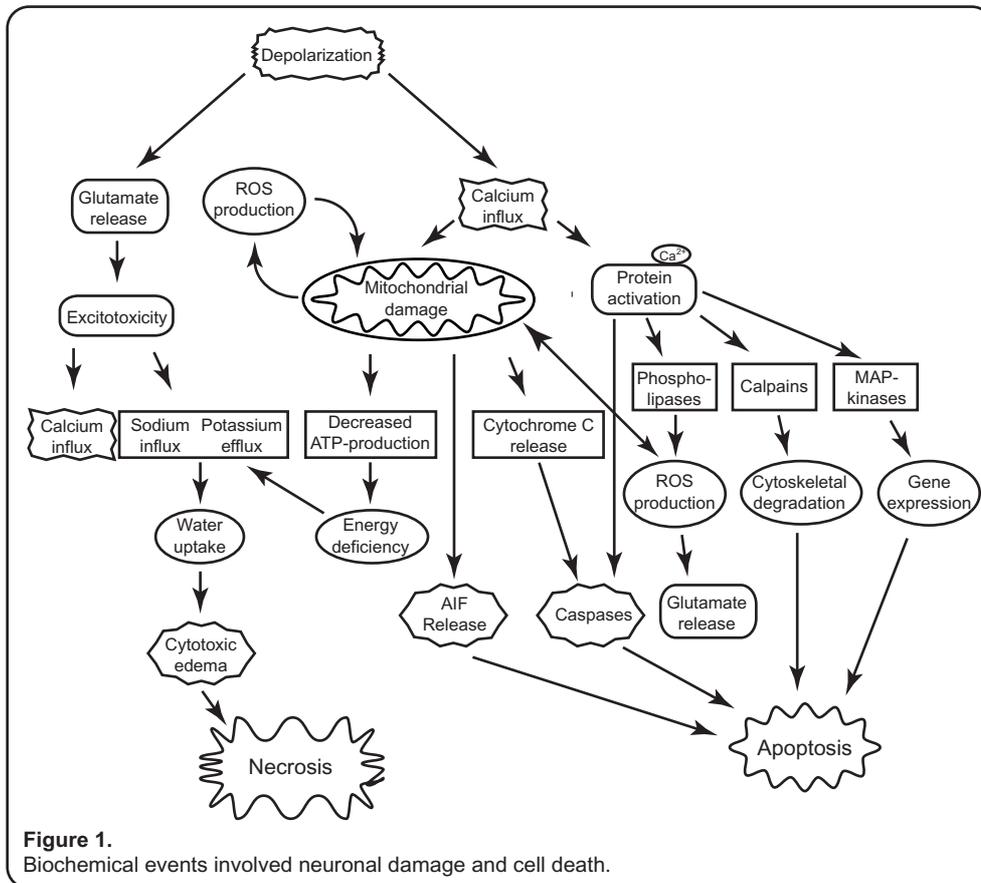
excitotoxicity as glutamate almost constitutively stimulates its receptors. One group of glutamate receptors is the N-methyl-D-aspartate (NMDA)-receptors, which are coupled to a calcium channel. When they are activated, Ca^{2+}, sodium, water, and chloride enters the cell, causing cell swelling and possibly cytotoxic oedema (346). Increased levels of glutamate in the brain interstitium have been shown both experimentally (231) and in TBI patients (246, 348).

Glutamate excitotoxicity with increased intracellular Ca^{2+} as a result can cause irreversible damage to the mitochondria (90). Calcium ions adhere to the mitochondrial membrane and enter the organelle via a specific calcium transporter. Abnormal intramitrochondrial calcium levels disturb the electron transport chain, severely disturbing the energy production (335, 336). This causes a deficiency of ATP at a time when the cell desperately needs it for protection, restoration of ion homeostasis and repair. This event has been reported to occur in axonal mitochondria as well and may be responsible for axonal perturbations after TBI (236). Injured mitochondria are also potent activators of apoptosis and generators of ROS (described below).

Astrocytes interact with the brain endothelium to form the blood-brain barrier (BBB).

The barrier is formed by tight junctions that restricts the passage of molecules into the brain parenchyma (38), as well as the infiltration of inflammatory cells (116). After TBI there is a breakdown of the BBB as early as one hour after trauma after which it transiently closes again around six hours after injury (126). During the breakdown of the BBB molecules can enter the brain parenchyma relatively freely, which has implications for potential drug delivery of substances that normally can not cross the BBB.

Inflammatory response

The inflammatory response to TBI is generally thought of as a double edged sword: certain mechanisms seem to attenuate and other exacerbate the damage (217, 315). Neutrophils are found lining the microvasculature as early as two hours after injury and starts infiltrating the parenchyma with a maximum at around 24-48 hours post trauma (290). Macrophages are present at 24 hours, but the peak within the lesion is reported between 3-5 days after trauma (138, 290). Infiltrating leukocytes release vasoactive mediators to alter cerebral vasoreactivity and cytotoxic substances such as enzymes and free radicals (111). Abnormal platelet activation has been reported to occur after TBI and accumulation in smaller blood vessels can lead to microthromboses and reduced blood flow (76). This can be ameliorated by treatment with the anti-platelet agent prostacycline, resulting in improved cortical perfusion and decreased contusion volume (27).

The production of cytokines is upregulated after TBI, especially IL-1, IL-6, and TNF-α (139, 309), inducing an inflammatory response and acting as chemoattractants to leukocytes (175). Upregulation of IL-1 and TNF-α synthesis can be found in the injured hemisphere up to three months after CCC in rats, suggesting participation in the chronic degeneration after TBI (136).

The pro-inflammatory IL-1 has two subtypes (α and β), and in the brain mainly IL-1β is induced by systemic or local insults (263), with an early upregulation of mRNA after trauma (309), which has been linked to glutamate excitotoxicity after experimental TBI (245). IL-1β has a wide range of effects that can influence cellular fate, including activation of glia, upregulation of adhesion molecules, damage to the vasculature, and release of nitric oxide and free radicals. IL-1 has an endogenous inhibitor called IL-1 receptor antagonist (IL-1ra), and treatment with recombinant IL-1ra is neuroprotective after FPI (312). However, in small and regulated amounts IL-1β can protect cells by inducing neurotrophins (262, 317).

TNF-α is also regarded as pro-inflammatory and is together with IL-1β secreted from glia cells after injury in an autocrine manner, stimulating proliferation and activation, leading to astrogliosis (317). TNF-α also mediates BBB-breakdown and increases leukocyte adhesion (280) and inhibition of TNF-α after CHI reduced oedema and protected hippocampal neurones (278). Both cytokines are also involved caspase

dependent apoptosis and oedema formation after TBI (137) and have been shown to co-stimulate inducible nitric oxide synthase (iNOS) (194). However, studies on TNF-α knock-out mice have shown that there may be a temporal shift from a damaging to a protecting effect of the cytokine, as the knock-outs show less injury one to two days after trauma and shows more deficits two to four weeks after injury (268).
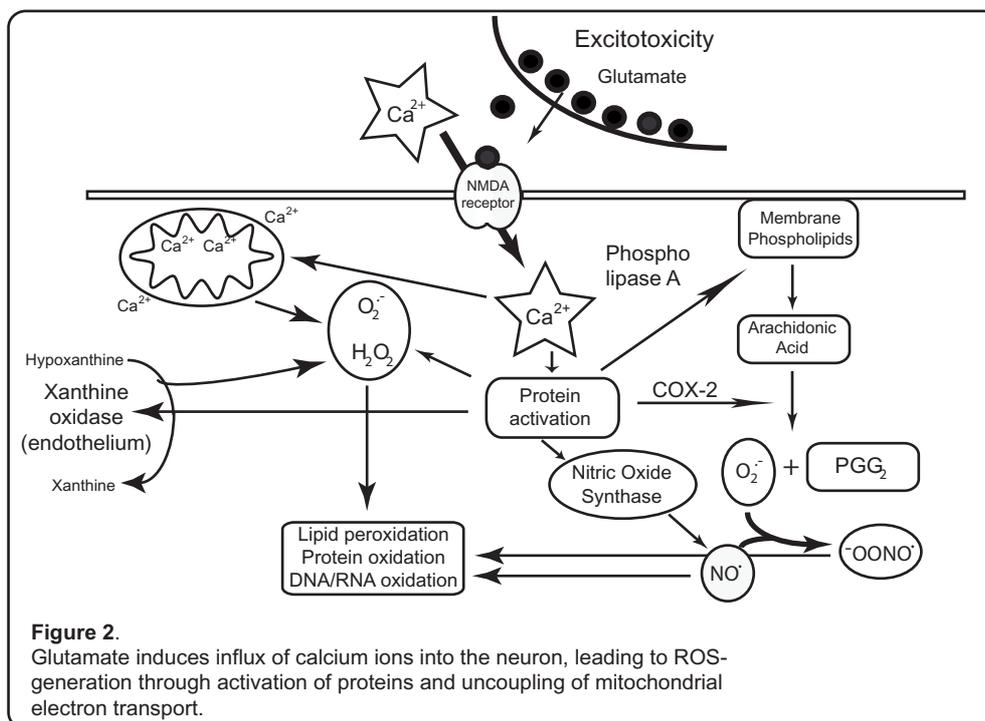
Studies on the involvement of IL-6 after ischemia have suggested neuroprotective effects. Patients with elevated IL-6 levels either in the brain measured with micro-dialysis (330) or in CSF (283) showed improved outcome. In experimental TBI IL-6 knock-out mice had a higher mortality rate, slower recovery rate and more extensive BBB-breakdown after CHI compared to the wild type (293). Possible explanations to improved outcome could be due to the ability to induce NGF-production or inhibiting TNF-α activation (217).

The complement system is activated after TBI in humans and has been found in serum (21), in ventricular CSF (165) and in brain tissue (26). The effects of the complement system are increased vascular permeability, cytokine production and facilitation of phagocytosis (315). Fragments of the complement system have been found two hours and persisting up to seven days after FPI in rats (154).

Reactive oxygen species

One prominent secondary injury mechanism after TBI is the overproduction of reactive oxygen species (ROS, Figure 2). ROS, or free radicals as they are more commonly called, can be defined as any ion or molecule that has one or more unpaired electrons (99). Species with one unpaired electron include the hydrogen atom ($H^·$), superoxide ($O_2^{·-}$), hydroxyl radical ($OH^·$), nitrous oxide ($NO^·$), and transition metals such as iron and copper. The diatomic oxygen molecule (O_2) actually qualifies because it has two unpaired electrons in two separate orbitals. However, the configuration of the unpaired electrons makes O_2 highly unlikely to react with non-radicals. It readily reacts with radicals by accepting electrons and becoming the reactive superoxide radical (81).

Normally, superoxide ($O_2^{·-}$) is generated through the cell metabolism in complex II and III of the mitochondrial electron transport chain, metabolism of arachidonic acid, nitric oxide synthase (NOS), NADPH oxidase in phagocytic cells, and xanthine oxidase in the endothelium (70). Superoxide is in itself not harmful for DNA, proteins or lipids, but it readily reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), which may damage cells by lipid peroxidation and protein tyrosine nitration. NOS synthesises both NO and superoxide from the substrate L-arginine. Under normal physiological conditions, endothelial (eNOS) and neuronal (nNOS) nitric oxide synthase are only activated in short bursts when intracellular Ca^{2+} concentration is elevated (141). After TBI however, there is a massive influx of Ca^{2+}, suggesting that NOS is

Figure 2.
Glutamate induces influx of calcium ions into the neuron, leading to ROS-generation through activation of proteins and uncoupling of mitochondrial electron transport.

continuously activated in neurones and endothelial cells, a condition that leads to increased NO and superoxide synthesis. This produces (333) and activates both eNOS and nNOS after CCC in the rat (103). The third variant of NOS, called inducible NOS (iNOS), is primarily found in inflammatory cells, but has also been found in neurones, glia and oligodendrocytes early after human TBI (104). When iNOS is activated it continuously produces NO in large amounts (117).

Superoxide also produces hydrogen peroxide (H_2O_2), which is a quite stable but oxidising agent. H_2O_2 is involved in modulation of kinases and phosphatases and is only harmful if produced in non-physiological concentrations. Combined with superoxide, hydrogen peroxide forms the Haber-Weiss reaction, resulting in the production of the hydroxyl radical ($OH^{\cdot}$), which is reactive with most known molecules, and hydroxide anion (OH^-). This reaction is normally quite slow, but transitional metals such as iron (Fe^{2+}) or copper (Cu^{2+}) ions can accelerate it, a phenomenon called the Fenton reaction. Lipid peroxidation by oxygen radicals leads to the formation of the lipid peroxyl radical ($LOO^{\cdot}$), which attacks a second unsaturated fatty acid, starting a chain reaction.

Depletion of antioxidants occurs after TBI

The ROS production after trauma is believed to be due to multiple events such as increased intracellular levels of Ca^{2+}, phospholipid degradation, increased glutamate, activation of enzymes, and mitochondrial dysfunction, causing an overload of the

endogenous free radical scavenger enzymes (Table 3), superoxide dismutase (SOD), and glutathione (GSH). Low molecular weight antioxidants (α-tocopherol (vitamin E), ascorbate (vitamin C), uric acid, melatonin, and histidine-related compounds are important antioxidants and may be depleted in damaged tissue (15, 19, 331). Though the brain has a large pool of ascorbate, in fact only the adrenal glands have a higher concentration (16), other important antioxidants such as catalase, SOD, and glutathione peroxidase are produced in lower amounts than in other organs (208). After TBI the antioxidant reserves are depleted for at least seven days (19). The defence against free iron is also worse as CSF contains far less transferritin than plasma and the transferrin present is more or less saturated under normal conditions (121). The brain endothelium contains the enzyme xanthine dehydrogenase which can be converted to xanthine oxidase contributing to the production of superoxide (29). Finally, the brain is rich in monoamine neurotransmitters (dopamine, epinephrine and norepinephrine), which are oxidised by monoamine oxidase with hydrogenperoxide as a by-product. All this means that the brain is rather poorly protected against pathophysiological levels of ROS (279).

Cellular effects of increased ROS-formation

Lipid peroxidation of cell membranes of neurones and glia is catalysed by free iron released from haemoglobin, transferrin, and ferritin by either lowered pH or oxygen radicals (326). If this process is not stopped, the lipid peroxidation will progress over the surface of the cell membrane, causing damage to phospholipid-dependent enzymes, disruption of ionic gradients, and, if severe enough, membrane lysis. The CNS is particularly susceptible to lipid peroxidation, becuase the membrane lipids of the brain are rich in polyunsaturated fatty acids, which readily participate in both initiation and propagation of peroxidation (172).

Hydrogen peroxide has been recognised as a second messenger in intracellular signalling; there is evidence that an increased level of ROS triggers gene activation of immediate early genes (IEG) such as c-fos and c-jun, heat shock proteins, cytokines, growth factors, adhesion molecules, apoptosis-related proteins and proteases (60, 129).

Spin trapping

To study ROS *in vivo,* a number of methods have been used. One indirect technique uses salicylate (2-HBA) treatment that reacts with free radicals, forming the adducts 2,3-DHBA and 2,5-DHBA. These new compounds can be detected by HPLC (93) and this method has been used to study ROS formation in brain tissue using microdialysis after experimental stroke (42, 157) and TBI (164). Phenylalanine and 4-hydrobenzoic acid (4-HBA) have also been used to detect hydroxyl radicals (100, 301).

Antioxidants	Mechanism	Location
Endogenous enzymes		
Direct antioxidants		
Superoxide dismutase (SOD)		
Copper/zinc-SOD (CuZnSOD)	Superoxide dismutation	Cytosol
Manganese-SOD (MnSOD)	Superoxide dismutation	Mitochondria
Extracellular-SOD (ECSOD)	Superoxide dismutation	Extracellular space
Catalase	Hydrogenperoxide degradation	Cytosol
Glutathione peroxidase (GSH-Px)	Hydrogenperoxide removal	Cytosol
Low molecular antioxidants		
Endogenous		
Glutathione	Substrate for GSH-Px	Cytosol
NADH		
Carnosine, homocarnosine, anserine	Chelating agents	
Melatonin	Scavenger for peroxyl and hydroxyl radicals	
Uric acid	Scavenger for peroxyl, hydroxyl and singlet oxygen radicals	
Exogenous		
Ascorbic acid (vitamin C)	Reducing agent, recycles tocopherol	Cytosol
Tocopherol (vitamin E)	Reducing peroxyl radicals	
Lipoic acid	Scavenger for peroxyl, peroxynitrite,, hydrogen peroxide and singlet oxygen radicals, chelating agent	
Other important proteins		
Transferrin	Iron binding	
Ceruloplasmin	Copper binding	Produced in astrocytes, excreted into the EC-space
Hemoglobin	Iron binding	
Albumin	Iron binding	
Metallothionein I, II and III	Scavenger for superoxide and hydroxyl radicals	Cytosol and nucleus

Table 3. Endogenous antioxidants and associated proteins in the cellular defense against ROS

Substances that react with free radicals and become fluorescent have also been discovered. 2,7-dihydrochlorofluorescein diacetate reacts with peroxynitrite (249), lucigenin detects superoxide anion and hydrogen peroxide (12, 247) and dihydroethidine reacts with superoxide (213). Other methods use protein- (286) or DNA-oxidation products (98, 332) as markers for oxidative damage.

Neuroprotection by ROS scavenging

ROS-inhibition has been proposed as a possible neuroprotective treatment after TBI. This idea has been tried in clinical trials, albeit with no significant improvement of outcome for neither Tirilazad (203) or PEG-SOD (345). Nitrones are compounds with spin-trapping ability of free radicals and were originally developed as tools in the study of short lived free radical species. Alpha-phenyl-tert-N-butyl nitrone (PBN) was originally used as a detector for free radicals when it was discovered that due to the

stable adducts it forms with free radicals it could be neuroprotective (47, 48, 237). PBN is lipophilic and pass easily through the blood-brain barrier (BBB) and into brain parenchyma (56) and has been reported to decrease the concentration of hydroxyl radicals (274) and attenuate phospholipid breakdown (178) after TBI. PBN treatment have been shown to attenuate the lesion size after experimental ischemia (347, 351) and TBI in rat (196). A neuroprotective effect has been shown with a 12 hours post injury treatment after focal ischemia (45).

PBN has two sulfonated analogues that have been found equally protective in cerebral ischemia models. Sodium-2-sulfophenyl-N-tert-butyl nitrone (S-PBN) and disodium 2,4-disulfophenyl-N-tert-butylnitrone (NXY-059,) differ from PBN in the important aspect that due to their hydrophilic character, they do not pass the blood-brain barrier easily. S-PBN is capable of free radical scavenging (193), reducing excitotoxic injury (270) and decrease infarct volume after focal ischemia (343). NXY-059 treatment four hours after injury reduces the lesion with 44% after transient focal ischemia in rat (304) and has significant effects on motor function and lesion volume in a primate model of permanent focal ischemia (202). These results and other prompted AstraZeneca to start clinical trials on NXY-059 (*Cerovive)*, which are currently in phase III (256) after a successful phase II trial (174).

The neuroprotective efficacy of S-PBN and NXY-059 found in experimental ischemia despite the poor BBB-penetration may be due to effects in the blood-endothelial interface. Another possibility could be metabolites of the substances, but none have been detected *in vivo.*

Apoptosis versus necrosis

"There is no field of basic cell biology and cell pathology that is more confusing and more unintelligible than is the area of *apoptosis versus necrosis*" (85). Apoptosis is a construction from the Greek words apó (from) and ptósis (fall), illustrating the falling leaves of autumn trees and was coined by Kerr in 1972 (155).

Necrosis can be tracked as far back as 1858 and Lecture XV of Virchow's Cellular Pathology. It was used to describe an advanced state of tissue breakdown, although the Greeks used it (*nécrõsis*) in ancient texts for the same phenomenon (190). Since necrosis *per se* is not visible in the tissue until 12-24 h after injury, there is an ongoing discussion about its use. The phrase "accidental cell death" has been put forth as a replacement (28). However, in the field of TBI research the term necrosis is accepted. Necrosis is characterised by the swelling of cytoplasmic organelles and disruption of mitochondrial and plasma membranes (Figure 3)(184). The disrupted cell spills dangerous components (e.g. lysozymes), normally stored in subcellular compartments, into its immediate environment, often eliciting an inflammatory response (72). This will affect neighbouring cells and could induce cell death to them

(140). In the secondary injury phase both necrotic and apoptotic cells can be found. If the cell maintains a certain level of ATP-production it will enter apoptosis. If no energy is available it will go into necrosis (102, 227). However, it has been reported that there are cells that show both necrotic and apoptotic signs after TBI (257), suggesting an ATP-dependent shift from apoptosis to necrosis (Figure 4).

Apoptosis

Apoptosis was first proposed in the early 1970s as a controlled form of cell death (155), although ultrastructural studies after experimental TBI in cats had previously described the morphological signs of apoptosis (271). It is often used synonymously with programmed cell death (PCD), something that is inaccurate. The term apoptosis refers to certain changes in morphology and biochemistry that also occur in PCD during development of the embryo. These include cell shrinkage, condensation and cleavage of chromatin, membrane blebbing and the formation of cytoplasmic structures called apoptotic bodies (Figure 3). The resulting membrane enclosed vesicles are recognised and removed by phagocytes, avoiding the activation of a inflammatory response (72). Biochemical markers of apoptosis include the activation of endonucleases, DNA-

Figure 3.
Apoptotic versus necrotic cell death.

Figure 4.
The energy state of the cell partly determines the mode of cell death

degradation and the activation of caspases. The process is energy dependent.
In developmental apoptosis research, several proteins and pathways have been found occurring before or during apoptosis (Figure 5). These includes cysteinyl
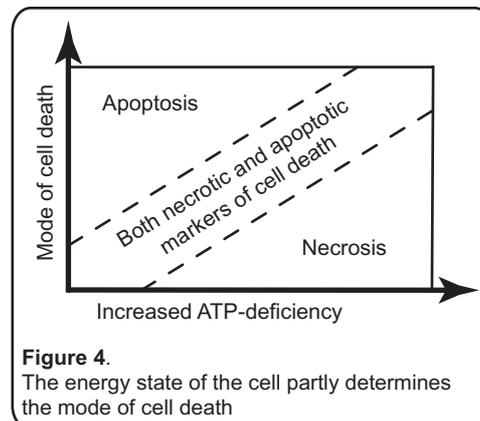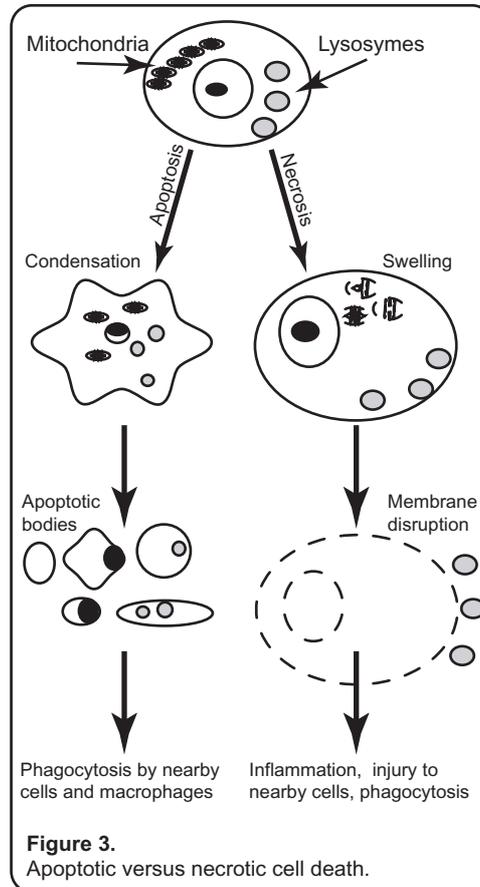
aspartate-specific proteases, commonly called caspases, tumour necrosis factor-α (TNF- α), apoptosis inducing factor (AIF), apoptosis activating factor-1 (apaf-1), various bcl-2 family members (i.e. Bax, Bcl-X$_s$ and Bid), and cytochrome C (189, 204). Caspases can be divided into initiator and effector caspases. Initiator caspases (i.e. caspase -2, -8,-10, and -12) start the apoptotic pathway and activate effector caspases (i.e. caspase -3,-6, and -7), which in turn activates the biochemical processes leading to cell death. Caspase dependent cell death is divided into two pathways. The intrinsic pathway is activated by hypoxic stress, growth factor withdrawal or irradiation, which can lead to a shift in the balance between pro- and anti-apoptotic Bcl-2 family members. This rapidly elicits a loss of mitochondrial membrane potential, leading to release of cytochrome C, therefore also known as the mitochondrial pathway, and eventually activating caspase-3 (341). This event is also seen after oxidative or calcium induced injury to the mitochondria (177, 335, 337).

The extrinsic pathway is dependent on extracellular stimulation of the death receptors (Fas or TNFR1) to send the signal downstream to caspase-8, which activates caspase-3, -6, and -7 as well as Bid.

There are also caspase-independent pathways. Apoptosis inducing factor (AIF) is released from the mitochondrion in the same manner as cytochrome C, if the membrane is damaged. AIF enters the nucleus and induces cellular apoptosis via chromatin condensation and large-scale DNA-fragmentation (302). Other proteins proposed to induce caspase-independent apoptosis are calpains, cathepsins, and granzymes (149).

Mitochondria and apoptosis

Obviously, mitochondria play a pivotal role in apoptosis. The organelle is the centre of the Bcl-2 family of pro- and anti-apoptotic proteins and if the membrane integrity is compromised, the mitochondrion releases cytochrome C and AIF, two potent activators of apoptosis (72). In the cytosol of the mitochondria there is also a protein called Smac/Diablo which acts by binding to and inactivating a group of anti-apoptotic proteins called inhibitor of apoptosis proteins (IAPs) (51). IAPs inhibits apoptosis by binding to active caspases, for instance the X chromosome linked IAP (XIAP) binds to caspase-9, an event that is blocked by Smac/Diablo (292).

The location of these proteins within the mitochondria probably reflects how important it is for the cell to have a fully operational "power-house". If too much of the cells capability to produce ATP is damaged, it has to enter a cell death program, since a poor energy state will lead to several problems, with the inability to duplicate or repair DNA properly as the most dangerous aspect (285). This could lead to the activation of oncogenes and transform the cell into a budding tumour growth, which would threaten the entire organism (140).

Figure 5.
Apoptotic pathways
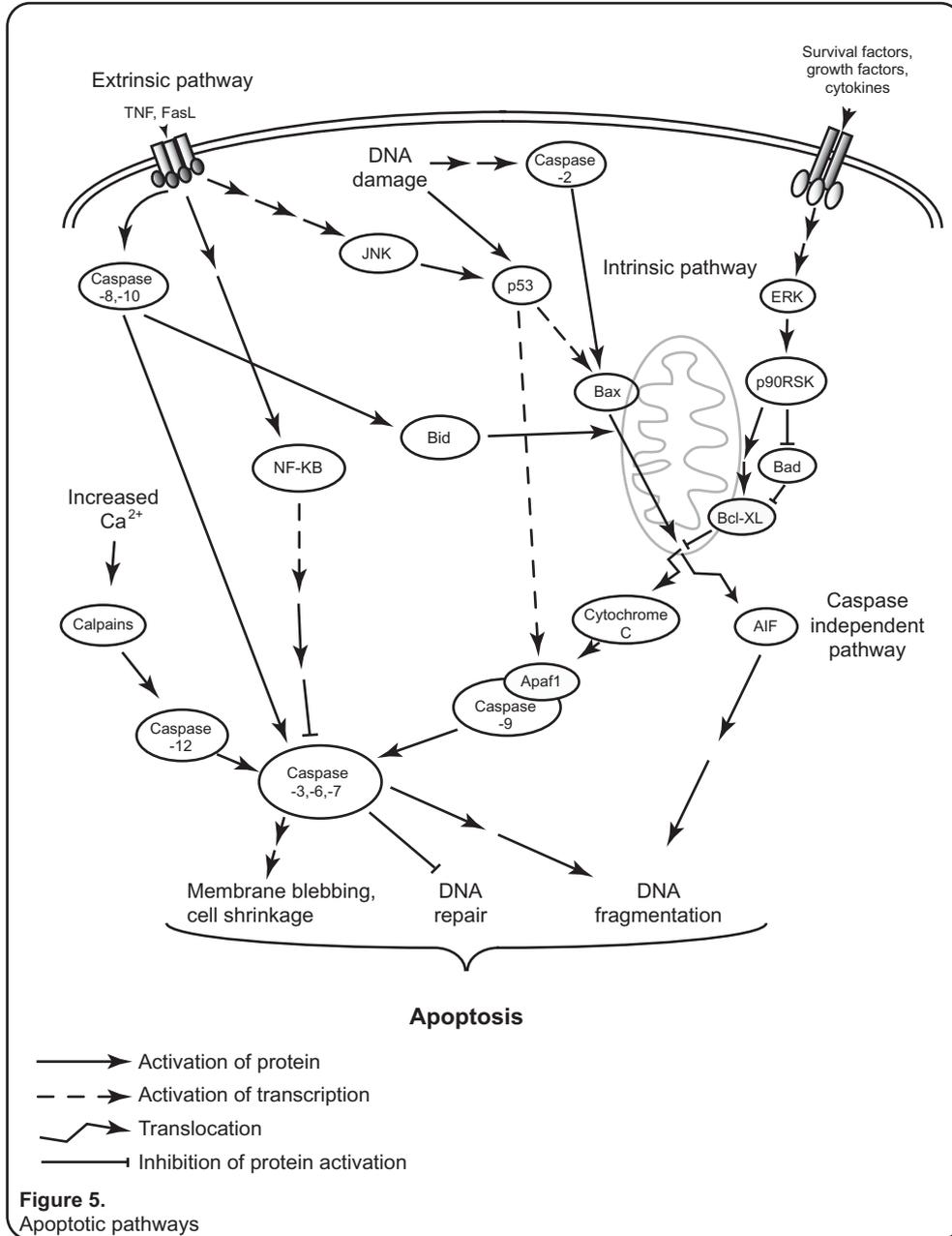
Apoptosis after TBI

Kaya et al. (153) showed the "apoptotic" timecourse using several apoptotic markers after TBI. They reported that apoptosis begins as early as 2 hrs after injury and peaks around 48 hrs post trauma, but persists for at least 2 weeks. Conti et al. (1998) reported that at two months after TBI in rats the apoptosis reached baseline levels in most brain regions (62).

All cell types of the brain have been shown to undergo apoptosis after TBI (62, 153, 226). In general neurones seem to be the first to succumb, followed by astrocytes, whereas too little is known about the temporal profiles of oligodendrocytes, microglia and endothelial cells to conclude their place in the order (184). All three pathways have been identified after TBI (23, 40, 338, 349), suggesting that there are several different stimuli to apoptosis after TBI.

Intrinsic pathway

The release of cytochrome C from mitochondria into the cytosol has been detected six hours after CCI in rats and is further increased at 12 and 24 hours after trauma (300). Cytosolic cytochrome C binds to apoptotic protease-activating factor-1 (Apaf-1) which allows the recruitment and activation of caspase-9. Activated caspase-9 cleaves pro-caspase-3, thereby activating it. Evidence of upregulation of Apaf-1 expression and caspase-9 activation have been found after FPI (340). Cleaved caspase-3 has been found in neurones, astrocytes and oligodendrocytes from 6-72 h after injury in rats (24). Activated caspase-3 is responsible for several biochemical effects, such as disintegration of actin and α-spectrin (266) and inhibiting DNA repair by proteolysis of poly(ADP-ribose) polymerase (PARP) (171).

Extrinsic pathway

TNF-α is increased in tissue and CSF after TBI (113, 309). It is, however, possible that TNF-α plays a dual role after TBI (see above under inflammatory response). Fas and Fas ligand (FasL) on the other hand has been found in the damaged cortex from 15 minutes up to 72 h after trauma (23, 24) and has been shown to form the death-inducing signalling complex (DISC) in mice (253). Subsequent activation of caspase-2 or -8 by DISC would provide a pathway to activate caspase-3 (55).

Caspase-independent pathways

Nuclear translocation of AIF has been shown within hours after CCI, accompanied by large-scale DNA-fragmentation (349). The DNA-fragmentation after AIF translocation differs from that seen in caspase dependent apoptosis as the fragments are much larger, hence the denomination large-scale fragmentation. Earlier studies on apoptosis after trauma have reported the presence of cells that did not adhere to the proposed apoptotic morphology (57, 257). This, and the fact that caspase inhibition only partially attenuates apoptosis after TBI (58, 339) supports that caspase-independent apoptosis occurs post trauma.

*Mitogen activated protein kinases (*MAPKs*) and apoptosis*

Programmed cell death after TBI is a response based on the balance of pro- and anti-apoptotic proteins as well as stress-activated signalling pathways (184). The latter includes MAPK cascades (ERK, JNK and p38). Both ERK and p38 were upregulated after CCI in mice (220), whereas FPI in rats increased ERK and JNK (242).
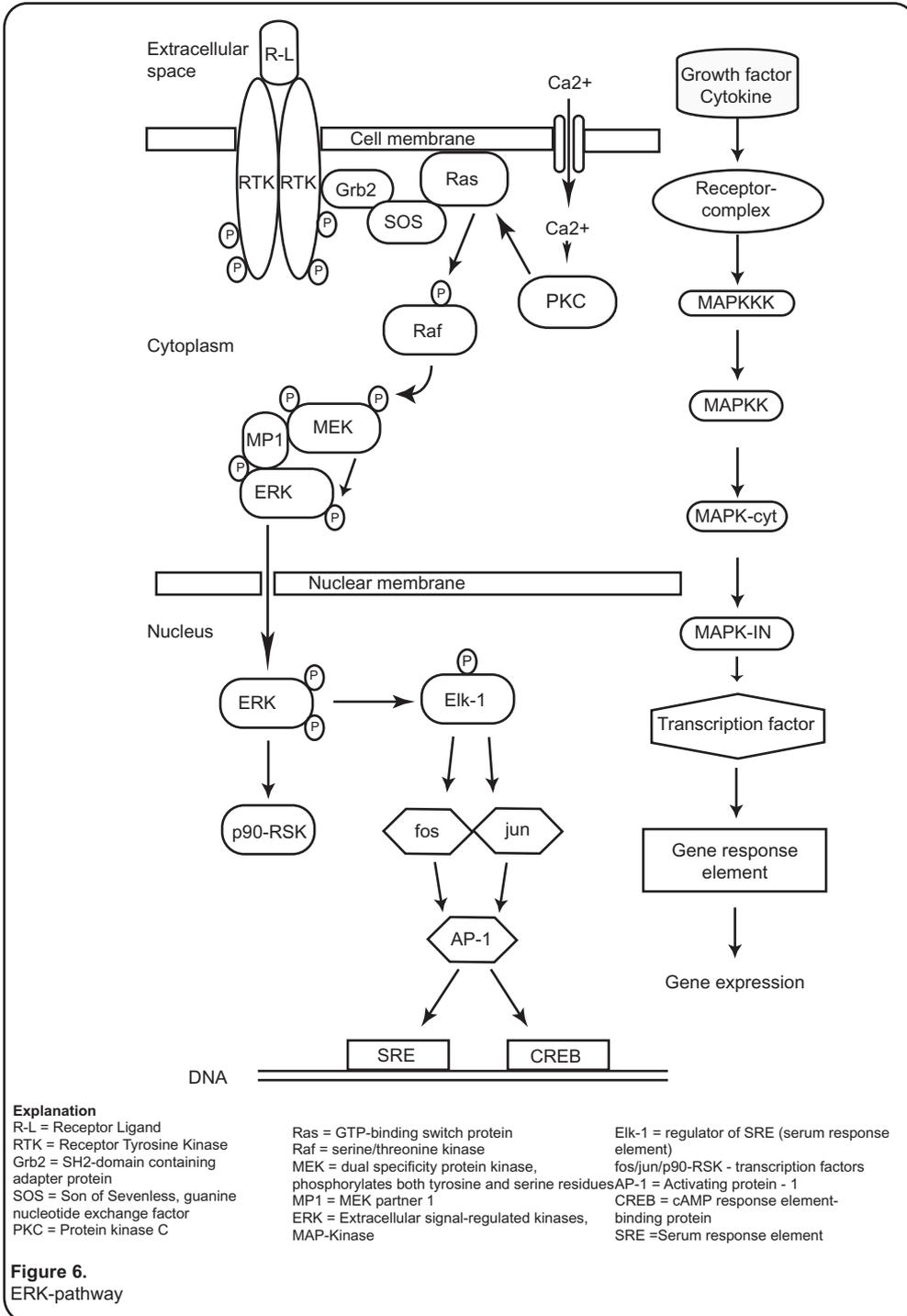
Extracellular-signal regulated kinase

Recent studies have shown that MAPKs have a role in the secondary injury process. MAPKs are involved in signalling between the cell membrane and several transcription factors in the nucleus. One of the MAPK-cascades is the Ras-Raf-MEK-ERK-pathway (Figure 6), often referred to as the extracellular signal-related kinase 1 and 2 (ERK 1/2) pathway. This pathway is primarily activated by tyrosine kinase receptors such as the epidermal growth factor receptor, Trk A (the high affinity receptor for nerve growth factor), and cytokines such as transforming growth factor beta (TGF-β). However, protein kinase C (PKC), activated by increased intracellular calcium, can also activate Ras (61). ERK has effects downstream on cell proliferation, differentiation (303) and memory (2). Two other important MAPK-pathways are the c-Jun N-terminal kinase (JNK) and p38 cascades, both mainly involved in stress responses (260).

MEK, a dual specificity kinase, is considered to be the executive step in the pathway by phosphorylating ERK on a threonine and a tyrosine residue (Figure 7) (44). After phosphorylation ERK forms homodimers (156) and passes the nuclear membrane to transfer the signal into the nucleus, where it can activate other proteins (p90RSK and Elk-1) or transcription factors (c-fos and c-jun). ERK can be dephosphorylated by several phosphatases. Dual specificity phosphatases can dephosphorylate both the tyrosine and threonine sites. Two members of this family are called MAPK phosphatases (MKP)-1, which has been proposed to act within the nucleus and MKP-3, which acts in the cytosol (254). Protein tyrosine phosphatases (PTP) can dephosphorylate the tyrosine residue and serine/threonine protein phosphatases can remove the phospho-group from the threonine site, inactivating ERK (43).

Although each MAPK-cascade has unique targets, there is a substantial amount of cross talk crucial to the co-ordinated response of the cell (260). For instance both ERK and JNK activation is required for T-cell activation and IL-2 production (101, 299).

The most common way to inhibit the activation of ERK is to inhibit the activation of MEK. There are several MEK-inhibitors available, but the most frequently used are PD98059 and U0126, because of their high specificity (86). Owing to the involvement in proliferation of ERK 1/2 pathway, the MEK-inhibitors were originally developed as anticancer drugs for solid tumours (272).

Two major secondary injury mechanisms after TBI are glutamate toxicity and oxidative stress (195, 211, 232) and both have been linked to increased activation of ERK *in vitro* (1, 4, 168, 215, 264). Inhibition of MAPKs has been shown to decrease caspase-3 activation and cell death after oxidative stress injury in neuronal cultures (167, 235). An early and transient phosphorylation of ERK has been linked with neuronal ischemic preconditioning (112), but if the ERK activation is prolonged it

Explanation
R-L = Receptor Ligand
RTK = Receptor Tyrosine Kinase
Grb2 = SH2-domain containing adapter protein
SOS = Son of Sevenless, guanine nucleotide exchange factor
PKC = Protein kinase C

Ras = GTP-binding switch protein
Raf = serine/threonine kinase
MEK = dual specificity protein kinase, phosphorylates both tyrosine and serine residues
MP1 = MEK partner 1
ERK = Extracellular signal-regulated kinases, MAP-Kinase

Elk-1 = regulator of SRE (serum response element)
fos/jun/p90-RSK - transcription factors
AP-1 = Activating protein - 1
CREB = cAMP response element-binding protein
SRE =Serum response element

Figure 6.
ERK-pathway

can promote cell death (284). Continuous stimulation of the EGF-receptor has been found to induce ERK-dependent cell death in cortical cultures (50). It has also been

reported that an increase in ROS in the cells leads to inhibition of protein tyrosine phosphatases (PTPs), the proteins responsible for dephosphorylation of proteins upstream of ERK (89, 173) and of ERK itself (43). It is currently not known if the MKPs are regulated similarly.

One proposed mechanism is the nuclear retention of activated ERK 1/2 (294) which can influence downstream effectors of cell death and the resulting apoptosis that seems to be caspase-dependent (167, 294, 296). ERK activation after TBI has been reported previously in both cortex and hippocampus early after trauma (46, 241, 242) and treatment with MEK-inhibitors have shown neuroprotection in experimental stroke (5, 318). The robust tissue protection shown in these studies suggest that MEK-inhibition could be a possible target for pharmacological treatment. However, phosphorylation of ERK is a crucial step in memory formation through long term potentiation and treatment with U0126 has been found to impair long term recognition memory (35). MEK-inhibition with PD98059 in rats subjected to CCI has been reported to lead to poorer performance in the MWM two weeks after trauma (67). The possible

Figure 7.
Regulation of ERK

negative effects on memory could be transient, but if the condition persists for longer times it is doubtful that MEK-inhibitors would become clinically accepted. Nevertheless, they are useful tools in experiments that attempt to describe the mechanisms behind neurotrauma.
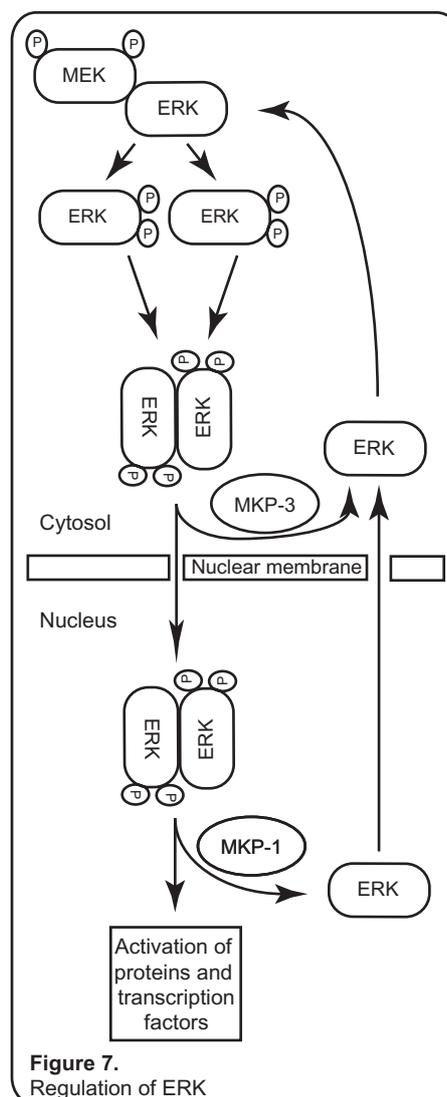
Aims

The general purpose of my studies has been to extend the studies on the involvement of reactive oxygen species (ROS) after traumatic brain injury. To this end, the ROS scavengers PBN and S-PBN have been used as tools to study ROS mechanisms in study II, IV, V, and VI.

The specific aims of this thesis are as follows:

- To study whether or not the extracranial measurement of the pressure pulse routinely performed in FPI accurately reflects the intracranial pressure. The intracranial pressure in CCC and CCI were measured for comparison. *Study I*

- To investigate if the two nitrones PBN and S-PBN offer neuroprotection in a post-treatment paradigm after TBI, with morphology and spatial learning as outcome measures.
Study II

- To evaluate if microdialysis with 4-HBA is a feasible method to analyse free radical generation in the cortical brain parenchyma after TBI.
Study III

- To study the effects of PBN and S-PBN on ROS formation in the cortical brain parenchyma after TBI.
Study IV

- To investigate if changes in hippocampal morphology correlates to spatial learning impairment after TBI and if ROS influence these outcome measures.
Study V

- To study if ERK activation increases after TBI, if ROS- or MEK-inhibition can reduce ERK-activation, and if there is a connection between ERK-activation and delayed cell death
Study VI

MATERIALS AND METHODS

Animal experiments

The local animal ethics committee of Uppsala County approved all animal experiments. All efforts were made to reduce any animal suffering during and after the surgical procedures. A mix of isoflurane/N_2O/O_2 (1.2-1.4 in 70/30 mix) was used as general anaesthesia during surgery, and local anaesthesia (Xylocain, AstraZeneca, Sweden) was used in all cuts and sutures. Animals were kept together in groups of three or four in the animal facility to keep the social rigor to a minimum.

Physiological monitoring included core and brain temperature, blood pressure and blood gases (pH, pCO_2, and pO_2).

Surgical procedures

The animals in *Study I, II, III,* and *IV* were intubated with a PE 205 tube and connected to a mechanical ventilator. The animals in *study VI* were allowed to breathe spontaneously using a nose cone with the anaesthetic gas mix.

To monitor blood pressure, the tail artery was cannulated with a catheter and connected to a pressure transducer. From the catheter, blood was drawn for the arterial blood gas analysis. In *Study II, IV,* and *VI,* the external jugular vein was cannulated to deliver the PBN and/ or S-PBN drugs.

In *Study VI,* an intrathecal infusion of U0126 was administered by drilling a hole (diameter 0.5 mm) at − 1 mm from

Figure 8.
Landmarks of the exposed skull bone for invasive procedures.

bregma and 1.5 mm lateral to the midline (Figure 8) and inserting a thin needle (diameter 0.4 mm) 3.5 mm into the lateral ventricle according to Paxinos atlas (244). The same technique was used in *Study I* to insert a thin intracranial pressure probe (diameter 0.34 mm) into the lateral ventricles.

Controlled cortical contusion injury

In *Study I, III, IV, V,* and *VI* the CCC model was used to produce a cortical contusion injury. The technique has been used in our lab for more than a decade (133, 231). Briefly, the scalp was cut along the midline and, using a dental drill, a craniotomy 6 by 9 mm centred at bregma -3.5 mm and 3.5 mm lateral to the midline was made (Figure 9). The 21 g weight was dropped 35 cm through the guiding tube and onto a piston of 4.5 mm diameter, which was resting on the exposed and intact dura (Figure 10). The pistons were made to produce a compression between 1.5 and 2.5 mm: 2.5
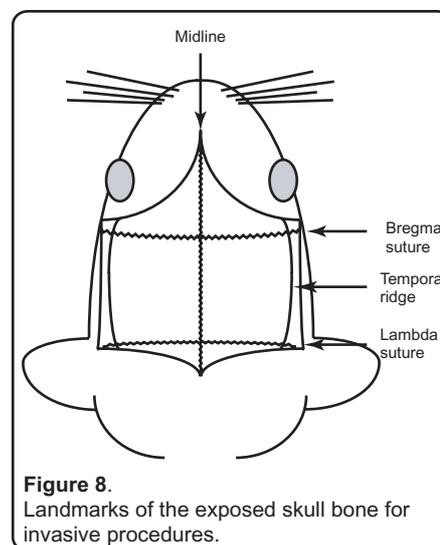
mm depth produces a necrotic cavity and hippocampal and thalamic damage (severe injury); 2.0 mm compression induces a moderate injury, leading to spongiosis in the shear stress zone (SSZ) around the rim of the impact site but no cavity formation (moderate injury); 1.5 mm results scattered cell death mainly in the SSZ and axonal damage in the subcortical white matter (mild injury) and only transient changes in the hippocampus (176, 230, 231). After the trauma the bone piece was replaced and the scalp sutured.

Controlled cortical impact

In *Study I* CCI was included to compare the intracranial pressure generated during impact with CCC and FPI. CCI relies on a pneumatically driven piston to compress the exposed brain (Figure 11). The parameters for calibration of the injury severity are the velocity of the piston as it hits the dura and the depth and duration of the compression. Severe injury was defined as a piston velocity of 4 m/s and a compression depth of 2.5-3.0 mm. The CCI injury was performed using a pneumatic device (made at the VCU Biomedical Engineering Facility, Virginia, USA). The animal was put asleep the same way as in the CCC procedure, then placed in a stereotactic frame to prevent movement. The scalp was cut open, and a 6 by 8 mm craniotomy was made (Figure 9). The bone fragment cut out was put in isotonic saline until replaced. The dura was kept intact to improve reproducibility of the injury. The velocity of the piston was calibrated to 4 m/s and monitored using an oscilloscope connected to a PC with LabView software. The piston was centred 4.5 mm behind
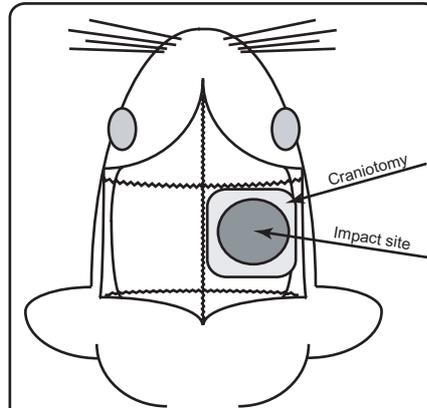

Figure 9.
Location of craniotomy in the CCC and CCI.

Weight drop injury device

A = weight
B = tube
C = piston housing
D = piston
E = weight stopper
F = exposed brain

When the weight hits the piston a compression of the brain is produced

Figure 10.
Main features of the CCC model.

Controlled cortical impact device

A = control unit
B = oscilloscope, monitoring the piston velocity
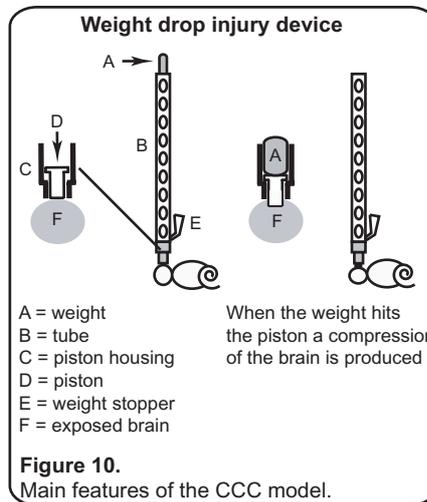C = computer connected to oscilloscope
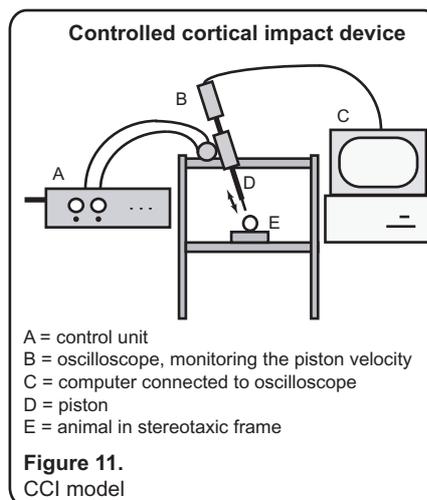D = piston
E = animal in stereotaxic frame

Figure 11.
CCI model

bregma and 4.5 mm lateral to the midline on the exposed dura mater. The duration of the compression was set to 100 ms and the compression depth to 2.5 mm.

Fluid percussion injury

In *Study I* and *II,* FPI was used as a trauma model. To connect the rat to the FPI device, a 4.8 mm craniotomy situated at bregma -2 to -7 mm and along the temporal ridge was made (Figure 12) using a trepanoma. A modified female luer-lock coupling was fitted into the hole and fixed with tissue adhesive. An anchoring screw was fastened into the skull bone contralaterally. Dental cement was poured around the coupling and the screw and allowed to harden.

Then the animal was connected to the FPI device and a pulse of saline was projected onto the dura with a pressure of 2.2-2.9 atm (Figure 13). The length of apnoea and loss of toe-pinch reflex were monitored. After resumption of breathing, the animal was reconnected to the ventilator.

Sacrifice

In *Study II, IV,* and *V,* the animals were sacrificed with an overdose of chloral hydrate and pentobarbital (300 mg/kg). Then they were either transcardially perfused with phosphate buffered 4% formaldehyde and the brains prepared for embedding in paraffin, or they were put into a cryo-protective solution (isotonic saline solution with 25% glucose) and then snap frozen in iso-pentane (temperature -55°C) and stored in -70°C.

In *Study IV*, the animals were sacrificed by rapid decapitation immediately after the microdialysis was done and while they were still under isoflurane anaesthesia. Trunk blood, bilateral samples from the cortex, hippocampus and the cerebellum were collected for analysis of 4-HBA, PBN and S-PBN concentrations. Animals without the MD experiments were included to determine the brain concentrations of PBN and S-PBN, with or without trauma.

Figure 12.
Preparation of the rat skull for FPI.
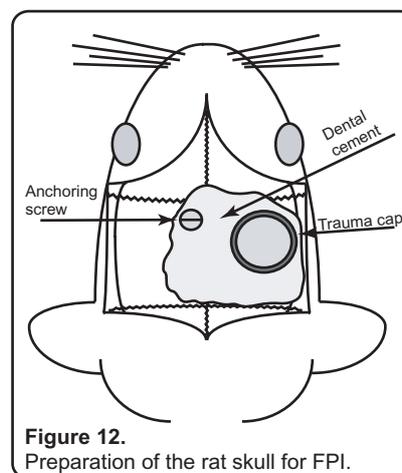

Fluid percussion injury device

A = pendulum hammer
B = piston
C = saline filled cylinder
D = nozzle head with extracranial pressure sensor
E = rat coupled to the nozzle
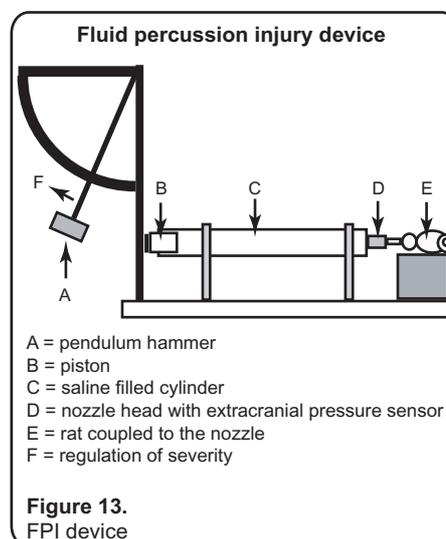F = regulation of severity

Figure 13.
FPI device

For Western blot studies in *Study VI*, the animals were sacrificed under deep haloth-
ane anesthesia and the brains removed. Brain tissue samples weighing 30-35 mg
were made, using a 2.5 mm biopsy device, from the centre of the lesion, the region
directly affected by the cortical impact, eventually developing into a cortical cavity
(196). Samples were also taken from the perifocal cortical region.
Paraffin embedded brains were sectioned to 5-7 µm using a slide microtome, and
freeze sections were sectioned to 12-14 µm using a cryotome.

ROS and MEK inhibition treatments

The ROS scavengers α-phenyl-N-tert-butylnitrone (PBN) and 2-sulfophenyl-N-tert-butyl-
nitrone (S-PBN) were administered as an i.v. post-treatment in *Study II,* using a cannu-
lation to the external jugular vein (Figure 13)*.* Both treatments were started as a bolus
dose 30 minutes after trauma as equimolar doses (PBN 30 mg/kg; S-PBN 47 mg/kg).
A continuous i.v. infusion was then started (PBN 30 mg/kg/h; S-PBN 47 mg/kg/h) and
continued for 24 hours with a rate of 0.5 ml/h. Injured control and sham injured animals
received saline solution in the same manner.
The MEK-inhibitor U0126 or the inactive homologue (U0124) was administered via in-
fusion to the contralateral lateral ventricle, as described above. Using a syringe pump,
the drug was infused either as pre- or post-treatment at a rate of 1 µl/minute and total
volume of 100 µl. Pretreatment experiments began 160 minutes before trauma and con-
tinued for 100 minutes. After the infusion the rats were left for 60 minutes to let the drug
diffuse into the parenchyma. The post-treatment regime was applied to four sub-groups
using intrathecal infusion for 100 min (see Table 4 for details). To study the involvement
of oxygen free radicals we included a group of animals treated with S-PBN (47 mg/kg
dissolved in saline), which was administered via i.v. infusion 15 minutes before trauma.
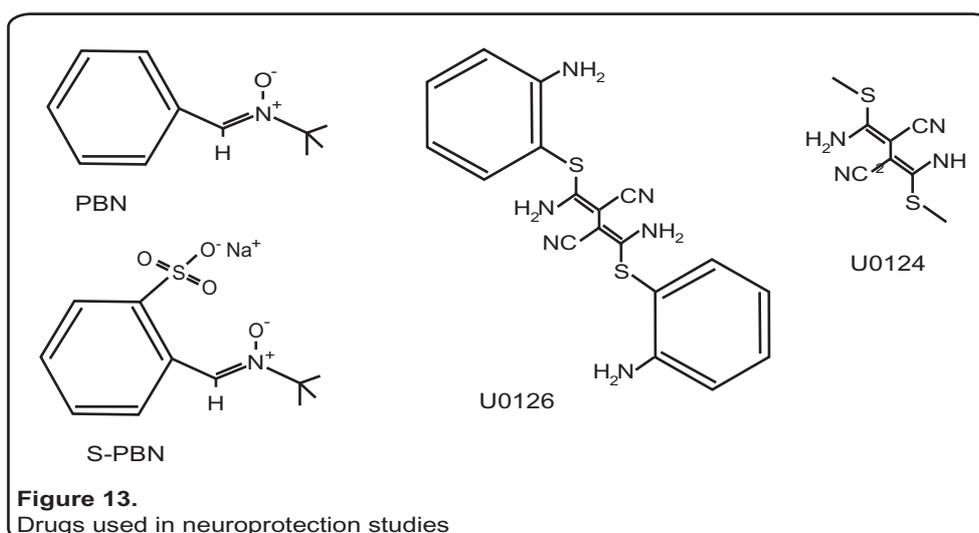

Figure 13.
Drugs used in neuroprotection studies

Group	1	2	3	4
Number of animals	6	6	6	6
Treatment	U0124	U0126	U0126	U0126
Dose	100 nmol	100 nmol	100 nmol	10 nmol
Timepoint (post injury)	30 min	30 min	24 h	30 min

Table 4.
Treatment groups in the post-treatment experiments with the MEK-inhibitor U0126 and the inactive homologue U0124.

Intra- and extracranial pressure measurement during experimental TBI

The intracranial and extracranial pressure probes and their monitoring systems were provided by Samba Sensors (Gothenburg, Sweden). The probes were 0.34 mm in diameter and had a pressure range of 0.90 to 5.0 atm. The probe consists of a small pressure sensor mounted on the end of an optical fibre coupled to a pressure monitor connected to a computer that continually registers the pressure. The system had a sample frequency of 500 Hz. The intracranial probes were modified to protect them by inserting and securing them in a modified needle tip, leaving 3.5 mm of the probe tip free to insert into the ventricle. The extracranial probe replaced the original pressure probe of the FPI device (situated in the nozzle of the device, 50 mm from the tip).

The intracranial pressure probes were stereotactically inserted into the ipsilateral or contralateral lateral ventricles depending on the treatment group. The stereotactic co-ordinates (Paxinos, 1998) were 1.5 mm lateral to the midline suture, -1 mm to bregma, and 3.5 mm below the dura. The probes were secured with tissue adhesive and dental cement around the modified needle part of the probe to allow movement of the animal from the stereotactic frame in the FPI experiments. In CCI, tissue adhesive and dental cement was used to secure the probe, because of the force of the impact which was not necessary in the WDI experiments where the probe was held in place using an arm attached to the stereotactic frame.

The pressure probes were calibrated to the ambient air pressure (1 atm) immediately before injury, using the internal calibration system of the pressure monitors. The data recording was started and the injury induced.

Immunohistochemistry

DAB-stainings

The paraffin embedded sections were rehydrated and microwaved in a citrate buffer to unveil antigens and endogenous peroxidase activity was blocked with H_2O_2. The sections were blocked for unspecific binding with normal horse or goat serum after which the primary antibody was applied and incubated in a humidity chamber overnight. The secondary antibody was applied before the avidin-biotin-complex was added. The stainings were visualised using di-amino-benzidine as a chromatic agent and counterstained with Mayer's hematoxylin, a nuclear stain.

Cryosections were thawed and fixed in cold acetone instead of the rehydration and microwave treatment. Thereafter the procedure was as described above.

Immunofluorescense stainings

Double immunofluorescense stainings were used in *Stydy VI*. After an over-night incubation at 4°C with the first primary antibody (rabbit polyclonal), the slides were incubated for 30 minutes with a fluorescent secondary anti-rabbit antibody (fluorescein-conjugated). After rinsing, the second primary antibody (mouse monoclonal) was applied and incubated for 2 hours at room tempera-ture. Negative controls were made by omitting both primary antibodies. After rinsing, the second secondary antibody (anti-mouse, biotinylated) was applied and incubated for 2 hours. After rinsing, Texas Red Avidin was applied and incubated for 30 minutes. After rinsing the cover slips were mounted and the slides stored at -20°C.

Histochemistry

Cryosections were thawed and fixed in cold acetone and stained for eosin and Mayer's hematoxylin. The sections were dehydrated using ethanol and xylen and then covered using pertex.

Morphological evaluations

In *Study II and V*, for the volumetric part of the morphological investigation, we stained sections from five different anatomical levels throughout the lesion us-ing H&E or MAP2 stainings, beginning at bregma 0 level and caudally to about 6 mm behind bregma. The sections were digitised and software (Neurozoom, Scripps Research Institute New York, NY, USA) was used to measure and cal-culate the cortical volume in the injured area and the total cavity volume.

In *Study V,* the MAP2 stained sections were evaluated semiquantitatively by two blinded examiners. The CA1, CA2, and CA3 regions and the hilus of the dentate gyrus (HDG) were scored bilaterally. For each animal, we studied four slides from different anatomical levels, from bregma -1 to –6 mm. We scored between 0 and 3 according to the criteria in Table 5. The results from both ex-aminers were averaged and statistically analysed.

Score	Cell body	Dendrites
0	No visible changes	No visible changes
1	Slightly abnormal morphology	Abnormal morphology
2	Abnormal morphology	Loss of dendrites, abnormal morphology
3	Cell loss, severely abnormal morphology	Severe loss of dendrites, abnormal morphology

Table 5.
Scoring system for changes in hippocampal morphology to cellbodies and dendrites

Western blots

The biopsies were homogenised using a glass homogenizer in ice cold buffer. The samples were centrifuged at 8000 g for 10 minutes in 4°C to remove debris. The supernatant was frozen at -80°C before being analysed.

Sample protein concentration was measured using a protein assay kit. Twenty-five μg of sample and 1:2 sample buffer were heated at 70°C for 5 minutes. The samples were loaded to and separated by gel-electrophoresis for 35 minutes and then transferred to a nitrocellulose membrane for 100 minutes. Unspecific antigen binding was blocked by incubation with 5% dry milk in PBS-Tween20 for 60 minutes and then washed with PBS-Tween20. The membranes were subsequently incubated with primary polyclonal antibodies at 4°C overnight. After washing with PBS-Tween20, a secondary antibody (peroxidase conjugated monoclonal anti-rabbit) was applied at room temperature for 60 minutes. Western blot visualisation was done using chemiluminescense. The membranes were stripped from ERK-antibodies and antibodies for β-actin was applied and processed as described above, serving as a control for the amount of protein loaded.

The films were scanned and quantified using the NIH-image software (National Institute of Health, Bethesda, MD, USA). Densitometric values were corrected for background and normalised for protein loading using β-actin as an internal reference. Both the p44 and the p42 bands were included in the quantification.

Functional evaluations

Neurology

In *Study II,* using a modified scoring system, neurological function was tested on day 1, 4, and 8 after trauma (22). Forelimb flexion and resistance to lateral push was scored using a graded scale where 0 indicated normal, 1 indicated abnormal, but not marked deficit and 2 indicated marked paresis. The sum of the two scores was used for the analysis. An inclined plane test was also used by placing the rat with its right or left side downward while the plane was elevated. When the rats lost their grip, the angle of the plane was noted.

Morris water maze

This test was developed in the early 1980's and has since gained both many users and recognition(75, 221). It consists of a circular tank that's 1.4 meters in diameter and a 12 by 12 cm transparent platform hidden 1 cm below the surface. The rats are subject to four trials a day, starting at day eleven post injury and continuing to day 15. A trial is run by placing the rat in the tank at different starting points facing the wall, activating the video-based computer tracking system (HVS Image Ltd. U.K.) and stopping the system when the rat finds the platform. Then the rat is allowed to remain undisturbed on the platform for 15 seconds to acquire the visual cues of the

room. The animal is given 120 seconds to find the platform, if it does not, it is placed on the platform for 15 seconds. Several parameters were automatically recorded by the overhead video camera and by a computerised tracking system sensitive for contrast (i.e. a white rat in a dark pool). The most useful parameters in our hands were as follows: latency to find the platform, which indicates whether or not the animal learns to find it; swim speed, to evaluate whether the motor function was comparable between injured and sham operated animals; path length, how far the animal swims before it finds the platform. To further refine the test the platform was removed to perform a probe trial, a trial that examines the amount of time the animal spends searching for the platform in the correct quadrant of the pool.

In vivo free radical trapping using microdialysis

Intracerebral microdialysis (MD) is a method that uses a double-lumen cannula with a semi-permeable membrane tip to trap small substances in a fluid phase such as the interstitial fluid of the brain. It relies on simple diffusion driven by the difference in concentrations of the substances in the perfusion fluid and the tissue investigated. The membrane allows molecules up to 20 kDa to move out of the probe. At steady state the dialysatet concentration of a metabolite is proportional to the interstitial concentration, determined by the relative recovery of the MD probe used (314).

When 4-HBA is exposed to ROS, it reacts and forms the stable adduct 3,4-DHBA. By adding 4-HBA to the MD perfusate and collecting the dialysate, it is possible (using high performance liquid chromatography, HPLC) to detect how much of the 4-HBA has reacted with free radicals to form 3,4-DHBA (Figure 14) (195, 298).

Microdialysis was performed using a 3 mm membrane micro-dialysis probe connected to a microdialysis pump and perfused with 3 mM 4-HBA dissolved in artificial CSF (aCSF). A nick in the dura was made in the central part of the craniotomy and the probe was inserted into the brain paren-chyma. The probe was perfused at the rate of 2.0 µl/min and the samples were collected every 30 minutes and stored at -70°C be-fore analysis. Baseline samples were collected during two hours before trauma. Then samples
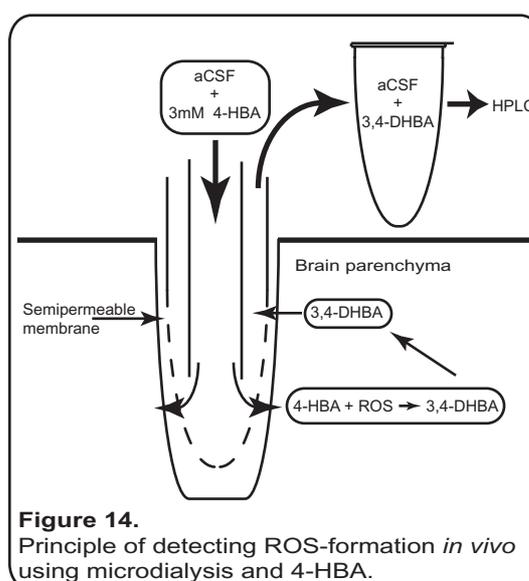

Figure 14.
Principle of detecting ROS-formation *in vivo* using microdialysis and 4-HBA.

were collected for three more hours after trauma. During the CCC procedure, the probe was removed and immediately replaced using the stereotactic co-ordinates. The relative recovery was evaluated in *Study III* and were 18% for 4-HBA and 12 % for 3,4-DHBA with no significant differences after TBI.

High performance liquid chromatography analysis

In *Study III* and *IV*, a metal free HPLC-system was used to prevent auto-oxidation of 4-HBA. The microdialysis samples were analysed for 3,4-DHBA using a reversed phase system coupled with electrochemical detection. Brain tissue samples were homogenised, centrifuged, and filtered before analysis of 4-HBA, PBN and S-PBN concentrations using a HPLC-system with UV-detection. Blood samples were analysed for PBN and S-PBN in the same manner. External standards of 3,4-DHBA and 4-HBA was used to create standard curves with 5 known concentrations. Standard curves for PBN and S-PBN were made by adding 5 known concentrations to rat plasma or brain tissue extracts.

HPLC made it possible to detect PBN concentrations in plasma above 0.35 µg/ml and S-PBN above 0.62 µg/ml. For the brain samples, the levels of detection were 2.0 µg/g for PBN and 0.3 µg/g for S-PBN.

Statistical analyses

In *Study I, II, III, IV,* and *VI* analysis of variance (ANOVA) was used for group comparisons, with subsequent post-hoc test (Bonferroni or Fischer's PLSD) if the ANOVA was statistically significant at p<0.05. For neurological data in *Study II,* non-parametric methods (Kruskal-Wallis and Mann-Whitney's U-test) were used. These calculations were made in the StatView software (Abacus Concepts Inc. Berkeley, CA, USA). In *Study V,* morphological data were analysed by repeated measures ANOVA with Sheffe's post-hoc test, and correlation statistics was done using conventional Pearson product-moment correlation coefficients in the statistical software SPSS (SAS Institute, Cary, NC, USA) and Statistica (Stat-Soft, Tulsa, OK, USA). Comparisons between correlated coefficients from *Study V* were made in PEPI (Sagebrush Press, Las Vegas, NV, USA).
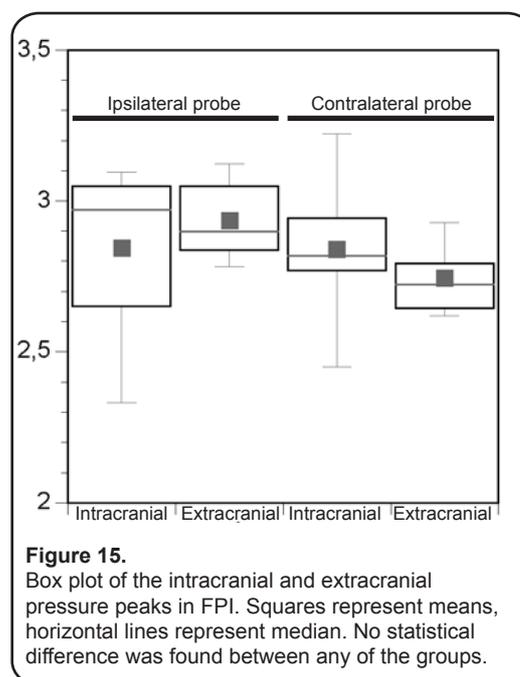
Results and discussion

Intracranial pressure registration, *Study I*

The study investigates how well the intracranial pressure (ICP) correlates with extracranial measurements normally recorded in FPI and if there were any differences in ICP between the ipsi- and contralateral lateral ventricle. We also measured the ICP during CCC and CCI for comparison. This is the first successful recording in rats of the intracranial pressure pulse during impact, because small enough high pressure probes have not been available until recently.

Our results showed that the intra- and extracranial pressure measurements in FPI had a high degree of correlation (ipsilateral intracranial probe and extracranial probe: $r = 0.992$, $p < 0.0001$; contralateral intracranial probe and extracranial probe: $r = 0.996$, $p < 0.0001$). No differences in peak ICP were found between ipsi- and contralateral recordings (Figure 14). Both CCC and CCI were shown to elicit an intracranial pressure pulse recordable in the contralateral lateral ventricle, albeit significantly smaller than in FPI. Using the data, we were also able to roughly estimate the time of global ischemia to between 100-200 ms in FPI and 2-4 ms in CCC and CCI. It is unclear whether this ultra-short ischemia has any effect on outcome. Ischemia shorter than one minute has been shown to produce preconditioning of the brain (186, 206), but this issue needs further evaluation.

The pressure monitored in the nozzle of the FPI-device is a good estimation of the ICP. The pressure pulse was equally strong in both lateral ventricles, supporting the theory that FPI has global impact on the brain. Both CCC and CCI sustained some mechanical impact to the contralateral side, although both models generally are considered focal models of TBI.

Figure 15.
Box plot of the intracranial and extracranial pressure peaks in FPI. Squares represent means, horizontal lines represent median. No statistical difference was found between any of the groups.

Effects of PBN and S-PBN on TBI; Morphological and functional outcome, *Study II*

Because a previous study (196) found that pre-treatment with PBN improved outcome, a study to discern if post-treatment had the same beneficial effects was done and was expanded to include S-PBN, a sulfonated analogue to PBN more closely resembling the clinical drug candidate NXY-059. The treatment started 30 minutes after trauma, a timepoint chosen because it reflects the earliest possible treatment after TBI in a human scenario, which would be by paramedics at the site of injury. FPI was used as injury model. A neurological scoring test, an inclined plane test, and MWM were used to evaluate functional outcome and hemispheric tissue loss for morphological outcome evaluation.

The neurological scoring showed that all traumatised groups differed compared to the sham operated animals. However, it did show a significant improvement at day 4 and 8 in the PBN-treated group compared to saline treated injured animals. In the inclined plane test, the S-PBN treated animals performed better than the saline treated on day 4. No other treatment effects were found in the neurological tests.

MWM results showed that S-PBN treatment significantly reduced both latency to find the platform and path length compared to either PBN or saline treatment. Swim speed did not differ between traumatised or sham injured animals, signifying that neurological deficits were not part of the increased latency.

The morphological evaluation showed that post-treatment with PBN or S-PBN did not attenuate the cortical cavity volume significantly, although there was a strong trend in the S-PBN treated animals (p=0.06). The hemispheric tissue loss was significantly lower in both nitrone treatment groups compared to saline.

Pharmacological neuroprotection by nitrones has shown robust effects in experimental stroke models (169, 170, 248, 351) and we have shown efficacy of pre-treatment with PBN after CCC in a previous study (196). It was interesting to find that S-PBN, a substance that poorly penetrates the BBB and has a shorter half-life (communication from the manufacturer), showed at least as good neuroprotective results as PBN, which is known to easily diffuse into the brain parenchyma (54). However, a disturbed BBB may leak S-PBN into the brain after TBI, since BBB breakdown is a known sequelae of experimental brain injury (269, 291). This issue led to the design of *Study IV*.

Free radicals are known to increase BBB permeability and one proposed mechanism of radical scavengers is to protect the BBB by protecting the microvascular endothelium from lipid peroxidation (326). Endothelial cells are suggested to be generators of ROS due to their high concentrations of mitochondria, polyunsaturated fatty acids and xanthine oxidase (170, 325). Xanthine oxidase has been reported to be involved in ROS induced injury to the cerebrovascular endothelium (162) and reperfusion

injury to the endothelium after experimental cerebral ischemia (25). The damage to the cerebrovascular endothelium probably contributes to the increase in hydroxyl radical production after TBI (120). Together this suggests that the endothelium of the brain is as much a potential target for radical scavenging as the neurones and glia cells. This may be part of the explanation for S-PBN:s neuroprotective effect despite its poor BBB penetration.

The concept of free radical scavenging after TBI has been explored previously experimentally and two clinical studies have been performed, PEG-SOD and Tirilazad (225), unfortunately without significantly beneficial effects. An inactive cannabinoid analogue, dexanabinol, which is primarily a non-competitive NMDA receptor antagonist, but also with radical scavenging properties, has shown promising results in a phase II clinical trial and is currently in phase III trial for TBI (18, 160). At the moment NXY-059 is in clinical trial for stroke and constitutes a strong candidate for further trials in TBI-patients.

Tracking ROS production in the brain parenchyma after TBI in rats, *Study III*

As mentioned in the introduction, several methods have been used to study the generation of free radicals after insults to the brain, and among them salicylate (2-hydroxybenzoate, 2-HBA) is particularly well suited for microdialysis experiments (42, 93, 157, 164). However, salicylate affects vascular tone, platelet aggregation, and inflammatory responses (9) in ways that make it unsuitable for use in human patients. Since microdialysis has become part of the multimodal monitoring of TBI-patients, it would be of interest to be able to measure the free radical production in humans after brain injury. 4-hydroxybenzoic acid (4-HBA) is a possible alternative since there are no known adverse side effects. 4-HBA forms one stable adduct, 3,4-dihydroxybenzoic acid (3,4-DHBA) after contact with ROS and has been previously used in detection of free radicals after focal ischemia with good results (106). However, before any experiments can be done in human patients, the possible toxicity of 4-HBA has to be ruled out.

The results showed a graded increase of ROS formation after TBI. 1.5 mm cortical compression led to a small and transient increase of free radicals, whereas 2.5 mm compression elicited a marked increase of 3,4-DHBA that persisted for 90 minutes after trauma. The results were compared with control experiments using 2-HBA as the trapping substance and the dialysates were analysed for the resulting 2,3- and 2,5-DHBA adducts. The concentrations of both adducts were increased similarly to 3,4-DHBA after trauma, a result that agrees with previous work (7, 120).

4-HBA is subject to spontaneous 3,4-DHBA formation in aqueous solution and a control experiment showed that prolonged exposure to room temperature increased the formation of the adduct. Since the microdialysis protocol demands that the solu-

tion be exposed to room temperature for six hours, some of the 3,4-DHBA could be due to spontaneous formation. It is also of utmost importance that the solution has as little as possible exposure to metal surfaces as this increases hydroxyl radical formation in water (122); however, some metal contact in the microdialysis system was unavoidable, which also can have contributed to the 3,4-DHBA concentration of the samples.

In vivo effects of ROS scavenging with PBN and S-PBN after TBI in rats, *Study IV*

The effects seen in *Study II* warranted further studies into the scavenger effects of PBN and S-PBN and their pharmacokinetics in brain tissue. After establishing a method of *in vivo* radical trapping using 4-HBA and microdialysis after TBI in *Study III*, the technique was put to use in studying the effect of the nitrones on free radical generation after CCC.

Traumatised animals showed an increase of over three times in ROS-formation compared to sham injured controls, at 30 and 60 minutes after trauma. Pretreatment with PBN significantly reduced the overall increase of 3,4-DHBA concentration; however, it did not significantly reduce it compared to saline treatment at the individual time points. S-PBN pretreatment significantly attenuated 3,4-DHBA formation at 30 minutes after trauma compared to saline treated injured controls. Both nitrone treatments lowered the concentration of 3,4-DHBA to the extent that the groups were not significantly different from sham operated controls.

PBN concentrations remained high in brain and plasma up to 210 minutes after injection, the end of the experiment. PBN was found in all brain samples and there were no statistically significant differences between the injured and uninjured parietal cortices. S-PBN was not found in plasma or brain 210 minutes after trauma. At 30 and 60 minutes after trauma or sham injury, no S-PBN was detected in the brain samples. In the plasma samples S-PBN was cleared with an estimated half-life of 9 minutes.

Blood-brain barrier

BBB-penetration has been described as an important feature that is necessary for successful pharmacological neuroprotection (80). However, a number of substances with poor ability to enter the brain parenchyma have shown neuroprotection in experimental studies. (119, 170, 201, 202). The BBB protects the CNS from the compositional fluctuations that occur in the blood in order to maintain homeostasis in the CNS. This is essential for proper function of the nervous system. The BBB is so effective that it establishes a nearly complete separation of the CNS to the rest of the body. The result is a tight control over the penetrability of macromolecules and immune cells (107). The latter is termed immune privilege and is thought to be

maintained by the tight endothelial junctions of the BBB and the presence of an immunosuppressive environment (6).

Possible explanations for the neuroprotective efficacy of S-PBN found in *Study II* and the successful reduction of 3,4-DHBA formation in this study despite its apparent failure to penetrate into the brain could be due to that the scavenger effect is restricted to the blood-endothelium interface or that S-PBN is metabolised into smaller compounds not detected by the current HPLC protocol.

Blood-endothelial interface

The suggestion that S-PBN acts at the blood-endothelial interface is supported by circumstantial evidence. As mentioned above, endothelial cells are proposed to be important generators of ROS (161), but the cerebrovascular endothelium is also exposed to high oxygen tension and free radical forming neutrophils and platelets (163, 281, 282) (Figure 16). Kuroda et al. (1999) suggested that the S-PBN analogue NXY-059 exerts its neuroprotective effects by trapping extracellular free radicals produced by endothelial and inflammatory cells.

The free radicals can cause local damage to the endothelium by lipid peroxidation, leading to BBB breakdown (308) and in a recent *in vitro* study it was reported that PBN protected the brain endothelium from oxidative stress (33). BBB breakdown is observed almost instantly after TBI and persists for up to 24 hours in the lesion site (74, 307). The initial breakdown is probably due to a transient increase of the arterial pressure in the moment of trauma, which forces apart tight junctions in larger arteries and arterioles, resulting in extravasation of blood components (110). There is also evidence of a secondary BBB breakdown 24-48 hours after trauma in the ipsilateral hippocampus (17). During BBB breakdown, it could be possible for S-PBN to enter the parenchyma, but in an *in vitro* model of the BBB showed very low uptake of S-PBN by brain endothelial cells at normoxic conditions (71) and we were unable to trace any amount of S-PBN one hour after treatment in the brain.
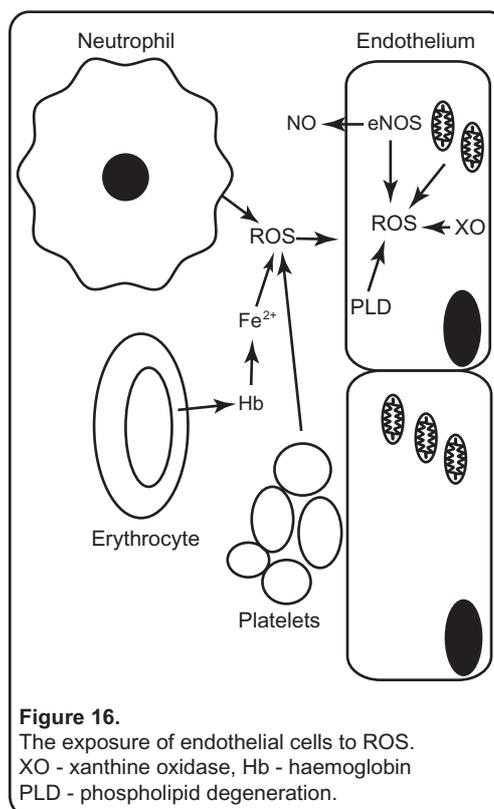

Figure 16.
The exposure of endothelial cells to ROS.
XO - xanthine oxidase, Hb - haemoglobin
PLD - phospholipid degeneration.

Damage to the endothelial cells could lead to decreased efficiency in transporting nutrients and oxygen to the glia cells and neurones they normally support. The ROS generated at the blood-endothelial interface could diffuse into the tissue to injure other cells.

Nitrone decomposition

In aqueous solutions, it is possible for PBN to decompose to benzaldehyde and N-tert-butyl hydroxylamine (NtBHA) (39) through hydrolysis (Figure 17), which is catalysed by the presence of Fe^{3+}-ions (53). NtBHA can then be further oxidised into tert-butyl hydronitroxide, which in turn can be decomposed into a NO-donor (52). It is possible for PBN to decompose in storage over time (39) and especially commercially attained batches seems to be prone to this phenomenon (77). This is rumoured to enhance the efficacy of PBN (13). The resulting substance NtBHA, has been shown to delay senescence of cultured lung fibroblasts (13) and serve as an antioxidant that reverses age-related changes in mitochondria in rats (14). If there is a corresponding decomposition of S-PBN, it might explain why a non-penetrating substance offers robust neuroprotection. Unfortunately, data on whether or not S-PBN or its double-sulfonated analogue NXY-059 shares this feature with PBN is not available, but worth studying. According to AstraZeneca representatives there is no evidence of any *in vivo* decomposition of S-PBN or NXY-059 (personal communication).

The *in vitro* evidence for PBN as a possible source of NO is interesting, since it has been reported that PBN and S-PBN treatment increases regional cerebral blood flow (rCBF) early after TBI (197). However, it has been reported that PBN-treatment inhibits NO breakdown, thereby increasing rCBF (144). The effect on rCBF observed with nitrone treatment could be partly due to reduced platelet aggregation, since hydroxyl radicals and hydrogen peroxide is involved in platelet aggregation (273). NO is a known vasodilator (69, 142) and could potentially facilitate increased blood flow, inhibit platelet aggregation and leukocyte adhesion (141). However, it could just as well react with superoxide to form peroxynitrite and facilitate iron loss, thus inducing lipid peroxidation through the Fenton reaction (117).

Several sets of data points toward that the nitrones are effective in protecting mito-
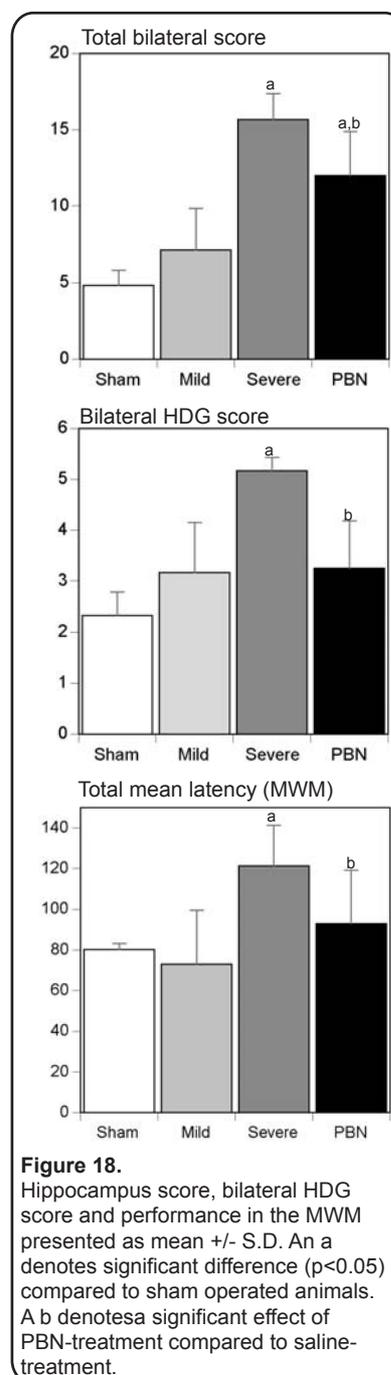

Figure 17.
Hydrolysis of PBN.
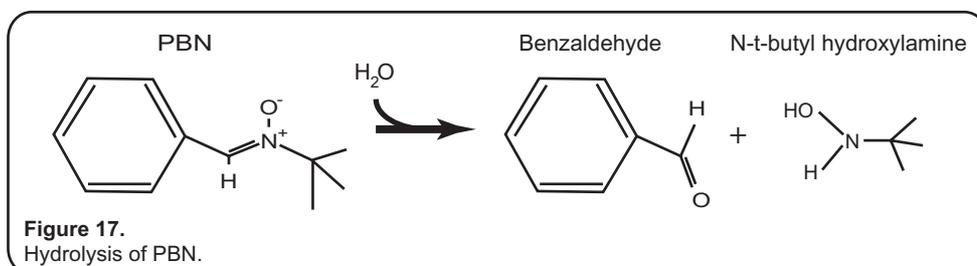
chondrial function (169, 344), which also seem to be true for NtBHA (14), but so far no reports on hydrolysis of nitrones *in vivo* have been presented, leaving this issue open for further research.

Bilateral hippocampal changes and effects of PBN pre-treatment, *Study V*

In an earlier study (196) we found that pre-treatment with PBN both reduced morphological damage and improved the performance in the MWM using two injury levels of brain injury (mild and severe CCC). In the severe groups we found improved spatial learning and attenuation of the cortical cavity volume after PBN treatment. However, in the groups with mild damage there were no treatment effects on the MWM performance.

Since the hippocampus is involved in the process of spatial learning, the need arose to further evaluate the graded response to trauma on hippocampal morphology and the treatment effects of PBN. Thus, the study focused on the relationship between the MWM memory dysfunction, cortical lesion volume, and regional hippocampal morphological changes following CCC, as well as the influence of pre-treatment with the nitrone radical scavenger PBN.

Rats were subjected to mild or severe CCC and tested on days 11-15 in a MWM task. The hippocampi of the same rats were evaluated using immunostainings for the dendrosomatic protein MAP2 by employing a semiquantitative scoring system as used by other investigators (96, 143, 145). The cortical lesion volume was previously determined morphometrically. The correlation between the MWM task and the morphological data was statistically evaluated. Loss of MAP2 staining is due to degradation of the protein by calpains, which are activated by free intracellular Ca^{2+} ions (214, 216). Previously, it has also

Figure 18.
Hippocampus score, bilateral HDG score and performance in the MWM presented as mean +/- S.D. An a denotes significant difference (p<0.05) compared to sham operated animals. A b denotesa significant effect of PBN-treatment compared to saline-treatment.

been shown that the neuronal soma hyperstains for MAP2 after CCC (179). The potentially pathophysiological effect of hyperstaining is presently unknown.

In the mildly traumatised group, we only found significant difference of the score in the ipsilateral HDG compared to the sham-operated animals. These data correspond well with the lack of spatial learning impairment in MWM found in the earlier study. The severely injured group showed changes in all subregions, except in the contralateral CA2, which correlated well to the earlier MWM-results. PBN treatment of severely injured animals led to significantly lower changes in three out of eight subregions. This group also showed significantly less cortical damage and navigated the MWM better than the saline treated injured animals. This suggests that PBN protects both the cortex and the structure of the hippocampus after severe TBI. Figure 18 shows some of the scores used for correlation analyses between the different hippocampal scores: total score, ipsi- and contralateral total scores, and bilateral HDG scores, the cortical lesion volume and total latency in the MWM showed that the total hippocampal score had the highest correlation coefficient ($r = 0.67$) and the cortical lesion volume the lowest ($r = 0.48$) when plotted against total latency. However, the difference failed to reach statistical significance ($p = 0.086$).

Bilateral hippocampal injury – Is it necessary for spatial learning deficits?

The hypothesis that bilateral damage to the hippocampus is necessary for impaired performance in spatial learning tasks has been discussed in the literature previously. Some studies of experimental TBI have shown deficits in spatial learning without having found cell loss in the contralateral hippocampus (108, 114, 158, 258), whereas other have reported bilateral injury (188, 288, 329). In behavioural neuroscience, it has been shown that a bilateral injury significantly worsens spatial learning (105) or is even necessary for deficits (322, 323). The result of the correlation analyses suggests that the total bilateral hippocampus score correlates more strongly to MWM deficits than the other parameters.

To conclude: Mild CCC-injury did not lead to significant changes of the hippocampus or deficits in the MWM, whereas severe injury level led to significant bilateral morphological changes and impaired spatial learning in severe CCC. Treatment with the free radical scavenger PBN attenuated morphological changes and reduced the total latency in the MWM significantly compared to saline treatment.

ROS dependent ERK activation and its relation to apoptosis, *Study VI*

Generally, the role of extracellular signal-regulated kinase has been described as pro-survival, especially since it is involved in cell proliferation and differentiation in the growing organism (303) and is activated by the stimulus of receptors for epithelial growth factor (EGF) and NGF (187). However, recent reports implicate ERK-activation as a culprit in apoptosis-like cell death in neurones after glutamate induced

excitotoxicity *in vitro* (284, 294, 296) and inhibition of ERK-activation protects cells from oxidative stress induced injury (267) and okadaic acid induced neuronal death (265). Reduction of ERK-activation using MEK inhibitors (U0126 or PD98059) *in vivo* have shown neuroprotective efficacy in ischeamia models (5, 224) and TBI (218, 219).

The first part of this study examined the effects of pretreatment with the MEK-inhibitor U0126 or the free radical scavenger S-PBN on ERK activation after CCC in rats. This part included Western blots for protein quantification of ERK and p-ERK, immunohistochemistry for p-ERK, and double immunofluorescense for cell type specific markers and p-ERK. Double stainings for the cell death marker TUNEL and p-ERK and immunostainings for TUNEL and cleaved caspase-3 were made to look for any connection between prolonged ERK-activation and apoptosis. Biopsies and sections were made from both the central and the perifocal parts of the lesion for the protein quantification. The rationale behind this was that earlier results point towards a difference in pathophysiological mechanisms (230). The central part is subject to mechanical impact, whereas the perifocal part is thought to be damaged by secondary injury mechanisms.

The second part the study focused on the effects on morphological outcome of posttreatment with U0126, administered in two different doses at 30 minutes after trauma or at high dose at 24 hours after injury. The injured controls were treated with the analogue U0124 30 minutes after trauma. The cortical lesion volume and cortical tissue volume were calculated using immunohistochemically stained sections.

ERK is activated in the cortex after TBI

This study showed early and prolonged activation of ERK after TBI. In the Western blots we found a twofold increase of p-ERK 10 minutes after injury, a finding that agrees with what others have published (67, 218, 219, 241). At 24 hours the activation persisted, but had decreased somewhat in the perifocal part of the injury and increased to threefold in the centre of the lesion. At neither time point was there any significant increase in unphosphorylated ERK noted. p-ERK positive cells were counted in the perifocal part of the injury just to get a rough estimate of how many cells were p-ERK positive (Figure 19).

The double immunofluorescense for Neu-N (neuronal marker), GFAP (astrocytic marker), Vimentin (astrocytic marker) or OX-42 (microglial marker), and p-ERK showed that all cell types that had activated ERK in the cytosol and nucleus at 10 minutes and 24 hours after injury (Figure 20). This has been reported in an earlier TBI study (242), whereas others have shown mainly neuronal staining after TBI (219). However, Vimentin and p-ERK double staining were only present at 24 hours post injury. OX-42 and p-ERK co-staining was more evident at 24 hours after trauma, but was present at 10 minutes.

ERK-activation can be found in several cell types after TBI

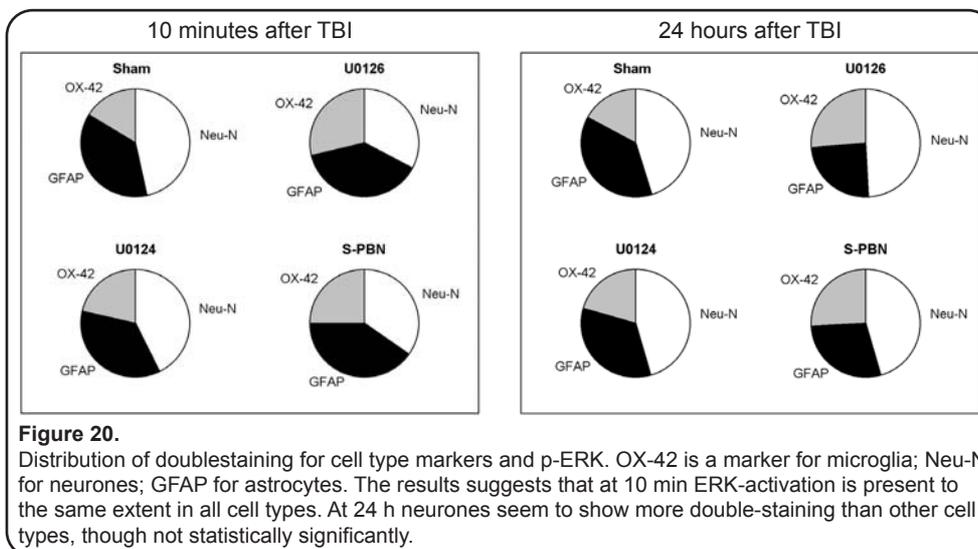The activation of ERK may be found in all three cell types studied, but it is not certain that the activation leads to the same cellular effects (Figure 20). There is evidence that constitutive ERK activation is present in reactive astrocytes involved in astrogliosis (192) and ERK is also needed to activate matrix metalloproteinase 9 (11, 218), which is a part of the cascade that degrades most extracellular matrix molecules, resulting in BBB breakdown, tissue injury, and inflammation (127). In neurones p-ERK and the pro-apoptotic molecule cytochrome C co-stain early after transient focal ischemia (181) and prolonged retention of p-ERK in the nucleus facilitates caspase dependent cell death (295). However, ERK-activation has been linked to cell survival after hypoxia in neuronal cell cultures (148) and coupled to ischemic precondi-

Figure 19.
Number of p-ERK positive cells in the perifocal part of the lesion. Error bars indicate S.D.; asterisk indicates statistically significant difference from the other groups. However, the cell count was restricted to 2 sections from 3 animals per group.

tioning in global ischemia in gerbils (118). Activated ERK has also been found in microglia in the border zone of the lesion after neonatal hypoxia-ischemia (319), suggesting involvement in the reactive gliosis process or in phagocytosis of injured cells.

TUNEL and p-ERK co-stains after TBI

TUNEL and p-ERK showed a strong co-localisation 24 hours after trauma in cells that had condensed and fragmented nuclei, both morphological criteria for apoptosis. This suggests that ERK activation is involved in the execution of cell death programs after TBI. In a previous study using 2.0 mm CCC, we reported that most cells that were TUNEL positive at 24 hours after injury were Neu-N positive (180). However, it must be pointed out that the 2.5 mm compression used in this study causes more mechanical disruption to the brain parenchyma, which probably results in some differences in the injury mechanism.

Figure 20.
Distribution of doublestaining for cell type markers and p-ERK. OX-42 is a marker for microglia; Neu-N for neurones; GFAP for astrocytes. The results suggests that at 10 min ERK-activation is present to the same extent in all cell types. At 24 h neurones seem to show more double-staining than other cell types, though not statistically significantly.
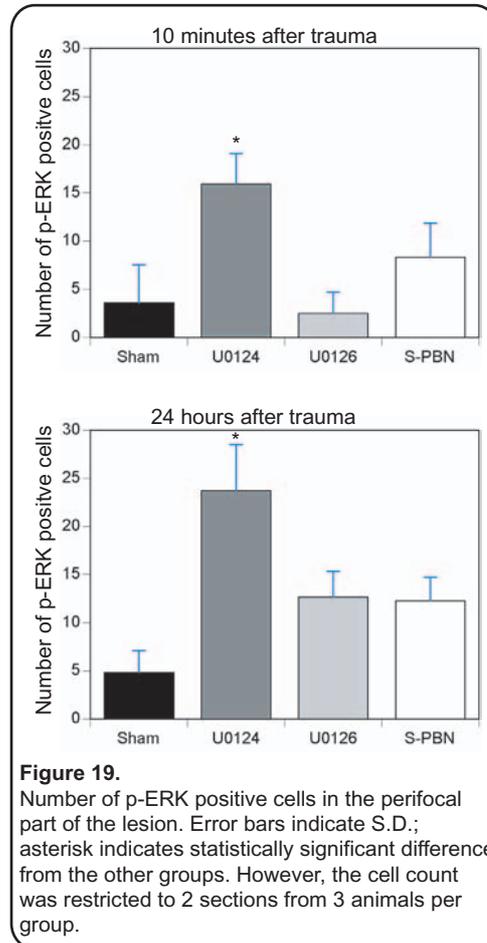
MEK-inhibition efficiently reduces ERK-activation after TBI

Treatment with the MEK-inhibitor U0126 decreased ERK activation in both the central and perifocal parts of the injury 10 minutes after trauma, but at 24 hours post trauma only the perifocal part showed decreased ERK activation. In immunohistochemical stainings for p-ERK made from animals sacrificed 30 minutes and 6 hours after trauma, we found a treatment effect at 30 minutes after trauma on the number of cells positive for p-ERK. At 6 hours post injury, we saw no effect of U0126 treatment compared to U0124, which conflicts with the results from the 24 hour post injury Western blots where there was an effect in the perifocal part of the lesion. This result could be because the cell counts were calculated as means from both the central and peripheral part of the lesion for methodological reasons.

ERK-activation is prolonged after TBI

ERK-phosphorylation is normally tightly controlled and the duration of the activation is a crucial component of the particular biological result (200). NMDA-receptor activation by glutamate and subsequent Ca^{2+} influx are known to activate ERK, possibly by activation of PKC (61, 321, 334), with simultaneous activation of a PTP that dephosphorylises ERK (243). Under normal conditions this process is involved synaptic plasticity, long term potentiation, and memory formation in neurones (303). However, after TBI the presence of glutamate excitotoxicity and prolonged increas of intracellular Ca^{2+} may disturb the balance of this system with sustained ERK activation as a result.

MEK-inhibition attenuates cell death after TBI

U0126 pretreatment significantly reduced the number of cleaved caspase-3 and TUNEL positive cells 24 hours after injury and early post-treatment with U0126 with either a 100 nmol or a 10 nmol dose significantly reduced the lesion volume at 14

days after injury as well as increased the cortical volume ratio. The late treatment, a full day after trauma, showed no efficacy on cortical lesion volume or cortical volume ratio. The decrease in apoptotic cells after pretreatment and improved morphological outcome after posttreatment strongly suggests that MEK-inhibition and subsequent reduction of ERK-activation is neuroprotective after TBI. Similar reduction of the cortical lesion has been shown after experimental ischemia (5, 224) and TBI (219), but this is the first study to show an effect on apoptosis-like cell death after MEK-inhibition in TBI, though it has been reported after experimental transient focal ischemia (318).

ERK-activation is involved in the induction of several cellular programs and there are many possible mechanisms of neuroprotection after MEK-inhibition (Figure 21). It could be due to a reduction of neuronal apoptosis by reduced nuclear retention of p-ERK (294), protection of the BBB by decreased activation of matrix metalloproteinase-9 in astrocytes (218), or attenuation of astrogliosis by inhibiting astroglia from becoming reactive astrocytes (191, 192). Some of these protective events could be due to the reported reduction of IL-1β mRNA expression after MEK-inhibition (320), although this conclusion may be somewhat farfetched. However, cytokines have been implicated to induce apoptosis through the extrinsic caspase dependent pathway (82), IL-1 is known to break down the BBB and facilitate the infiltration of lymphocytes and monocytes (262) and IL-1β is proposed to be necessary for astrogliosis (130).

The possible involvement of ERK in apoptosis

The potential role of ERK in neuronal apoptosis could be due to that activated ERK phosphorylates Elk-1, which in turn activates the transcription factor c-fos. c-fos attaches to c-jun, which is activated by the MAPK JNK to form the activating protein-1 (AP-1) complex (151). One of the targets for AP-1 is the gene encoding Fas ligand (FasL) (277), an activator of the extrinsic caspase dependent apoptotic pathway (240). Upregulation of AP-1 (68, 342) and FasL (23, 350) have been reported to occur after TBI. In T-cells activation induced cell death is regulated by ERK and executed by FasL (316). It would be valuable to examine the possible link between FasL and ERK after TBI more closely.

ROS-scavenging decreases ERK-activation after TBI

S-PBN pre-treatment significantly attenuated the ERK-activation 10 minutes after trauma in both the central and perifocal part of the injury. At 24 hours after trauma, there was no difference compared to U0124 treated injured animals, which was expected because S-PBN has a very short half-life (*Study IV*).

The effect of a free radical scavenger on ERK-activation is a novel find, but mice that overexpress Cu-Zn-SOD show attenuated ERK activation and less cell death after focal ischemia (234). However, in a study of ischemia-reperfusion injury in gerbils an

Figure 21.
Possible effects of sustained ERK-activation.
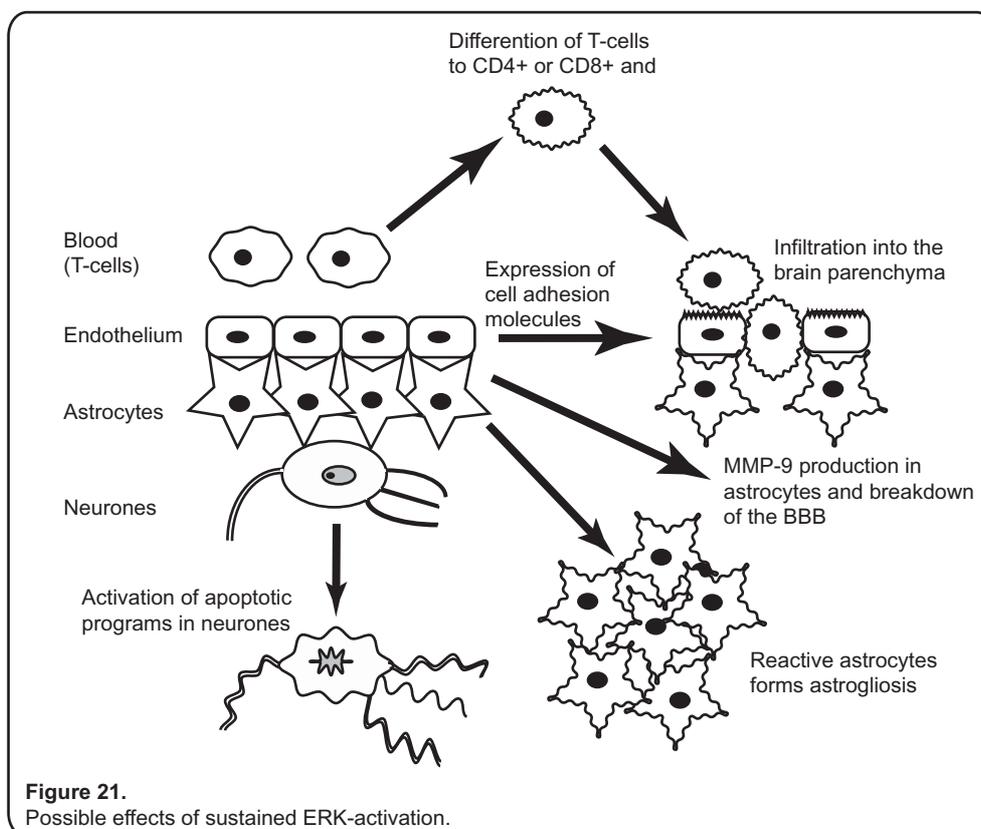
increase of p-ERK in the hippocampus was found six hours after PBN-treatment in control animals without the ischemic insult (313).

In vitro experiments have shown a connection between oxidative stress, ERK activation, and cell death after glutamate induced neurotoxicity (294-296) and antioxidant treatment decreases ERK activation after oxidative stress (150). However, it has been reported that PBN increased ERK activation after H_2O_2-treatment of PC12-cells (128), possibly by increasing intracellular calcium. In myocytes, ERK-activation after oxidative stress have been reported to increase cell survival (185).

H_2O_2 has been suggested to contribute to cellular signalling by inhibiting protein tyrosine phosphatases (PTPs) (89) and protein phosphatases such as 2A (PP2A) (95). These proteins are responsible for removing the phosphate groups from MAPKs so it stands to reason that in a situation of excess of ROS PTPs and PP2A would be inactivated. This may be part of the explanation to the decreased activation of ERK after S-PBN-treatment and it would be of interest to study the regulation of PTP:s after TBI. Experiments with PBN has shown protection of PTPs after H_2O_2-treatment of astrocyte cell cultures (259). Interestingly, H_2O_2 stimulates T-cells to proliferate the same way as activation of the T-cell receptor via activation of ERK and JNK

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(173). Another connection between free radicals, ERK-activation, and inflammatory response is the suggestion that peroxynitrite induces neutrophil adhesion to the endothelium via the ERK-pathway (353), a process that was inhibited to 80 % using the MEK-inhibitor PD98059.

The injury mechanisms of ROS have in general been thought of as more chemical than biochemical. The possibility that free radicals are involved in protein regulation leading to cell death could lead to the discovery of new potential targets for neuroprotective intervention. It also suggests that there may be more to free radical scavenging than just inhibiting ROS from injuring cells.

Conclusions

The results presented in this thesis have led to the following conclusions:

Characterisation of the intracranial pressure during trauma in three TBI-models in rat showed that FPI produces a global mechanical impact that can be accurately measured by extracranial pressure monitoring in the FPI device. The cortical compression models CCC and CCI elicit a pressure pulse of much smaller magnitude, explaining the lack of brain stem involvement in these focal models.

Post-treatment with the ROS-Scavengers PBN and S-PBN attenuated tissue loss and S-PBN improved cognitive function after FPI.

Microdialysis with 4-HBA trapping was found to be a useful tool for detection of ROS-formation *in vivo* after CCC.

Pre-treatment with PBN and S-PBN significantly attenuated ROS-formation after CCC, despite the observation that S-PBN did not penetrate into the brain tissue.

Severe CCC produced bilateral hippocampal damage which correlated to memory impairment in the MWM. PBN pre-treatment protected the hippocampus, especially the HDG subregion, known to be involved in memory function.

ERK-activation was increased in neurones, astrocytes and microglia early after severe CCC and the activation persisted for up to 24 hours. MEK-inhibition with U0126 and radical scavenging with S-PBN both decreased ERK-activation and protected the brain tissue. ERK-activation, normally involved in cell survival, may shift to a triggering mechanism for cell death in the injured brain a process that may be regulated by ROS after trauma. ERK-activation was found in TUNEL-positive cells and MEK-inhibition decreased apoptosis, suggesting that ERK is involved in delayed cell death.

To summarise, these studies have shown that ROS plays a pivotal role in the delayed cell death after TBI and that free radical scavengers can influence the outcome positively by protecting the brain tissue and attenuate cognitive impairment, suggesting a potential as a neuroprotective treatment. The 4-HBA trapping method is a useful tool to study ROS-formation *in vivo* and may have a clinical potential. MEK-inhibition and subsequently decreased ERK-activation resulted in protection of cortical tissue following TBI. The observation that ROS may regulate ERK-dependent delayed cell death mechanisms may help defining new targets for neuroprotective intervention after TBI.

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Summary in Swedish / Sammanfattning

Traumatisk hjärnskada (THS) genom våld mot skallen är en vanlig orsak till dödsfall och livslånga handikapp. Vanliga följder är motoriska störningar, försämrat minne, inlärningssvårigheter och personlighetsförändring. Andra patologiska följder av THS är epilepsi, ökad risk för demens och långsam hjärnvävnadsförlust. Unga människor drabbas oftast i samband med fordonsolyckor, emedan äldre mest drabbas genom fall. Säkrare bilar och hjälmtvång för motorcyklister har minskat antalet hjärnskador i den industrialiserade världen, samtidigt som vården blivit bättre. Genom neurointensivvårdens införande har antalet döda efter svåra hjärnskador minskat radikalt. Trots detta kvarstår THS som ett dominerande hälsoproblem, särskilt i befolkningen under 45 års ålder.

I skadeögonblicket utsätts hjärnan för mekaniska krafter som leder till vävnadsskada, blödning och brott på nervbanor. Efter den primära skadan kan hjärnan svälla och trycket inne i skallen stiga så mycket att vävnaden drabbas av syrebrist och skadas. Det är en av neurointensivvårdens viktigaste uppgifter är att övervaka och behandla högt intrakraniellt tryck. På cellulär och molekylär nivå ändras jonbalansen i skadeögonblicket vilket leder till att celler sväller och deras ämnesomsättning störs. Detta ger upphov till sekundära skademekanismer vilket utgör en stor del av skadan och har lett till att ett antal läkemedel tagits fram för att skydda hjärnans celler. Flera av dessa substanser har visats kunna skydda hjärnan efter THS i djurmodeller, men hittills har inget läkemedel varit effektivt nog att införas som klinisk behandling. Det

är därför av högsta intresse att studera de sekundära skademekanismerna för att utröna vilka som utför de bästa måltavlorna för läkemedelsbehandling. Ett exempel på sekundära skademekanismer är att mer fria radikaler än normalt bildas.

Fria radikaler är molekyler som kan reagera med andra ämnen genom att donera en elektron, kallat oxidation. Därigenom kan detta ämnes struktur förändras och dess funktion förstöras. Fria radikaler produceras normalt under cellens ämnesomsättning och vid aktivering av vissa enzymer. Eftersom överproduktion av fria radikaler kan vara skadligt så finns det flera naturliga skyddsmekanismer i kroppens organ. Efter THS stiger dock produktionen av fria radikaler till så höga nivåer att skyddsmekanismerna inte räcker till, vilket leder till en kedjereaktion som skadar många känsliga ämnen i viktiga delar av cellen, t.ex. cellmembran, proteiner och DNA.

Efter THS dör celler både okontrollerat, vilket kallas nekros, och på ett kontrollerat sätt genom s.k. apoptos. Det senare innebär att celler med skadade proteiner, cellmembran eller DNA krymper ihop och packar sina beståndsdelar i mindre enheter som lätt kan tas upp av andra celler. Fördelen med det är att inga farliga ämnen läcker ut i vävnaden, vilket kan leda till inflammation och skador på andra celler.

Celler styrs av komplicerade proteinsystem som aktiveras av att receptorer stimuleras eller att förhållandena inne i cellen förändras. Viktiga signalvägar mellan cellmembranet och generna i cellkärnan är proteinkinas-kaskaderna, De aktiveras av att tillväxtfaktorer binder till receptorer på cellytan vilket leder till att ett flertal proteiner i följd aktiveras, därav namnet kaskad. Ett intressant proteinkinas kallas ERK (extracellular signal-regulated kinase) och när det aktiveras påverkar det cellen så att nya proteiner tillverkas. Normalt leder ERK-aktivering till celldelning och cellutveckling, men om aktiveringen blir för långvarig kan det leda till att cellen går i apoptos och dör.

Det främsta målet för den här avhandlingen har varit att öka kunskapen om hur fria radikaler skadar vävnaden efter THS. För att kunna studera förloppet efter THS i människa så har man utvecklat djurmodeller av THS på gnagare som simulerar skadan. Genom djurförsöken skapar man en vävnadsskada som vid THS som kan undersökas för att bättre förstå vilka mekanismer som är negativa eller positiva för hjärncellernas och hjärnvävnadens överlevnad. Genom att använda de fria radikalhämmande substanserna PBN och S-PBN kan man minska mängden fria radikaler i hjärnan efter THS markant och därigenom utröna hur stor skada radikalerna bidrar till och genom vilka mekanismer de verkar.

I ett delarbete i avhandlingen utvärderades efterbehandling med PBN och S-PBN. Båda substanserna skyddade hjärnvävnaden och S-PBN förbättrade även råttornas inlärningsförmåga efter THS. Att S-PBN var bättre på att skydda minnesförmågan var ett intressant fynd eftersom S-PBN till skillnad från PBN har mycket svårt att lämna blodbanan och gå in i hjärnvävnaden.

I två delarbeten utvärderades en metod för att mäta produktionen av fria radikaler i hjärnvävnaden efter THS och även PBN:s och S-PBN:s förmåga att hämma radikalbildningen. Resultaten visade att det bildas höga nivåer av fria radikaler i hjärnvävnaden efter THS. PBN och S-PBN minskade likvärdigt mängden fria radikaler, trots att S-PBN inte kunde spåras i hjärnvävnaden. Dessa fynd tillsammans med fynden i det tidigare delarbetet, ledde till slutsatsen att S-PBN eventuellt kan minska mängden fria radikaler i hjärnans blodkärl och därigenom skydda hjärnans försörjning av näring och syre.

Ett annat delarbete fokuserades på de fria radikalernas inblandning i skada på den cellulära strukturen i ett område i hjärnan som är delaktigt i inlärningsprocessen, hippocampus. Eftersom inlärningsproblem är vanligt efter THS är hippocampus ett intressant område att studera noggrant. Den strukturella skadan i hippocampus korrelerades till djurens inlärningsförmåga och resultaten visade att en större skada ledde till att djuren presterade sämre i inlärningstestet. Fri radikalhämning med PBN visade sig skydda delar av hippocampus och det korrelerade med förbättrad inlärningsförmåga.

I det sista delarbetet studerades ERK-aktivering efter THS och om den kunde kopplas till apoptos. En substans kallad U0126 användes för att hämma aktiveringen av ERK efter THS och dess eventuella förmåga att skydda vävnaden utvärderades. Även de fria radikalernas effekt på ERK-aktiveringen efter THS undersöktes m.h.a. S-PBN-behandling.

Resultaten visade att ERK aktiveras efter THS och ERK-aktivering kunde kopplas till apoptos. Både ERK-aktivering och apoptos kunde minskas efter förbehandling med U0126 och S-PBN och U0126 som efterbehandling visade sig vara vävnadsskyddande.

De resultat som redovisas i avhandlingen leder till följande slutsatser:

Fria radikaler spelar en stor roll i skadeutvecklingen efter THS. PBN- och S-PBN-behandling minskade fri radikalbildningen i hjärnbarken efter THS, vilket ledde till minskad vävnadsdöd i både hjärnbarken och hippocampus samt förbättrad inlärningsförmåga.

S-PBN:s förmåga att fånga radikaler och skydda hjärnan trots att substansen inte lämnar blodbanan kan förklaras med att hjärnans blodkärl i sig är en viktig del att skydda efter hjärnskada.

Fria radikaler bidrar till förlängd ERK-aktivering, en process som kan leda till apoptotisk celldöd. Kunskapen att fria radikaler är inblandade i intracellulär signalering kan leda till identifikation av nya angreppspunkter för hjärnskyddande behandling efter THS.

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