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EXPERIMENTAL FOCAL CEREBRAL ISCHEMIA

PATHOPHYSIOLOGY, METABOLISM AND PHARMACOLOGY OF THE ISCHEMIC PENUMBRA

by

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Copenhagen, Denmark
2007
Denne afhandling er i forbindelse med de på side 2 anførte tidligere offentliggjorte afhandlinger af Det Sundhedsvidskabelige Fakultet ved Københavns Universitet antaget til offentligt at forsvares for den medicinske doktorgrad.

København, den 6. juni 2007

_Ulla Wever_
Dekan

Forsvaret finder sted fredag den 16. november 2007 kl. 14.00 i auditorium A, Teilumbygningen, Frederik V’s vej 11, 2100 København Ø.
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This overview is based on the following previously published studies which are referred to in the text by their roman numerals:


IV. Christensen T, Ebert B, Bruhn T, Diemer NH. Ketobemidone, an opioid analgesic with NMDA antagonist properties, does not improve metabolism or reduce infarct size after middle cerebral artery occlusion in rats. *Neuroscience Research Communications* (2000) 26:77-86.


LIST OF ABBREVIATIONS

2-DG: 2-deoxyglucose
AMPA: $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ADC: Apparent diffusion coefficient of water
CBF: Cerebral blood flow
CNS: Central nervous system
CSD: Cortical spreading depression
CPu: Caudate putamen
Ctx: Cortex
DC: Direct current
ETC: Electron transport chain
GFAP: Glial fibrillary acidic protein
HE: Hematoxylin-eosin
IEG: Immediate-early gene
KA: Kainic acid
$\text{lCMR}_{\text{glc}}$: Local cerebral metabolic rate of glucose
MCAO: Middle cerebral artery occlusion
tMCAO: Transient middle cerebral artery occlusion
pMCAO: Permanent middle cerebral artery occlusion
MK-801: Dizocilpine maleate, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate
MPT: Mitochondrial permeability transition
MRI: Magnetic resonance imaging
NBQX: 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline
NMDA: N-methyl-D-aspartate
NO: Nitric oxide
NOS: Nitric oxide synthase
PET: Positron emission tomography
PID: Periinfarct depolarization
$\alpha$-PBN: $\alpha$-phenyl-$N$-tert-butyl nitrone
rCBF: Regional cerebral blood flow
lCBF: Local cerebral blood flow
ROS: Reactive oxygen species
ROCC: Receptor-operated calcium channels
VOCC: Voltage-operated calcium channels
PREFACE AND ACKNOWLEDGMENTS

During the last almost three decades insight into the complex pathogenetic mechanisms involved in ischemic cell death in the central nervous system has been greatly augmented. Since the first publication of useful rodent model of ischemic stroke in 1981 almost 4000 studies have been published dealing with a wide range of important aspects of experimental focal cerebral ischemia. It is beyond the scope of the present thesis to attempt to comprehensively summarize all of this tremendous amount of data. Instead focus is concentrated primarily on pathogenetic, metabolic and pathophysiological events which are important in the acute phase of focal cerebral ischemia. The thesis is based on seven original papers published from 1993-2005 during my various employments as a member of the scientific staff at the Laboratory of Neuropathology, Institute of Molecular Pathology, University of Copenhagen. I am deeply indebted to my two closest collaborators professor, DMSc Nils H. Diemer and Torben Bruhn, DMSc for interesting and fruitful scientific discussions as well as true friendship and numerous invigorating and humorous moments.

I am very grateful to Berit Jensen, Marianne Nielsen and Lisbeth Thatt-Jensen for close collaboration and expert technical assistance. I also wish to thank associate professor Flemming Fryd Johansen, the coauthors of the papers and all my other colleagues during the past years who contributed to an inspiring scientific milieu at the laboratory.

The financial support from University of Copenhagen, the Danish Medical Research Council, The Michaelsen Foundation and several other generous danish foundations is greatly appreciated.

Above all I wish to thank my wonderful girls Nina, Nicoline and Cecilie for their support, extreme patience and never waning belief in the project.

Organization of the thesis

The present overview is organized into three parts. In the first important pathophysiological and biochemical characteristics of the ischemic penumbra are reviewed and discussed in order to provide a background for the author’s own experimental investigations. In part two the results of these investigations are summarized and commented upon. Part three is devoted to a more general discussion and review of the relevant literature concerning the pathogenetic mechanisms investigated in the papers I-VII.

Copenhagen, June 2006
BRIEF SUMMARY

Focal cerebral ischemia due to occlusion of a major cerebral artery is the cause of ischemic stroke which is a major reason of mortality, morbidity and disability in the populations of the developed countries. In the seven studies summarized in the thesis focal ischemia in rats induced by occlusion of the middle cerebral artery (MCAO) was used as an experimental model of ischemic stroke. MCAO produces an acute lesion consisting of an ischemic core or focus with severely reduced blood flow surrounded by a borderzone or ischemic penumbra with less pronounced blood flow reduction. Cells in the ischemic focus are irreversibly damaged after only 15-30 minutes of ischemia. In contrast, cells in the penumbra may – although threatened and functionally impaired – remain viable for several hours following arterial occlusion but will eventually also succumb if ischemia persists. In this way the initially viable tissue in the penumbra is recruited in the infarction process leading to a progressive growth of the infarct. The penumbra hence constitutes an important target for pharmacological treatment because of the existence of a therapeutic time window during which treatment with neuroprotective compounds may prevent recruitment of penumbra to infarct resulting in mitigation of the final ischemic brain damage.

The pathogenetic mechanisms involved in ischemic cell death in the penumbra encompass excitotoxic mechanisms mediated by activation of ionotropic glutamate receptors, loss of cellular calcium homeostasis and accelerated generation of damaging free oxygen radicals.

The overall aim of the studies was to elucidate the pathogenetic and pathophysiological mechanisms involved in recruitment of the penumbra in the acute phase of focal ischemia. In the three first studies, it was specifically addressed how the glutamate receptor antagonists MK-801 and NBQX influence expression of Fos protein, a product of the immediate-early gene c-fos, and changes of general protein synthesis and glucose consumption in the penumbra in the acute phase following MCAO. The effect of treatment with ketobemidone, an opioid receptor agonist and weak NMDA glutamate receptor antagonist, upon protein synthesis and glucose metabolism in the penumbra and infarct volume was investigated in a fourth study. In the fifth study, transient perinfarct depolarizations were recorded and the effect of treatment with the free radical scavenger α-PBN on the perinfarct depolarizations and infarct volume was investigated. In study number six, the activity of the mitochondrial electron transport complexes I, II and IV was evaluated histochemically during reperfusion after MCAO in order to assess the possible role of mitochondrial dysfunction in focal ischemic brain damage. Finally, the effect on infarct volume one week after MCAO of treatment with Pinokalant, a new broad-spectrum cation channel blocker, was examined in the seventh study.

The results of these studies are presented and discussed in relation to the current knowledge of the pathogenetic and pathophysiological mechanisms involved in the acute phase of the infarction process.
PART ONE: BACKGROUND

INTRODUCTION

Clinical background

Stroke is a major cause of mortality, morbidity and disability in the populations of the developed countries. In European countries the overall annual incidence has been estimated to be 8.72/1000 in the age group from 65 to 84 years. Prevalence in the same age group is about 5% [70]. Both incidence and prevalence increase markedly with advanced age. In Denmark the annual incidence of stroke is 10000-12000. According to the Danish Reference Programme for Stroke, 30000-40000 danes live with more or less severe sequelae following a stroke [335]. Stroke is the third leading cause of death surpassed only by ischemic heart disease and cancer in the industrialized world. Mortality is up to 20% within the first month after the onset of stroke. It is estimated that socioeconomic expenses directly related to stroke amount to 2.7 billion danish kroner each year, equivalent to 4% of the annual health care budget in Denmark. Stroke thus poses a significant clinical and socioeconomic problem.

The majority of strokes (approximately 80%) is caused by sudden focal ischemia, i.e. a complete or partial interruption of blood flow to part of the brain due to arterial occlusion. This eventually results in development of a brain infarct. The remainder of the stroke cases is caused by hemorrhage. Although significant progress has been made in the recent years in preventing ischemic stroke by reducing risk factors there is currently no cerebroprotective treatment clinically available aiming at preventing irreversible damage and salvaging brain cells once occlusion of a cerebral artery has occurred. Basic research employing animal models of ischemic stroke has tremendously increased our knowledge of the mechanisms involved in ischemic cell death and infarction in the brain at the cellular and molecular level. It is hoped that insight into the pathogenetic mechanisms obtained in these models eventually will provide the basis for development of a clinically available cerebroprotective treatment paradigm.

Ischemic thresholds and the concept of an ischemic penumbra

In a seminal study from 1977 Astrup and co-workers examined the relationship between regional cerebral blood flow (rCBF), extracellular potassium ion concentration and cortical somatosensory evoked potentials in brains of baboons subjected middle cerebral artery occlusion [17]. They observed that two CBF thresholds for loss of neuronal function exist, one for loss of electrical activity, i.e. synaptic transmission measured as an abolition of the evoked potentials, and one for loss of ion homeostasis evidenced as release of potassium from the intracellular to the extracellular space. The CBF threshold for cessation of electrical activity was clearly higher than the threshold loss of ion homeostasis. In focal ischemia, tissue with blood flow between these two thresholds, i.e. the upper threshold for electrical failure and the lower threshold for energy failure and ion pump failure, was shown to form a ring around a more densely ischemic center [17,38]. In analogy with the half-shaded zones on the Earth bordering each side of the path of totality during a solar eclipse ischemic tissue in this condition was
descriptively termed “the ischemic penumbra” [16]. Earlier neuropathological studies had shown that morphological damage, i.e. infarction, was confined to areas where blood flow in the acute stage was reduced to values below the threshold for deranged ion homeostasis [308-310]. The penumbra concept therefore had important clinical implications since it was hypothesized that cells in the penumbra, although electrically silent, had not suffered irreversible damage and were thus potentially viable and capable of regaining normal function by even small improvements of blood flow [16,308].

Subsequently critical flow thresholds for disturbance of other important features of brain function such as Ca^{2+} homeostasis [122,246], excitatory neurotransmitter release [246], breakdown of cellular energy production [211], changes in glucose consumption [268], tissue acidosis [162], and protein synthesis [211] have been established. It is evident from this work, which has been thoroughly reviewed previously [134,289], that a hierarchy of critical flow thresholds exists. Figure 1 shows a simplified attempt to summarize this.

**Current definitions of the ischemic penumbra**

In 1981 Tamura et al. [317] published an experimental model of focal ischemia in rats employing surgical middle cerebral artery occlusion (MCAO). Since then rodent models have become widely used to study focal cerebral ischemia experimentally. In these models occlusion of the middle cerebral artery produces an acute “stroke lesion” which can be considered to consist of two components: 1) A central focus of densely ischemic tissue surrounded by 2) perifocal or penumbral areas of less dense ischemia [33,235,318,321] (fig. 2).

Cells in the ischemic focus rapidly suffer irreversible damage because of the severely reduced blood flow which is inadequate to sustain the cells beyond approximately 15-30 minutes of ischemia [69,96,97,205,227]. In contrast, cells in perifocal areas may – although stressed and at risk – remain viable for several hours after occlusion because of collateral blood supply from the anterior and posterior cerebral artery. However, if arterial occlusion persists cells in perifocal areas
will usually also succumb and suffer irreversible damage with time. In this way perifocal tissue is gradually recruited in the infarction process. The ensuing final histological lesion is a cerebral infarct, i.e. a pannecrotic lesion characterized by death of all cells and cell types within the ischemically affected area. Cerebral infarcts evolve and mature in a characteristic pattern over days to weeks [69,97,176]. Morphological damage first occurs in the ischemic focus in the dorsolateral part of the caudate putamen and in the adjacent lower part of the somatosensory cortex [69,205]. From here damage spreads into perifocal areas in the caudate putamen and neocortex, and will, in the frontal plane, eventually encompass the entire caudate putamen, except a small medial rim, and most of the neocortex sparing the superomedially located cingulate cortex which is supplied by the anterior cerebral artery [59,205]. In the sagittal plane damage extends to the watershed zones between the middle cerebral artery and the anterior and posterior cerebral artery. Reperfusion of the occluded artery in due time or appropriate pharmacological treatment may prevent perifocal areas from being recruited in the infarction process. In rat MCAO models the therapeutic window for reperfusion is up to three hours [145,205] whereas the therapeutic time window for pharmacologic treatment may be considerably longer depending on the type of drug used (discussed in [115] and [75]).

Although the original definition of the penumbra as critically hypoperfused regions containing functionally depressed but potentially viable cells with preserved ion homeostasis is a very precise one it is also from an operational point of view a restricted one because it is difficult to physically delineate the penumbra in the MCAO models according to this definition. Consequently, the penumbra concept has been modified somewhat over the years, and other definitions of the penumbra have been proposed that are focused on blood flow, metabolism and the potential for recovery in perifocal areas. The proposed definitions are listed in table 1.

### Table 1: Proposed definitions of the ischemic penumbra

<table>
<thead>
<tr>
<th>No.</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>A region of reduced CBF with absent electrical activity, but preserved ion homeostasis and transmembrane potentials [16].</td>
</tr>
<tr>
<td>2</td>
<td>A region of reduced CBF where energy metabolism is preserved [134].</td>
</tr>
<tr>
<td>3</td>
<td>A region of reduced CBF where energy metabolism is intermittently compromised [108].</td>
</tr>
<tr>
<td>4</td>
<td>A region of reduced CBF with suppressed protein synthesis but preserved ATP metabolism [134].</td>
</tr>
<tr>
<td>5</td>
<td>A region of reduced CBF with suppressed protein synthesis but continuing glucose consumption [paper III].</td>
</tr>
<tr>
<td>6</td>
<td>An ischemic region with potential reversibility of function [118].</td>
</tr>
<tr>
<td>7</td>
<td>An ischemic region that is destined for infarction, but one that is potentially salvageable with timely pharmacological intervention [289].</td>
</tr>
</tbody>
</table>

The common denominators inherent in these definitions are the reduction of cerebral blood flow and the potential for tissue recovery within a certain time limit. In the experimental models of focal ischemia precise topographic identification of the penumbra at any given time after MCAO is difficult due to the penumbra’s dynamic nature with respect to spatial and temporal changes. In many studies the distinction between normal tissue, the ischemic focus and the penumbra is not made objectively on grounds of visualization or measurements but often has to rely only on the
investigator’s own experience and knowledge of the model. For these reasons the terms “penumbra”, “perifocal areas” and “ischemic borderzone” are often all used as synonyms in the literature.

CHARACTERISTICS OF THE ISCHEMIC PENUMBRA

Metabolism in the penumbra

Glucose consumption

Glucose is considered the only substrate for in vivo production of ATP in brain [2]. Much attention has therefore been directed to how glucose consumption is changed in experimental focal ischemia [105]. Regional rates of glucose consumption can be visualized and computed using the autoradiographic technique developed by Louis Sokoloff in which $^{14}$C-2-deoxyglucose is used as a radioactive tracer of glucose utilization [301]. Application of this method to experimental models of focal ischemia has demonstrated that glucose utilization is affected locally in a heterogeneous pattern following arterial occlusion.

Ginsberg et al. [109] were the first to study acute alterations of glucose metabolism incurred by 90 minutes of unilateral MCAO plus common carotid occlusion in cats. They observed a central zone of greatly diminished glucose utilization in the caudate nucleus, i.e. in the ischemic focus of the acute lesion, which was surrounded by a variable rim of enhanced $^{14}$C-2-deoxyglucose uptake. This topographic pattern of changed glucose consumption in cats was confirmed by Welsh et al. [330] who correlated changes in glucose utilization to tissue levels of high-energy phosphates and lactate 1 hour after permanent MCAO and found that in the ischemic focus, which was depleted of ATP and phosphocreatine, uptake of $^{14}$C-2-deoxyglucose was markedly depressed whereas in adjacent regions, with less severe reductions in high-energy phosphates, $^{14}$C-2-deoxyglucose uptake was increased. Hossmann et al. [136] used multiparametric imaging techniques to study local cerebral blood flow and metabolism in cats two hours after MCAO and observed an inhomogenous pattern of changes local cerebral blood flow (ICBF) and local cerebral metabolic rate of glucose (ICMR$_{glc}$) with regions of reduced, normal and increased rates of both. In some regions there was an uncoupling between blood flow and metabolism which was most prominent in the periphery of densely ischemic foci resulting in ring-like enhancements of $^{14}$C-deoxyglucose uptake.

In rat models of focal ischemia similar heterogeneous changes in the pattern of glucose consumption have been observed [232,234,235,237,254,268,286,287] which are consistent with and exemplified by the observations that Nedergaard et al. made in a triple-tracer autoradiographic study of glucose utilization, glucose content and ICBF in rats subjected to permanent proximal MCAO [235]. $^{14}$C-deoxyglucose uptake was suppressed in the lateral two-thirds of the caudate putamen and the adjacent lower convexity of the cortex corresponding to the ischemic focus of the lesion 15 minutes after initiation of MCAO. The focus of suppressed glucose consumption was surrounded by a wide border of enhanced $^{14}$C-deoxyglucose uptake in the ischemic penumbra. CBF was severely reduced in areas without $^{14}$C-deoxyglucose uptake (<8 ml/100g/min) and moderately reduced (CBF 20-30 ml/100g/min ~ 25-30% of normal) in the border with apparent hypermetabolism of glucose (Fig. 3). In other groups of rats the

Fig. 3: $^{14}$C-deoxyglucose autoradiograph showing the regional glucose consumption in the third hour after permanent MCAO. The ischemic hemisphere is on the right side of the image. Increased optical density reflecting increased glucose metabolism is noted in the penumbra (from Paper III).
The penumbra in experimental focal cerebral ischemia  
Part one: Background

borderzone with increased glucose consumption had disappeared in half of the animals after 6 hours and in all of them 20 hours after MCAO. Thus, in both cats and rats a consistent pattern of completely depressed glucose consumption in severely ischemic foci surrounded by perifocal areas with increased glucose consumption has been observed during the initial hours of arterial occlusion. These penumbral areas of enhanced glucose consumption are of particular interest because continuing glucose metabolism in ischemically stressed tissue indicates that cells within this hypermetabolic zone are still viable. Several hypotheses to account for the perifocally elevated glucose consumption have been presented. Since the deoxyglucose method does not discriminate between aerobic and anaerobic glucose consumption, it has been a question whether the increased glucose consumption in marginally perfused tissue could be accounted for by a switch from aerobic to anaerobic glycolysis (Pasteur effect) resulting in lactate production as it was tentatively suggested by Ginsberg et al. [109]. A flow threshold at which the increased glucose utilization begin to appear was established by Sako et al. [268] in a correlational study of rCBF, glucose utilization and tissue pH one to three hours after permanent MCAO in rats. In moderately ischemic areas with CBF below 38% of control, glucose utilization was increased and below 10-20% of normal flow glucose utilization had almost ceased. This CBF threshold of 38% of normal at which glucose utilization increased was coincident with the flow values at which a decline in tissue pH was observed suggesting anaerobic glycolysis and lactate accumulation. Topographically this suggestion has been supported by observations of Peek et al. [254] who, in a double-tracer autoradiographic study of glucose consumption and local pH, reported that penumbral areas with increased glucose consumption showed moderate acidosis, as compared to regions within the ischemic focus which exhibited decreased glucose consumption and were severely acidotic, possibly indicating an admixture of aerobic and anaerobic glycolysis in the penumbra. However, since tissue acidosis is not solely determined by lactate accumulation, but is also governed by other mechanisms such as proton production resulting from ATP hydrolysis and proton-clearance mechanisms [251], the lactate content in the penumbra has also been determined in a number of studies which have reported a substantial lactate accumulation in the penumbra occurring concurrently with enhanced glucose utilization [74,90,91,279-282,331].

Nedergaard et al. also correlated glucose utilization to the occurrence of transient deflections in the direct current (DC) potential (i.e. periinfarct depolarizations, see later) in the penumbra in normo- and hyperglycemic rats subjected to MCAO [232]. In normoglycemic rats, transient recurrent depolarizations were detected in the same penumbral areas that displayed a 200% increase in 2-deoxyglucose phosphorylation. In rats rendered hyperglycemic, both the transient depolarizations and the elevation of glucose consumption were abolished indicating that periinfarct depolarizations stimulate glucose consumption. It was speculated that the increased glucose consumption occurred in the glial compartment reflecting increased Na⁺/K⁺-ATPase activity for restitution of the transmembrane ion gradients disrupted during the transient depolarizations. Taken to together, the above observations indicate that the enhanced glucose consumption in the penumbra during the first hours of permanent MCAO is likely attributable to a) a switch to anaerobic glycolysis requiring conversion of more glucose molecules to meet the cellular energy needs than under aerobic conditions and/or b) stimulation of (anaerobic) glycolysis, probably primarily in astrocytes, in order to buffer potassium and neurotransmitters released during perinfarct depolarizations or from the ischemic focus. The reduced glucose utilization seen in the densely ischemic focus may in contrast be explained by a blood flow restricted
substrate supply or irreversible cell damage developing early after onset of ischemia.

**Protein synthesis**

Protein synthesis in the brain is very sensitive to ischemia and suppression of protein synthesis occurs after even minor reductions in CBF [336]. In autoradiographic studies of radioactive amino acid incorporation into proteins in rats subjected permanent MCAO, Mies and colleagues have established that the CBF threshold for inhibition of cerebral protein synthesis, defined as flow rates at which protein synthesis is half-maximally suppressed, is about 50% of normal flow [163,211,212]. This flow threshold is distinctly higher than those at which perturbation of glucose metabolism and ATP depletion occur [211,235,268] (Fig. 1). Accordingly, overall protein synthesis is reduced in large parts of the ischemic hemisphere following MCAO in rats. Topographically, protein synthesis is totally inhibited in the lateral part of striatum and part of the adjacent frontoparietal cortex corresponding to the ischemic focus after proximal MCAO in rats. Surrounding the focus a metabolic penumbra is identified which exhibits a gradient of reduction extending from total inhibition in the ischemic focus towards normal protein synthesis bordering tissue outside the MCA territory (Fig. 4) [211]. In the metabolic penumbra absolute protein synthesis rates are on average reduced to approximately 50% of non-ischemic control regions [paper II].

If focal ischemia is permanent the amount of brain tissue affected by suppression of protein synthesis does not change during the first 12-24 hours after arterial occlusion [126,211,211]. One to three days after permanent MCAO in mice protein synthesis seemed to recover somewhat in the most peripheral parts of the middle cerebral artery territory [126] but this may very well reflect synthetic activity in proliferating astrocytes [97], activated microglia and macrophages [176] and polymorph nuclear leukocytes engaged in an inflammatory response at the margin of the infarct [66,350].

Protein synthesis is an energy-requiring process and total inhibition of protein synthesis in the ischemic focus is likely caused by the rapid energy depletion in this part of the lesion. It has been suggested that periinfarct depolarizations and the associated rise in intracellular calcium levels are responsible for the reduction of protein synthesis in the penumbra during focal ischemia. This assumption is based on observations that glutamate receptor antagonists, in particular the NMDA receptor antagonist MK-801, not only block periinfarct depolarizations [102,131,140] but also improve protein synthesis in perifocal areas [212,213, paper II]. In addition, induction of cortical spreading depressions in normallyperfused rat brains by topical application of KCl leads to suppression of protein synthesis [28,208]. At the molecular level the mechanisms involved in regulation of protein synthesis in focal ischemia are not clear. Despite the reduction of overall protein synthesis rates some proteins, such as for instance the protein products of immediate-early genes [paper I], are selectively expressed in perifocal areas during focal ischemia and reperfusion (for a review see [284]) showing that both transcription and translation can take place in cells of the penumbra. Structural damage to the protein synthesis apparatus is therefore not likely and can not account for the overall suppression of protein synthesis. Instead mechanisms regulating initiation and
The penumbra in experimental focal cerebral ischemia

Part one: Background

Elongation of polypeptide chain synthesis may be involved. Changes in the phosphorylation state of several eucaryotic initiation factors and elongation factors have been observed during reperfusion after MCAO in mice and may contribute to suppression of protein synthesis [6,6]. Data obtained in neuronal cultures in vitro suggests the existence of a direct link between NMDA receptor, increased cytosolic calcium levels, phosphorylation of eucaryotic elongation factor eEF-2 and depression of protein synthesis [197]. Such a link could possibly explain suppression of protein synthesis in the penumbra observed in vivo and that the NMDA antagonist MK-801 improves protein synthesis in the penumbra [212, paper II].

Energy metabolites

The immediate consequence of any form of ischemia, if severe enough, is perturbed energy metabolism because of failure of synthesis and consumption of ATP and other energy metabolites. The best and most detailed description of changes in levels of energy metabolites in rats subjected to MCAO and reperfusion has been provided by Folbergrová et al. [90,91]. During MCAO major losses of ATP (to ~17-26% of non-ischemic control values) and phosphocreatine (~9-25% of control) were observed in tissue sampled from the neocortical focus at intervals ranging from 5 to 120 minutes after arterial occlusion. Glucose content was greatly reduced (~10-28% of control) and glycogen was almost depleted (~ 3-12 % of control) in the ischemic focus. These changes occurred rapidly and were manifest and stable at these low levels already after 5 minutes of occlusion. In perifocal regions a similar pattern of changes were noted but these were not as pronounced as in the ischemic focus. During occlusion penumbral ATP levels were reduced to approximately 50% of non-ischemic control values, phosphocreatine was moderately affected (~43-74%) as was glucose levels (~21-65%) whereas glycogen was substantially reduced (~8-41%). Reperfusion for 1-2 hours following 2 hours of MCAO led to a substantial but incomplete recovery of energy state both in the focus and the penumbra. ATP in the focus and penumbra thus recovered to approximately 40% and 70% of non-ischemic control values. Of note is that when the reperfusion period was extended to 4 hours signs of a secondary deterioration of all energy metabolites were seen. Lactate accumulation was substantial both in focal and perifocal regions during MCAO and persisted, albeit at a lower level, during reperfusion. Results of other studies that have examined tissue energy state in focal ischemia are in overall agreement with the findings of Folbergrová et al. [279-282,331]. Thus, severe perturbation of tissue energy state occurs in the ischemic focus and to a lesser extent in the penumbra during MCAO, and energy metabolism is only incompletely recovered during reperfusion after 2 hours of transient MCAO [91,279].

Periinfarct depolarizations - characteristics

Electrophysiologically the penumbra is characterized by transient, recurrent depolarizations which to some extent resemble the phenomenon of cortical spreading depression (CSD) originally described by Leão as a wave of depressed electrical activity spreading in cortex in response to a variety noxious stimuli [174]. The depolarizations propagate in the penumbra and each of them is associated with a transient disturbance of the transmembrane ion gradients for Na⁺, K⁺ and Ca²⁺. Several synonyms for this electrical disturbance have been used. Spreading depression [231], repeated negative DC deflections [140], penumbral depolarizations [228], periinfarct DC shifts [210], periinfarct depolarizations [135] in the setting of experimental focal cerebral ischemia all cover this phenomenon. Because the wave of depolarization sometimes propagates outside the blood flow defined penumbra, and in rats subjected to middle cerebral artery occlusion may be detected in the cingulate cortex supplied by the anterior cerebral artery.
The penumbra in experimental focal cerebral ischemia

Part one: Background

The most descriptive term is periinfarct depolarizations and will therefore be adopted in the following.

The periinfarct depolarizations may be detected as negative deflections in the direct current (DC) potential [232] or with ion sensitive microelectrodes measuring extracellular levels of K⁺ [236] or Ca²⁺ [101,102]. More recently transient reductions in the apparent diffusion coefficient (ADC) of water measured by magnetic resonance imaging has been reported to indirectly reflect depolarizations in the penumbra [265]. Changes in NADH fluorescence emitted through a cranial window in the ischemic territory was also found useful for this purpose [304].

Different types of periinfarct depolarizations have been described which differ in duration, amplitude and probably also in the mechanism by which they are triggered [102,228,236]: 1) Short depolarizations with a mean duration of less than 5-10 minutes; 2) Long depolarizations lasting for more than 5-10 minutes and 3) depolarizations of reduced amplitude or even suppression of EEG activity without any significant deflection of the DC-potential (Fig. 5). In a study by Nedergaard and Hansen [236] the PIDs were characterized with respect to changes in the extracellular potassium ion concentration [K⁺]ₑ during the depolarizations. During a short PID, [K⁺]ₑ rose in a steep, monophasic fashion sharing the characteristics of potassium ion movements during a “classical” cortical spreading depression of Leão whereas biphasic increases of [K⁺]ₑ were observed during long PIDs resembling the changes during anoxic depolarization [120]. Furthermore, in dual-electrode recordings depolarizations sometimes occurred simultaneously at both electrodes in the penumbra [236]. Taken together these results suggested that PIDs may be evoked in two ways. Short PIDs are likely initiated by the sustained elevation of the extracellular levels of potassium [236] and/or glutamate [44,130] in the ischemic focus from which waves of depolarization spreads out into perifocal areas. In contrast, long PIDs, representing transient anoxic depolarizations, may be triggered in discrete foci of low blood flow within the ischemic penumbra with no preferential direction of spreading [228,236]. The mechanisms involved in the propagation of PIDs in moderately ischemic penumbral tissue are less well understood but involves depolarization of a critical mass of adjacent nervous tissue induced by glutamate and/or K⁺ [198] and probably activation of glutamate receptors of the NMDA subtype since both PIDs and CSD in the non-ischemic brain are suppressed by NMDA-receptor antagonists [102,173,238]. Recently, gap junctions between astrocytes were suggested also to be implicated in the propagation of periinfarct depolarizations [263].

The frequency of the spontaneous periinfarct depolarizations depends on the species being higher in lissencephalics (e.g. rats) than in gyrencephalics (e.g. cats and monkeys) [305], the model of focal ischemia, e.g. photothermbotic vs. surgical MCAO [72], the type of anesthesia used [253,267] as well as physiological parameters such as body temperature and plasma glucose concentration [305]. Hypothermia [36,342] and

![Fig. 5: Examples of different types of periinfarct depolarizations. In Paper V three types of periinfarct depolarizations (PIDs) could be distinguished on basis of their duration and amplitude. I: Short PID (duration < 10 min, amplitude >10 mV). II: Long PID (>10 min and >10 mV). III: Abortive PID (< 10 min and < 10 mV). IV: KCl-induced depolarizations recorded in the non-ischemic rat cortex shown for comparison.](image-url)
hyperglycemia [232] have been shown to reduce PID frequency. The first depolarization typically occurs within a few minutes after arterial occlusion and is followed by a varying number of depolarizations whose frequency decrease with time as long as vascular occlusion is maintained [18,166,228,229, own observations from paper V]. Although the majority of PIDs occur early following induction of focal ischemia transient DC-deflections have been detected more than 14 hours after permanent MCAO in halothane anesthetized cats as reported by Saito et al. [267]. In rat studies such long observation periods have not been employed but it was reported that PIDs may still be detected 5-7 hours after MCAO [19,232].

The duration of each periinfarct depolarization is determined by the residual blood flow in the penumbra. Mies et al. [209] found that at flow values below 40% of control the depolarization time of the periinfarct DC shifts increased significantly compared to the duration of these at higher flow rates. Nallet et al. [229] specifically studied the hemodynamic correlates of penumbral depolarizations after MCAO in rats and also found the duration of the intraischemic depolarizations to be significantly dependent on residual blood flow. In line with these findings, periinfarct depolarizations recorded close to the ischemic focus were observed to be of longer duration than those recorded at more distant sites in the ischemic territory where blood flow is better preserved [166].

As discussed later in this overview periinfarct depolarizations and the associated disturbance of transmembrane ion gradients are believed to be contribute to expansion of the infarct by a gradual recruitment of the penumbra in the infarction process.

**Gene expression – molecular markers of the penumbra**

Focal cerebral ischemia incites extensive complex spatial and temporal changes in gene expression in the affected hemisphere. Recent DNA microarray studies have identified several hundred genes that are either up- or downregulated at the mRNA level during and after MCAO in rats [154,156,191,192,276]. The differentially expressed genes belong to different functional groups. In the early phase, i.e. within hours after MCAO, immediate-early genes, heat-shock proteins and other stress-proteins such as heme-oxygenase and hypoxia-inducible factor are induced [192,276]. This is followed by mRNA expression of genes related to apoptosis [191] and inflammation such as cellular adhesion molecules, cytokines and chemokines [177,284]. Even later, i.e. within days following the ischemic insult, expression of various growth factors begin to occur [192]. In addition, several other genes involved in e.g. metabolism and intracellular signal transduction as well as genes coding for ion channels and neurotransmitter receptors are regulated [192]. The significance of these extensive mRNA regulations with respect to infarct pathogenesis is largely unclear at present. Some genes may, if translated into proteins, promote damage. Others may be induced as part of activation of endogenous protective pathways [76,186].

In perifocal areas probably only a fraction of the upregulated genes are expressed as proteins because of the overall reduction of protein synthesis in the penumbra. Despite this some mRNAs are selectively expressed as proteins. As an example of this, early studies showed that genes such as c-fos and c-jun, belonging to the family of immediate-early genes (IEGs), are selectively and rapidly expressed both at the mRNA level and as proteins in perifocal areas after MCAO in rats [7,137,323,332, paper I]. The physiological function of Fos and Jun proteins is to act as transcription factors in a cellular stimulus-transcription coupling cascade which converts extracellular signals into alterations of cellular function by regulating late response or target genes [218]. IEGs are expressed diffusely throughout the ischemic hemisphere except in the ischemic focus [323]. IEG expression extends outside the vascular territory.
supplied by the middle cerebral artery and the IEG response is therefore thought to be caused by calcium influx associated with the occurrence of periinfarct depolarizations spreading throughout the hemisphere [284,329].

The dynamic nature of the penumbra and hence the difficulties to physically delineate its location and extension at a given time following arterial occlusion has prompted the search for potential molecular markers of the penumbra (see [284] for a review). In our early study of Fos protein expression in rats subjected to permanent MCAO we suggested that Fos might be used as an early and persisting molecular marker of the penumbra [paper I]. This suggestion was based on the observation that treatment with the NMDA glutamate receptor antagonist MK-801, known to block periinfarct depolarizations and known to salvage penumbral tissue, completely blocked Fos expression in perifocal areas. However, Fos expression was detected also in the cingulate cortex which is supplied by the anterior cerebral artery and does not infarct in our MCAO model. Therefore, as a molecular marker, Fos expression seem to “overshoot” the spatial extension of the penumbra. Subsequently other marker candidates have been suggested [284]. Of these particularly two deserve mention because they seem related to the some of the fundamental pathophysiologic events in the penumbra.

Heat-shock protein 70 (HSP70) belongs to a family of stress proteins which are induced by protein denaturation resulting from various types of cellular stress including ischemia [284]. As a molecular chaperone HSP70 assists in renaturing of denatured proteins [202]. Hence, induction of HSP70 in focal ischemic regions indicates some degree of injury. Schmidt-Kastner et al. studied HSP70 mRNA expression by in situ hybridization and pixel-based image analysis at various time points after 2 hours of transient MCAO in rats and found a strong statistical correlation between HSP mRNA expression, blood flow reduction at the end of the ischemic period and histological infarction three days following the ischemic insult [277]. In rat studies employing transient or permanent MCAO followed by 1-2 days of reperfusion HSP70 protein and HSP72, another member of the heat-shock protein family, are found in neurons in the penumbral zone but not inside the ischemic focus and hence – like the Fos protein – discriminates between focal areas and penumbral areas [157,158,176].

Hypoxia-inducible factor (HIF-1) is a transcription factor that is induced by reduction of tissue oxygenation. As a transcription factor HIF-1 regulates expression of several target genes which among others code for a glucose transporter, several glycolytic enzymes and inducible nitric oxide synthase making HIF-1 interesting with respect to a possible pathogenetic role in focal cerebral ischemia [94]. In the endovascular suture model of permanent focal ischemia HIF-1 mRNA was found to be upregulated within 4-7 hours in essentially the same penumbra regions as HSP70 but also in more peripheral areas such as the cingulate cortex which in this particular model also suffers some degree of ischemia and hypoxia [29]. In another study selective upregulation of HIF-1 protein was reported in areas surrounding the ischemic core between 3 and 24 hours following induction of permanent MCAO [200].

Whereas expression of Fos protein, heat-shock proteins and HIF-1 clearly reflects some of the complex pathophysiological and biochemical events in perifocal areas they probably do not precisely identify tissue destined to undergo infarction since these expression of these molecules were also detected in non-infarcted tissue at the periphery of tissue displaying overt histological infarction [157,158]. With respect to this, a molecular marker molecule that can confidently distinguish reversibly from irreversibly damaged brain tissue still remains to be identified.
PART TWO: OWN EXPERIMENTAL INVESTIGATIONS

AIM OF THE STUDIES

The overall aim of the work was to study aspects of the pathophysiological and metabolic changes in the ischemic penumbra in the acute phase following middle cerebral artery occlusion in rats in order to elucidate mechanisms of damage and protection. This was pursued, inter alia, by investigation of the effect of pharmacological treatment with different neuroprotective compounds on changes of metabolism, electrical function and morphological damage in the penumbra. The objectives of each of the studies included in the present thesis are stated in the results and comments section.

METHODOLOGICAL CONSIDERATIONS

Several methods were used in the studies. Of these only those employing novel approaches or modifications of previously published methods are mentioned in the following. Microelectrode detection of periinfarct depolarisations and measurement of local cerebral blood flow with laser-Doppler technique were performed using “conventional” techniques as described in a number of previous studies and will therefore not be considered further [100,173,228,232,311]. This is also the case for the immunohistochemical and enzyme histochemical methods used.

Rat models of focal cerebral ischemia

Several rat models of focal cerebral ischemia have been developed (reviewed in [106]). The vast majority of these models involves occlusion of the middle cerebral artery but differ with respect to how occlusion is performed, the precise anatomical site of arterial occlusion and whether occlusion is permanent or temporary, i.e. followed by reperfusion. The focal ischemia model used in our experiments is based on the model originally published by Tamura et al. in which the middle cerebral artery is surgically occluded in anesthetized adult rats through a subtemporal craniotomy [317,318]. Slight variations of the Tamura model were employed in order to best answer the specific questions posed in the individual experiments and to comply with the often technically demanding character of the experiments. Hence, both permanent MCAO [papers I-IV, VI] and transient MCAO [paper V] as well as occlusion proximal [papers I-IV, VI] and distal [papers VI-VII] to the origin of the lenticulostriate arteries were performed.

In proximal occlusion, the middle cerebral artery is occluded proximal to the lenticulostriate arteries close to its branching from the circle of Willis. Proximal MCAO renders both the ipsilateral basal ganglia and the cortex ischemic [33,237,318,321] and, if occlusion is of sufficient duration, the ensuing infarct will be located in the lateral part of caudate putamen and a considerable part of the adjacent neocortex (see Fig. 2). In distal MCAO, the artery is occluded distal to the origin of lenticulostriate arteries and hence blood flow to the basal ganglia is not blocked. Ischemia is therefore purely cortical and only cortical damage will be produced. Importantly, however, in both proximal and distal MCAO an acute lesion consisting of two components will be generated: 1) A severely ischemic focus surrounded by 2) an oligemic penumbra with less pronounced CBF reductions [49] which, at least partly, may be salvaged by
pharmacologic intervention or reperfusion in due time. Distal MCAO was chosen in some experiments because it is technically somewhat easier and quicker to perform than proximal MCAO, and because pharmacologic salvage of the penumbra in most instances can only be obtained in cortex (discussed in [186]). Hence, distal MCAO is a suitable model to test for infarct reducing properties of a potentially neuroprotective compound.

**Autoradiography**

In papers II and III quantitative autoradiography was used to investigate changes of protein synthesis and glucose metabolism in the acute phase after MCAO in rats. The theoretical basis of the technique has been thoroughly reviewed in publications by Sokoloff [301] and Smith [296]. In paper II intravenous injection of $^{35}$S-labelled methionine was used to trace protein synthesis in the rat brains according to the method described by Lestage et al. [180]. As a novel approach, a double-tracer bolus consisting of $^3$H-leucine and $^{14}$C-deoxyglucose was used in Paper III to simultaneously study protein synthesis and glucose consumption in parallel sections from each of the rat brains. This allowed correlation of the changes in protein synthesis [180] and the changes in glucose consumption caused by focal ischemia. The validity of this approach provided that $^{14}$C-deoxyglucose could efficiently be removed from the sections in which protein synthesis rates were determined. It was shown that this could be accomplished by washing the sections in a trichloroacetic acid solution.

**Volume measurements**

Measurements of different types of tissue volumes were made in all of the studies except in paper I and many of the conclusions drawn in the individual studies are based on these volume measurements. A brief description of the measurement methodology is therefore justified.

All volume measurements were done on digitized images of coronal rat brain sections using a computerized image analysis system equipped with a video camera.

**Infarct volumes**

Infarct volumes were determined either one day after MCAO on hematoxylin-eosin stained sections (paper V) or one week after MCAO on sections immunostained for glial fibrillary acidic protein (GFAP; papers V and VII). The latter choice of staining procedure and timing of the measurements is superior with respect to accuracy and practicability. Due to reactive astrogliosis one week after MCAO, GFAP-staining clearly demarcates infarcted tissue from non-infarcted brain tissue. The infarcts can therefore easily and unbiased be delineated on the images of the brain sections (fig. 6). On HE-stained sections one day after MCAO, the infarcts are not as sharply demarcated but can nevertheless be distinguished from non-infarcted tissue due to the loss of stainability, i.e. pallor, in infarcts.

Two methods for measurement of infarct volume were used: The direct method [paper IV] and the indirect method [papers V and VII]. In the direct method, the infarcted area on digitized images of the brain sections, collected at regular intervals during sectioning, is outlined and measured. The infarct volume is then calculated by multiplying the summed areas of infarction with the distance between the sections according to the principle originally conceived by the Italian mathematician and astronomer Francesco Bonaventura Cavalieri in 1635 [51]. Inherent in the direct measurement method is a risk of erroneously overestimating infarct volume due to cytotoxic and vasogenic edema in the lesion. It has been shown that with the direct method infarct volume fluctuates with the distance between the sections according to the principle originally conceived by the Italian mathematician and astronomer Francesco Bonaventura Cavalieri in 1635 [51]. Inherent in the direct measurement method is a risk of erroneously overestimating infarct volume due to cytotoxic and vasogenic edema in the lesion. It has been shown that with the direct method infarct volume fluctuates with the evolution of brain edema after MCAO in rats [185].

In the indirect method [306], the area of normal tissue, rather than infarcted tissue, is outlined on the sections and measured in corresponding regions of interest in the
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Part two: The experiments

Fig. 6: Images of coronal rat brain sections immunostained for glial fibrillary acid protein (GFAP) in reactive astrocytes. Due to the reactive gliosis at the infarct margin, GFAP staining is suitable for delineation of the infarct one week after permanent MCAO. A: Video-captured gray scale image used to measure infarct volume. B and C: Medium and high power colour microphotographs showing the GFAP-reactivity at the inner infarct margin in detail. Data from Paper VII. Ctx: Cortex. CPu: Caudate putamen. The asterisk indicates “lost tissue” in the infarcted cortex.

lesioned and non-lesioned hemisphere. The infarct volume can then be calculated by subtracting the volume of normal tissue in the region of interest in the lesioned hemisphere from the volume of the corresponding region in the unlesioned hemisphere. For example, the volume of a cortical infarct may be indirectly calculated using the equation:

\[
V_{\text{INF}} = V_C - V_L = d \times \sum_i A_C - d \times \sum_i A_L
\]

Where:
- \( V_{\text{INF}} \) = indirect infarct volume
- \( V_C \) = volume of normal cortex on non-lesioned side, i.e. contralateral to MCAO
- \( V_L \) = volume of normal cortex on lesioned side, i.e. ipsilateral to MCAO
- \( A_C \) = area of normal cortex on non-lesioned side on section \( i \)
- \( A_L \) = area of normal cortex on lesioned side on section \( i \)
- \( d \) = distance between measured sections.

Since remaining viable tissue is measured, the indirect measurements are not influenced by swelling of the infarct as shown by Lin et al. who compared the direct and indirect methods to the degree of brain edema up to three days after MCAO [185].

Long survival periods after MCAO, i.e. more than one week, preclude the use of the direct method due to the natural course of the healing of processes resulting in tissue loss and cystic cavitation in the infarct. The indirect method must be employed in this situation because it is impossible to delineate the lateral border of the infarct as illustrated by Fig. 6.

**Tissue volumes with normal and deranged metabolism**

In the quantitative autoradiographic studies reported on in papers II and III, volumes of tissue with normal and abnormal rates of protein synthesis and glucose consumption were measured. The volumetric methods used were based on the principles outlined by Swanson et al. [306] in a paper describing a semiautomated method for measuring brain infarct volume. Central to the method is the use of optical density thresholds to identify normal and abnormal brain tissue. In the protein synthesis study presented in paper II, the area with the lowest optical densities within the gray matter of the unlesioned hemisphere was identified by visual inspection. The mean densitometric gray value in this area was measured and served as a threshold value to identify gray matter with normal and reduced rates of protein synthesis in the lesioned hemisphere. Tissue with optical densities equal to or greater than the threshold value were considered to have normal rates of protein synthesis. The
volume of gray matter with normal protein synthesis in the ischemic hemisphere could then be calculated according to Cavalieri’s principle. In the double-tracer autoradiographic study (paper III), the procedure for threshold determination was further developed and refined. In brief, the entire cortex contralateral to MCAO was outlined on each autoradiograph of the coronal brain cryosections and the mean optical density and the standard deviation were measured. Normal metabolism was defined to be within the mean value ± 2 standard deviations. The resulting range of optical densities was then used to identify and calculate tissue volumes with normal (mean ± 2 SD), reduced (< mean – 2 SD) and increased (> mean + 2 SD) rates of protein synthesis and glucose metabolism in the ischemic cortex. In this way volume measurements were obtained that were stringently based on objective measurements of optical densities and therefore were completely unbiased by the observer’s judgement of the autoradiographic changes of metabolism.

**Tissue volumes with reduced enzyme activity**

In the MCAO study of the mitochondrial electron transport chain complex activity (paper IV), volumetry of cortex and caudate-putamen was performed essentially in the same manner as in paper III. However, as the histochemically stained brain sections by visual inspection did not show any signs of increased activity of any of the studied electron transport chain complexes, it was only necessary to measure tissue volumes with reduced activity (< mean – 2 SD). Furthermore, in the histochemical study, the workload on the investigator could be reduced by restricting measurements to coronal brain sections on which caudate-putamen was visible. This could safely be done without influence on the results because enzyme activity changes were not detected in any sections outside the anterior and posterior boundary of the caudate-putamen.

**Telemetric long-term measurement of body temperature**

It has been demonstrated in a substantial number of studies that intra- and postischemic body and brain temperature modulates the final infarct size in experimental models of focal cerebral ischemia (for reviews see [62,107,215]). Intra- or post-ischemic hypothermia reduces brain infarct size in rats [22,55,111,338], even if the hypothermia is mild [338,340,341] and if its induction is delayed for up two hours following MCAO [20,194]. Conversely, hyperthermia has been demonstrated to aggravate ischemic brain damage [54,56]. Moreover, some drugs that are neuroprotective in experimental brain ischemia may also induce mild hypothermia [242,274] or correct mild hyperthermia to normothermia [206] thus complicating any considerations regarding the mechanism of neuroprotection. Because of this it is crucial to monitor and control intraischemic and postischemic body temperature in experimental ischemia studies testing the potential neuroprotective effect of pharmacological compounds. In the experiments reported on in paper V and paper VII, in which the effect of respectively the free radical scavenger α-PBN and the broad-spectrum cation blocker pinokalant on infarct volume was tested, the body temperature of the rats was therefore carefully controlled during the operative procedures and for the following approximately 20 hours by means of a telemetric temperature recording system. A small temperature-sensing radio-transmitter was implanted into the abdominal cavity of the rats during the operative procedures. The temperature signal was transmitted to a radio receiver which connected to a personal computer controlling a heating lamp and a cooling fan. The spontaneous postischemic temperature course of untreated rats subjected to MCAO was established in a preceding series of experiments [71]. As a novel approach the temperature data from these experiments were loaded into the computer controlling the heating lamp and cooling fan. In this
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way it was possible to closely imitate the natural spontaneous postischemic temperature course of untreated rats in the rats treated with α-PBN or pinokalant and to obtain identical temperature curves in drug treated and vehicle treated rats.

Pharmacology of the compounds used in the studies

Below a brief description of the pharmacodynamic and pharmacokinetic properties of the compounds used in the studies are given since these properties could potentially have influenced the results.

Glutamate receptor antagonists

In papers I, II and III the effect of pharmacologic blockade of glutamate receptors on immediate-early gene expression and metabolism in the ischemic penumbra was investigated. Two prototype glutamate receptor antagonists were used: MK-801 and NBQX.

MK-801 (dizocilpine maleate, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate) is a highly selective non-competitive antagonist which binds to the ion channel in the NMDA receptor subtype of glutamate receptors in a use-dependent and voltage-dependent manner [333,334]. MK-801 is highly lipophilic and readily enters the brain after systemic administration [327]. In rats maximal concentrations in plasma and brain are reached within 10 to 30 min of intraperitoneal injection of 2 mg/kg. Half-life is approximately 2 hours in both plasma and brain [326]. The doses of MK-801 and the route of administration used in papers I-III have in several studies been shown to reduce infarct size in rats when assessed within 48 hours after initiation of permanent MCAO [32,41,93,127,266].

NBQX (2,3-Dihydroxy-6-nitro-7-sulfamoylbenzo(F)quinoxaline) is a selective competitive AMPA receptor antagonist devoid of activity at NMDA receptors [285]. Plasma half-life of NBQX has been reported to be approximately 1 hour after intravenous bolus injection of 10 mg/kg in rats [65]. A major disadvantage of NBQX is the low water solubility which causes nephrotoxicity encompassing precipitation of the compound in the renal tubules and acute tubular necrosis [240,337]. The nephrotoxicity of NBQX is not likely to have influenced the results of the short term experiments in papers I-III in which the rats only survived up to 3 hours after NBQX administration was commenced. As for MK-801 the parenteral doses of NBQX used in papers I-III have been reported to convey neuroprotection in rat MCAO models [104,113,299].

Ketobemidone

Ketobemidone is a potent opioid receptor agonist acting at central μ receptors and is used for treatment of severe pain. There are no published results concerning the pharmacokinetic behaviour of ketobemidone in rats. In humans plasma half-life is 2-3 hours after a single intravenous or oral dose [35]. In addition to the agonist effect at opioid μ receptors ketobemidone also possesses antagonist activity at the NMDA receptor [8,80-82]. Compared to MK-801 it is a weak NMDA antagonist. Ebert et al. reported that in an in vitro assay ketobemidone is four orders of magnitude less potent than MK-801 in binding to the NMDA receptor [81].

α-phenyl-N-tert-butyl nitrone

α-phenyl-N-tert-butyl nitrone (α-PBN), used in papers V and VI, is the parent compound of a family of spin-trapping nitrones which has been widely used in experimental ischemia studies because of its properties as a scavenger of free radicals (reviewed in [115]). After intraperitoneal injection in rats, α-PBN is rapidly distributed in blood and brain. Maximum concentration in blood and brain is reached within 20 minutes following an intraperitoneal bolus injection [57]. Terminal half-life in plasma is approximately 2 hours and plasma clearance is low averaging 12 +/- 4 ml/min/kg [320]. Steady-state concentration in brain is significantly greater than in plasma after i.p. injection indicating an excellent penetration
of the blood-brain barrier [57]. It has been shown in several studies that α-PBN significantly reduces infarct volume in rat models of permanent focal ischemia [48] and transient focal ischemia [13,275,278,347,354]. The neuroprotective effect of α-PBN in these models of focal cerebral ischemia is generally ascribed to the antioxidant properties of the compound although it also possesses anti-inflammatory effects which may contribute to protection [86].

**Pinokalant**

The effect of the broad-spectrum cation channel blocker pinokalant (LOE 908 MS) on infarct volume in rats subjected to permanent MCAO was tested in paper VII. The compound, which in vitro blocks several types of cation channels such as voltage-operated sodium and calcium channels, delayed rectifier K⁺-channels, NMDA and AMPA receptors and store-operated calcium channels (see fig. 14), was given as an intravenous infusion for approximately 24 hours. Leusch et al. [181] investigated the pharmacokinetic profile of pinokalant in rats and reported that after intravenous infusion the concentration-time profile of pinokalant in the brain paralleled that in plasma indicating a rapid but low penetration from plasma into the brain. Plasma half-life was reported to be 4.8 hours.

**RESULTS AND COMMENTS**

Below the results of the individual studies are briefly reviewed and commented upon.

**Paper I: Investigation of the Fos protein as a potential molecular marker of the penumbra**

The aim of this immunocytochemical study was to examine protein expression of the immediate-early gene c-fos in the infarct borderzone 2 hours after permanent MCAO. It was especially addressed whether the known neuroprotective effect of the glutamate receptor antagonists MK-801 and NBQX in models of focal cerebral ischemia is accompanied by a reduction of Fos protein production in the infarct borderzone in order to investigate whether the Fos protein can be used as molecular marker of penumbral tissue that may be salvaged after MCAO.

There were no Fos protein positive cells in the ischemic focus of the acute lesion in any of the groups. This is compatible with a complete overall arrest of protein synthesis in this part of the lesion which is not sensitive to treatment with the glutamate receptor antagonists [papers II and III]. In control rats synthesis of Fos protein was induced in neuronal nuclei in a zone surrounding the ischemic focus. Treatment with MK-801 almost totally suppressed Fos expression in both the striatal and the cortical part of the borderzone whereas

![Fig. 7: Semiquantitative score of Fos protein expression in the cortical and striatal infarct borderzone 2 hours after permanent MCAO in control rats and rats treated with MK-801 or NBQX. MK-801 significantly suppressed Fos expression whereas the effect of NBQX treatment was not statistically significant.](image-url)
NBQX did not significantly affect Fos protein expression as shown in Fig. 7.

It was suggested that Fos protein formation is induced in the borderzone by an increase in the intracellular neuronal calcium concentration known to accompany peri-infarct depolarizations [102,167]. This assumption was based on the finding that Fos positive neurons could also be detected in the cingulate cortex, which is outside the MCA territory, and the demonstration that MK-801 is an effective blocker of peri-infarct depolarizations [102].

It was concluded that the Fos protein may be used as early and persisting molecular marker of tissue with penumbral conditions which can be rescued by pharmacological intervention.

**Paper II: Effect of NMDA and AMPA receptor blockade on protein synthesis in the ischemic penumbra**

In this autoradiographic study of $^{35}$S-methionine incorporation it was investigated whether the neuroprotective effect of the glutamate receptor antagonists MK-801 and NBQX is reflected in an improvement of protein synthesis in the ischemic hemisphere in the acute phase after MCAO. Three hours after permanent MCAO the regional protein synthesis rates and the volume of tissue in cortex and striatum with reduced protein synthesis rates were examined in control rats and rats treated with either MK-801 or NBQX at the onset arterial occlusion.

In line with other reports a complete arrest of protein synthesis in the cortical and striatal ischemic focus was observed in the control and the glutamate receptor antagonist groups [164,211]. Surrounding the focus a metabolic borderzone with approximately 50% reductions in the protein synthesis rates was detected. The pattern of protein synthesis suppression was similar in the three groups but the localization of the transition between the cortical metabolic penumbra, i.e. tissue with reduced protein synthesis rates, and the cortical infarct, i.e. tissue with completely suppressed protein synthesis, was different. In MK-801 treated rats this transition on the coronal brain sections had moved ventrally compared to control and NBQX treated rats reflecting an improvement of protein synthesis in the penumbra (fig. 8). This difference was quantitatively reflected in significantly greater volumes of gray matter exhibiting normal protein synthesis rates in MK-801 treated rats than in control rats (fig. 9).

NBQX treatment did not improve protein synthesis in the ischemic hemisphere.

Based on the *in vivo* observation that spreading depression in the normally perfused brain inhibits protein synthesis [208] and *in vitro* observations from neuronal cultures that $K^+$-induced
Depolarization *per se* suppresses protein synthesis [138], it may be hypothesized that the mechanism by which MK-801 improved protein synthesis in the penumbra is related to the compound’s ability to inhibit periinfarct depolarizations and, hence, the transmembrane ion movements of especially potassium and calcium associated with these depolarizations. The exact molecular mechanism nevertheless remains unknown. In conclusion, the study showed that NMDA and AMPA receptor blockade with MK-801 and NBQX, respectively, affected the disturbed protein synthesis in the penumbra differently.

**Paper III: Double-tracer autoradiographic investigation of protein synthesis and glucose consumption in the ischemic penumbra – effect of MK-801 and NBQX**

In this study a double-tracer autoradiographic procedure was devised which made it possible to compare changes in protein synthesis and changes in glucose consumption within the same rat brain. It was hypothesized that the spatial distribution of suppressed protein synthesis is not congruent with that of aberrant glucose metabolism since the blood flow threshold for inhibition of protein synthesis is markedly higher than the flow threshold at which disturbed glucose consumption occurs [211,252,268].

The study showed that in the third hour after MCAO suppression of protein synthesis is spatially more widespread within the ischemic lesion than are disturbances of glucose metabolism. Thus, the volume of cortex ipsilateral to MCAO having normal rates of protein synthesis was significantly smaller than the cortical tissue volume with normal glucose consumption rates both in control rats and in rats treated with MK-801 and NBQX (fig. 10).

The difference in distribution of deranged protein synthesis and glucose metabolism within individual rats made it possible to identify a metabolic penumbra defined as the difference between tissue with reduced protein synthesis and tissue with reduced glucose consumption of which the latter was consistently located in the ischemic focus. Moreover, the method allowed us to quantitate the volume of this metabolic penumbra. In control animals the metabolic penumbra constituted 22% percent of the ipsilateral cortex. In MK-801 treated rats the size of the metabolic penumbra was reduced to 14% which was, however, not a statistically significant reduction. An effect of NBQX on the metabolic penumbra could not be demonstrated. With respect to glucose metabolism alone, MK-801 treatment significantly reduced the tissue volume with disturbed metabolism defined as both increased and reduced rates of glucose consumption.

It is inherent in the applied definition of the metabolic penumbra that protein synthesis is reduced in all of the metabolic penumbra. It was therefore interesting to note that only 16% of the metabolic penumbra in control rats had increased rates glucose consumption indicating that glucose metabolism was normal in most of the metabolic penumbra. Based on this observation it therefore seemed justified to speculate that tissue with reduced protein synthesis but normal glucose consumption might represent tissue with preserved ATP synthesis and may recover provided that appropriate therapeutic actions are taken.
Paper IV: Effect of ketobemidone on metabolism in the penumbra and infarct size

In 1995 it was discovered by Ebert et al. [80] that ketobemidone, in addition to being an opioid agonist at central μ receptors, also acts as a non-competitive antagonist at the NMDA receptor. NMDA receptor antagonists reduce infarct size in models of focal cerebral ischemia but unfortunately several promising NMDA antagonists, including MK-801, have had to be abandoned in clinical trials because of unacceptable side effects [175]. Since ketobemidone has been clinically available for decades for treatment of severe pain in humans, we found it relevant to explore whether ketobemidone has neuroprotective effects in our rat MCAO model and hence could be a potential candidate for further investigation in stroke.

Using the same double-tracer autoradiographic method as in paper III the effect of systemic ketobemidone treatment on the metabolic perturbations of protein synthesis and glucose consumption was investigated in the third hour after MCAO in one series of experiments. Compared to the control group ketobemidone did not affect the volume of the metabolic penumbra defined as in paper III but the volume of cortex with reduced glucose utilization was significantly diminished. This could be a sign that ketobemidone reduced the size of the ischemic focus in the acute phase. If so, such an effect does not seem to be a lasting one since ketobemidone did not reduce histological infarct volume measured one day after MCAO in another series of experiments (fig. 11).

In conclusion, ketobemidone did not ameliorate the disturbed metabolism in the penumbra in the acute phase; nor did it reduce infarct size 24 hrs after MCAO. The explanation for this is probably that ketobemidone is too weak an NMDA antagonist to effectively interfere with the pathogenetic mechanisms. Ketobemidone’s affinity for the NMDA receptor in cortical binding assays is some four orders of magnitude less than that of MK-801 [82]. Alternatively, a different dosing regimen may be necessary to convey neuroprotection in the rat MCAO model.

Paper V: Effect of α-PBN on periinfarct depolarizations and infarct size

As mentioned later in this overview, both generation of highly reactive free radicals and the occurrence of periinfarct depolarizations are considered of major importance in infarct pathogenesis. The purpose of paper V was to elucidate a possible interplay between periinfarct depolarizations and free radicals in our rat MCAO model. To that end we performed two separate series of experiments. In the first, we studied the effect of the free radical scavenger α-PBN on infarct size one week after permanent MCAO. In the second series of experiments the effect of α-PBN on the occurrence of periinfarct depolarizations in the acute phase after permanent MCAO was recorded and infarct size was measured one week later.

In the first series of experiments systemic treatment with α-PBN one hour after induction of MCAO did not reduce infarct volume measured one week later compared...
to vehicle treated control rats (fig. 12). A confounding influence of body temperature on infarct volumes could be excluded since telemetric measurement and control of core temperature was undertaken for 20 hours postocclusion in order to obtain identical temperature courses in α-PBN treated rats and control rats.

In the second series of experiments, α-PBN given at the time of occlusion significantly reduced the number and the summed duration of periinfarct depolarizations recorded with DC-electrodes located in the ischemic penumbra during the first three hours after MCAO (table 2). The mechanism by which α-PBN attenuated the occurrence of periinfarct depolarizations remains elusive. A blood flow dependent mechanism could be excluded by laser-Doppler flow measurements in close vicinity to the DC-recording electrodes. Nor are mechanisms common to those involved in propagation of cortical spreading depression in the normally perfused brain likely because α-PBN treatment did not influence the frequency KCl-induced transient depolarizations (table 2). It may be speculated that α-PBN stabilized the penumbra, perhaps owing to an improvement of energy status as previously reported [91], and that α-PBN thereby decreased the propensity of the penumbra to propagate transient waves of depolarization.

The most surprising finding of the study was, however, that the infarct volumes measured seven days later in the same rats were not smaller in the α-PBN treated group compared to the control group despite the significant attenuation of periinfarct depolarizations in the acute phase after MCAO (fig. 12). This latter result therefore question the hypothesis that periinfarct depolarizations per se are critical.

### Table 2: In experiment IIA the DC-recording electrode was located in the penumbra at a site close to the ischemic focus. In experiment IIB the electrode was also located in the penumbra but more peripheral to the ischemic focus. Treatment with α-PBN significantly reduced both the number of periinfarct depolarizations and the total depolarization time. There was no effect of α-PBN on KCl-induced transient depolarizations in the non-ischemic cortex (Experiment IIC). Original Table 3 from paper V.

<table>
<thead>
<tr>
<th>Number of depolarizations after treatment</th>
<th>Number of depolarizations per hour after treatment</th>
<th>Total duration of depolarizations after treatment (s)</th>
<th>Total duration of depolarizations after treatment in % of recording time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. IIA Control (n=13) 2 (1-5)</td>
<td>0.98 (0.24-2.79)</td>
<td>680 (305-1588)</td>
<td>8.9 (3.5-21.2)</td>
</tr>
<tr>
<td>α-PBN (n=9) 1* (0.75-1.25)</td>
<td>0.32** (0.24-0.4)</td>
<td>163** (120-293)</td>
<td>1.1* (1.0-2.7)</td>
</tr>
<tr>
<td>Exp. IIB Control (n=9) 4.2 ± 2.5</td>
<td>1.37 ± 0.27</td>
<td>708 ± 240</td>
<td>6.4 ± 2.5</td>
</tr>
<tr>
<td>α-PBN (n=5) 0.6 ± 0.9*</td>
<td>0.19 ± 0.13*</td>
<td>592 ± 363</td>
<td>5.3 ± 3.3</td>
</tr>
<tr>
<td>Exp. IIC Control (n=4) 40.0 ± 10.7</td>
<td>16.1 ± 1.9</td>
<td>3297 (2412-4181)</td>
<td>36.7 ± 5.9</td>
</tr>
<tr>
<td>α-PBN (n=4) 40.3 ± 11.6</td>
<td>16.5 ± 4.2</td>
<td>2561 (2304-2819)</td>
<td>29.7 ± 5.5</td>
</tr>
</tbody>
</table>

Data were tested for compliance with the normal distribution and values are accordingly presented as mean values ± standard deviation or median values with the 1st and 3rd quartiles given in parentheses.

*Significantly different from control (p<0.05, Student’s t-test).
**Significantly different from control (p<0.05, Mann–Whitney U-test).

Fig. 12: Cortical infarct volumes 7 days after permanent MCAO in Experiment I and Experiment II. Infarct volumes were not statistically different in saline-treated control rats and α-PBN-treated rats in either series of experiments. (Experiment I: p = 0.36, t-test; Experiment II: p = 0.08, Mann–Whitney rank sum test).
determinants of the final infarct size in rat models of permanent focal cerebral ischemia [135].

**Paper VI: Mitochondrial electron transport complex activity after transient MCAO**

A large number of studies have established an important pathogenetic role of mitochondria in ischemic cell death in the brain but the precise mechanisms has yet to be fully elucidated (reviewed in [294]). In this histochemical study, providing a high regional resolution, we examined the activity of the mitochondrial electron transport complexes I, II and IV in the ischemic hemisphere after a 2 hours focal ischemic insult followed by reperfusion ranging from 0 to 4 hours in order to elucidate if inhibition of mitochondrial electron transport complexes might be involved in the conversion the penumbra to infarct and perhaps could explain the secondary deterioration of the energy state and respiratory rates in the penumbra that has been observed by other investigators [91,169,170].

Topographically, MCAO induced a reduction of activity of the investigated electron transport complexes which was restricted to the ischemic focus, i.e. to the dorsolateral part of the caudate putamen and the most inferior part of the adjacent cortex. The time course of these changes produced by ischemia and reperfusion was different in the cortex and the caudate putamen.

In cortex (fig. 13), the tissue volume with reduced activity did not change significantly during reperfusion although a tendency towards a subtle improvement of the activity at 1 hour of reperfusion followed by a deterioration at 4 hours of reperfusion was observed which could be in line with the previous observations of secondary deterioration in energy state and respiratory rates [91,169,170].

In contrast, the tissue volumes with reduced electron transport chain complex activity progressively and significantly increased during reperfusion in the caudate putamen as shown in (fig. 13).

This difference between cortex and caudate putamen seems to reflect the faster maturation of the ischemic damage in the caudate putamen compared to the cortex [53,95]. This is supported by the observation that hematoxylin-eosin stained sections collected in parallel to the histochemical

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**Fig. 13: Volumes of tissue in cortex and caudate putamen with reduced activity of mitochondrial electron transport complexes I, II and IV after 2 hours of MCAO without reperfusion (n = 7) and 2 hours of MCAO followed by 1 hour (n = 8) and 4 hours of reperfusion n = 8).** **Significantly different from complex II in the no reperfusion group and the group with 1 h reperfusion. #Significantly different from complex II at 4 h of reperfusion. *Significantly different from complex I in the group with no reperfusion. ##Significantly different from complex II in the α-PBN–treated group. Groups were compared with either a one way ANOVA or a Kruskall-Wallis ANOVA on ranks followed by relevant multiple comparison procedures, level of significance p < 0.05.**
sections showed that brain areas with reduced electron transport chain complex activity also displayed early morphological signs of damage. A spatial and temporal congruency between loss of electron transport chain complex activity and morphological damage was thus evident. Complex II activity was reduced in a significantly larger part of the caudate putamen than was complex IV at 4 hours of reperfusion indicating that the electron transport complexes have different sensitivities to ischemia/reperfusion. The study gives no hints to the mechanism responsible for this other than free radicals do not seem to play a role in electron transport chain complex inhibition since treatment with the free radical scavenger α-PBN did not influence the activity of any of the studied complexes in general and complex II in particular (fig. 13) in the 4 hours reperfusion group. To summarize, reduction of electron transport chain complexes I, II and IV activity was only observed in the most central part of the ischemic focus in areas that already displayed morphological damage. The study therefore does not support a critical role for widespread inhibition of components of the mitochondrial electron transport chain in the pathogenesis of experimental brain infarcts.

**Paper VII: Effect of broad-spectrum cation channel blockade on infarct size**

Derangement of ion homeostasis is an important pathophysiological event in cerebral ischemia. Activation of cation ion channels conducting Na⁺, K⁺ and, in particular, Ca²⁺ plays a major role in the pathogenesis of the ischemic cell death involved in infarction of the brain (for reviews see: [42,168]). Pinokalant (LOE 908 MS) is a recently developed compound that *in vitro* blocks several subtypes of cation channels including voltage-operated sodium

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**Fig. 14:** Drawing showing the multiple pathways by which cytosolic calcium concentration may increase during ischemia and the proposed sites where the broad-spectrum cation blocker pinokalant acts to prevent an increase in intracellular calcium and sodium ion concentration.
and calcium channels, delayed rectifier K⁺- channels, NMDA and AMPA receptors as well as store-operated calcium channels (fig. 14). This broad pharmacologic profile of cation flux inhibition, and especially its action at store-operated calcium channels whose role in ischemic brain damage has previously only been investigated to a very limited extent, made it interesting for us to test if pinokalant could provide lasting neuroprotection in terms of reducing histological infarct volume one week after permanent MCAO in rats.

Intravenous infusion of pinokalant, starting 30 minutes after MCAO and continuing for 24 hours, resulted in a significant 28% reduction of cortical infarct volume compared to vehicle treated controls (fig. 14). The reduction of the infarct volumes was even more pronounced (47% compared to control, fig. 15) in a subset of animals which had plasma concentrations of pinokalant within an expected therapeutic plasma concentration interval that was established in other experiments.

As in paper VI body temperature was carefully controlled per- and postoperatively during the drug infusions to circumvent any temperature modulatory effect of pinokalant which could eventually have influenced final infarct size.

The study demonstrated that infarct reduction can be obtained by broad-spectrum inhibition of cation channels in rats. Considering the established pathogenetic role of calcium in ischemic cell death and the multiple pathways by which calcium concentration may rise intracellularly, it seems reasonable to pursue this approach of broad-spectrum cation flux inhibition also in future studies aiming at clarifying which mechanisms may be pharmacologically targeted in order to obtain neuroprotection.
PART THREE: GENERAL DISCUSSION

MECHANISMS OF FOCAL ISCHEMIC BRAIN DAMAGE

In the following the discussion will be restricted to concern only the pathogenetic and pathophysiological mechanisms involved in the acute phase of focal ischemia, i.e. the initial hours after MCAO.

Calcium and other cations

Calcium

Cytosolic calcium overload is a crucial step in the sequence of events leading to ischemic cell death (for a scholarly review on the role of calcium in brain ischemia, see [168]). Taking into account that Ca\(^{2+}\), as an ubiquitous intracellular messenger, governs a large number of cellular functions such as cell growth and differentiation, gene expression, membrane excitability, exocytosis and synaptic activity, calcium ion homeostasis need to be very tightly regulated. Several pathways leading to cytosolic calcium overload exist. These may be divided into 1) movement of Ca\(^{2+}\) across the cell membrane from the extracellular space to the intracellular compartment, and 2) release from intracellular stores such as mitochondria and the endoplasmic reticulum. These pathways are summarized in fig.14.

In the cells of the brain there is normally a very large concentration gradient of Ca\(^{2+}\) across the plasma membrane, the extracellular concentration being 10,000-fold higher than the intracellular concentration. This gradient, in conjunction with the electrical potential difference across the cell membrane, tends to translocate calcium into the cells. Calcium may cross the cell membrane through voltage-operated calcium channels (VOCC) which flux calcium secondary to membrane depolarization, and through receptor-operated calcium channels (ROCC) such as the glutamate receptors of the NMDA subtype. Furthermore, in the setting of ischemia and ATP depletion, calcium extrusion mechanisms such as the ATP-driven Ca\(^{2+}\)-pump and Ca\(^{2+}\)-Na\(^{+}\)-exchange may reverse causing intracellular accumulation of calcium. Capacitative calcium entry is a recently identified mechanism in which calcium entry occurs through store-operated cation channels located in the plasma membrane [258-260]. Opening of store-operated cation channels seem to be activated by depletion of calcium stored in the endoplasmic reticulum and under physiologic conditions subserves the function of enhancing cytosolic Ca\(^{2+}\) signals as well as refilling the intracellular pools [30,31]. This mode of Ca\(^{2+}\) influx, which was examined in paper VII, may become particularly important during prolonged ischemia since it allows a continuous replenishment of the intracellular calcium stores from which calcium can then be released adding to cytosolic calcium overload in a vicious circle.

Calcium sequestered by the endoplasmic reticulum may be released in response to agonist activation of metabotropic glutamate receptors located in the cell membrane. Activation of metabotropic glutamate coupled to activation of phospholipase C leads to formation of inositol 1,4,5-trisphosphate (IP\(_3\)) which by binding to IP\(_3\) receptors on the endoplasmic reticulum induce release of calcium from this organelle to the cytosol. Furthermore, when a substantial rise in the intracellular calcium concentration occurs, mitochondria begin to accumulate large amounts of calcium that, under special circumstances, may leave the
The penumbra in experimental focal cerebral ischemia

Part three: Discussion

mitochondria again due to opening of a large conductance pore, the mitochondrial permeability transition pore, which is located in the mitochondrial inner membrane [168,294].

Cytosolic calcium overload, in turn, activates a number of detrimental biochemical reactions that ultimately result in cellular malfunctioning and cell death. These reactions encompass activation of numerous enzyme systems, including lipases such as e.g. phospholipase A$_2$ resulting in breakdown of membrane lipids and generation free oxygen radicals, proteolytic enzymes degrading, for instance, cytoskeletal proteins and endonucleases causing DNA degradation (reviewed in [75,272,289,290]). In addition, elevated intracellular calcium levels may impair mitochondrial function by opening the mitochondrial permeability transition (MPT) pore which leads to mitochondrial swelling, cessation of ATP production, release of apoptotic factors such as e.g. cytochrome c and possibly a burst of oxygen free radical generation [294].

The data implicating deranged calcium homeostasis in ischemic cell death in the brain is to a large extent based on experimental data obtained in in vivo global ischemia models and in vitro model systems. In relation to focal ischemia this may therefore rise two simple but pertinent questions: First, does cytosolic calcium overload occur in areas of focal ischemia and second, if so, does calcium overload mediate cell death in these areas?

Concerning the first question, studies assessing the regional calcium content by atomic absorption spectroscopy or $^{45}$Ca autoradiography during MCAO have shown calcium accumulation in areas affected by ischemia indicating an accelerated influx of calcium from the blood into the brain [153,167,224,225,261,288]. The techniques used in these studies do not discriminate between intra- and extracellular calcium accumulation. Extracellular levels of calcium in in the ischemic focus and the penumbra have been measured with Ca$^{2+}$-sensitive microelectrodes during MCAO in rats [102,167]. In the ischemic focus the extracellular calcium concentration drops from around 1.2 mM to 0.1 mM and remains stable following induction of MCAO. In the penumbra transient calcium concentration reductions to levels about 0.1 mM occur irregularly in the penumbra in line with the presence of periinfarct depolarizations. These studies indicate that virtually all extracellular calcium is translocated to the intracellular compartment when the focus and the penumbra become depolarized.

Opening of voltage-operated calcium channels during a focal ischemic insult in rats has been demonstrated by autoradiographic visualization of $^3$H-nimodipine binding to activated calcium channels [119]. $^3$H-nimodipine binding increased earlier in more severely ischemic structures, corresponding to the ischemic focus, than in penumbral areas with more moderate reductions in perfusion indicating increasing $^3$H-nimodipine binding with increased depolarization.

Attempts to directly measure intracellular calcium levels have been made in cats subjected to MCAO by use of the fluorescent intracellular calcium indicator Indo-1 and fluorimetry of the ischemic cortex through a cranial window [12,322]. These studies reported that the fluorescence signal arising from intracellular calcium increased significantly during MCAO and reperfusion in cats with severe ischemia. It should be mentioned, however, that others have not been able to reproduce this result [184]. Similar studies utilizing fluorescent calcium indicators have not been performed in rat MCAO models. DeGraba et al. [68] used an indirect immunohistochimical method to explore the temporal relation between the duration of focal ischemia in rats and the functional activity of increased intracellular calcium measured by calcium-calmodulin binding. Loss of calmodulin staining, reflecting binding of calcium to intracellular calmodulin, was observed in the cortical ischemic focus one hour after induction of MCAO and became maximal after 4 hours of ischemia. Furthermore, penumbral areas, represented by a mild loss
of calmodulin staining surrounding the central focus with maximal loss of staining, gradually decreased in size and became incorporated into the ischemic focus after 4 hours of MCAO thus illustrating recruitment of the penumbra in process of infarct evolution. Although direct measurements definitively showing an increase in cytosolic calcium concentration in focal ischemia have not yet been obtained, the above-mentioned studies provide ample evidence to infer that intracellular calcium overload occurs also in focal ischemia.

With respect to the second question several studies have shown that pharmacologic blockade of calcium entry into cells provides neuroprotection in terms of a reduction in infarct size in rat models of focal ischemia. Blockade of agonist-operated calcium channels with NMDA receptor antagonists reduce infarct size in rat models of both transient MCAO [41,78,79,206,269] and permanent MCAO [32,34,37,77,102,125,127,140,149,248-250,266] provided that the antagonists are administered before or with delay of at most 1-2 hours after arterial occlusion [127,196,337]. In the majority of these studies the reduction of infarct size has been between 20% and 50%. A similar degree of neuroprotection is obtained with compounds that block voltage-operated calcium channels. A number of different voltage-sensitive calcium channel blockers have been reported to reduce infarct size in rat models of transient MCAO [36,40,45,160,165,348] as well as permanent MCAO [32,45,98,129,144,150,183]. As with NMDA antagonists, the therapeutic window for VOCC antagonists is narrow and the compounds must be given before or shortly after arterial occlusion in order to be efficacious. A critical issue when attempting make inferences concerning the pathogenetic role of calcium influx in these VOCC antagonist studies is that some of the compounds tested, due to a vasodilator effect, induce systemic hypotension [40] and improve cerebral blood flow [217,243]. Some calcium antagonists also reduce glutamate release to the extracellular space [316] thus hampering clear-cut conclusions on the exact mechanisms of neuroprotection. Despite these experimental restrictions the bulk of evidence supports a critical pathogenetic role for cytosolic calcium accumulation in the infarction process.

**Sodium**

During ischemia sodium ions are translocated down their concentration gradient from the extracellular to the intracellular compartment through voltage-sensitive sodium channels in the plasma membrane and through ionotropic glutamate receptors of the AMPA subtype. Entry of sodium ions results in cell membrane depolarization, opening of voltage-operated calcium channels, glutamate release and relief of the voltage-dependent magnesium block of the NMDA receptor resulting in entry of calcium ions and more sodium. Along with sodium osmotically obligated water enters the cells resulting in intracellular edema and ultimately disruption of the cell membrane due to swelling of the cells [289]. Hence, sodium entry into cells associated with anoxic depolarization in the ischemic focus and the transient depolarizations of the penumbra is an important early pathophysiological event. Although sodium entry per se does not cause molecular damage it contributes to the initiation of complex cascade of pathogenetic events involved in the infarction process [186]. This is underscored by studies showing that blockers of voltage-sensitive sodium channels reduce infarct size even when given 1 hour after initiation of permanent MCAO in rats [50,112,262,297,298,300].

**Excitotoxicity - glutamate, glutamate receptors and antagonists**

The non-essential acidic amino acid glutamate is the most abundant excitatory neurotransmitter in the central nervous system and is present in a variety of neurons throughout the brain and the spinal cord [92]. In addition to being the major excitatory transmitter vitally important for normal brain function, glutamate may also - particularly in conditions of cellular energy...
compromise - act as a neuritotoxin that can kill nerve cells by excessive stimulation of their excitatory receptors as it was first proposed by Olney who coined the term “excitotoxicity” to this killing action of glutamate and glutamate analogues [244,245]. Since then substantial experimental evidence supporting the involvement of excitotoxic mechanisms in various pathological conditions characterized by acute and chronic neurodegeneration has been produced (reviewed in [1,15,23,187]).

A detailed description of glutamate as a neurotransmitter and as a neurotoxin, including its anatomical distribution and compartmentation within the CNS, its synthesis, release and uptake mechanisms as well as the molecular biology of the glutamate receptors is beyond the scope of the present overview and the reader is referred to the extensive literature on the subject (see e.g. [3,4,14,203]). In the context of the work presented in this overview, it suffices to note that glutamate after being released to the extracellular space binds to and activates different types of glutamate receptors located in cell membranes of pre- and postsynaptic neurons as well as on glial cells. The glutamate receptors are broadly grouped into ionotropic receptors, which are agonist-operated cation channels, and metabotropic receptors that are G-protein coupled receptors linked to intracellular second messenger systems such as turnover of inositol phosphates and mobilization of intracellular calcium as mentioned above, activation of protein kinases, and modulations in cAMP levels. Three different types of ionotropic glutamate receptors have been characterized and are distinguished from one another on basis of their preferred agonists: n-methyl-d-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainic acid (KA). The latter two are often collectively called non-NMDA receptors because it has been difficult to distinguish pharmacologically between them [179,207]. The NMDA receptor is a ligand-operated slow gating ion channel which is highly permeable to Ca\(^{2+}\), Na\(^+\) and K\(^+\). Binding of glutamate to the receptor results in an influx of Ca\(^{2+}\) and Na\(^+\) and efflux of K\(^+\). A distinctive feature of the NMDA receptor is its voltage-sensitive block of the ion channel by Mg\(^{2+}\) which must be relieved for full activation of the receptor to occur. The Mg\(^{2+}\) block is operative at normal membrane resting potential but is removed by partial membrane depolarization. Another specific feature of the NMDA receptor is the presence of a modulatory site to which glycine or D-serine, acting as a coagonist, must be bound in order for glutamate to open the ion channel (reviewed in [214]). AMPA and KA receptors both mediate fast excitatory neurotransmission. The ion channel of these non-NMDA receptors is primarily permeable to Na\(^+\) and K\(^+\). However, AMPA receptors may also be permeable to Ca\(^{2+}\) if they have a subunit composition in which the GluR2 subunit is not present [132,256]. In situ hybridization studies of the rat brain have shown that in the brain regions affected by ischemia due MCAO in rats, i.e. primarily the neocortex and the caudate putamen, the GluR2 subunit is abundantly expressed [61,270] which indicates presence Ca\(^{2+}\)-impermeable AMPA receptors in these areas.

A key triggering event in excitotoxicity is overactivation of NMDA receptors leading to excessive neuronal calcium influx through the NMDA receptor channel. Cellular calcium overload in turn leads to mitochondrial dysfunction, intracellular production of damaging free radicals and activation of enzymatic processes contributing to cell death as described above. For this to take place the voltage-sensitive Mg\(^{2+}\) block of the NMDA receptor channel must be removed by depolarization of the neuronal membrane. This may occur either by activation of AMPA receptors fluxing sodium into the cells or by cellular energy shortage compromising the function of the Na\(^+\)-K\(^+\)-ATPase as it is thought to happen in ischemia and many chronic neurodegenerative diseases (reviewed in [24,289]). In addition, Na\(^+\) influx through AMPA receptors and an associated
osmotically obligated movement of water into cells is believed to contribute to intracellular edema and cell disruption. A plethora of studies have convincingly demonstrated that excitotoxic mechanisms are likely to be pathogenetically involved in the recruitment of the penumbra in models of focal ischemia. First, glutamate is excessively released to the extracellular space in the penumbra during MCAO in rats [44,112,114,204,219,246,312]. Second, during MCAO energy requiring uptake mechanisms responsible for clearance of glutamate from synaptic clefts and the extracellular space are impaired in the penumbra [39]. Third, NMDA and AMPA receptor antagonists administered close to the point of arterial occlusion reduce infarct size if this is assessed within a few days after MCAO (see for example [32,77,79,103,104,113,127,151,248,250,274,337]). Fourth, glutamate is involved in the generation and propagation of perifarct depolarizations inasmuch as these have been shown to be sensitive glutamate receptor blockade [102,210,213,319]. There is therefore a general consensus that exitotoxic mechanisms related to NMDA and AMPA receptor activation are operative in the early phase of infarct evolution. The role of KA and metabotropic glutamate receptors are at present largely unclear.

Perifarct depolarizations – role in infarct evolution

According to a widely accepted hypothesis perifarct depolarizations are not merely passive events evoked by focal ischemia but may, in flow restricted and energy compromised tissue, actively participate in the sequence of pathogenetic mechanisms involved in irreversible cell damage and thus contribute to the enlargement of infarcts [135]. This hypothesis was generated on the basis of several lines of evidence. First, the number of perifarct depolarizations was found to correlate linearly with the final infarct volume [210]. Second, increasing the frequency of perifarct depolarizations in MCA-occluded rats by intentional induction of repetitive cortical depolarizations in perifocal areas produced a significant increase in the total volume of ischemic injury evaluated histopathologically [18] and a stepwise increment of lesion volume measured by diffusion-weighted magnetic resonance imaging after passage of each induced depolarization [43,313]. Third, in the hemodynamically compromised penumbra each wave of depolarization is not coupled to an adequate flow response, i.e. a flow increase sufficient to match the increased energy demand required to repolarize cell membranes [19]. Transient but detrimental episodes of relative tissue hypoxia therefore arise which stimulate anaerobic glycolysis and hence impair efficient energy production [19]. Fourth, glutamate antagonists acting at both NMDA and non-NMDA receptors suppress perifarct depolarizations and also reduce infarct volume [102,140,213]. Although this perifarct depolarization hypothesis has gained widespread focus and acceptance there are observations from other studies which are not easily compatible with the hypothesis that PIDs per se contribute to the gradual infarct expansion at the expense of penumbral tissue and thus should be considered the only critical determinants of final infarct size. For example, hyperglycemia suppresses PIDs almost completely but also significantly increases infarct size compared to normoglycemia [230,232]. More importantly, in two studies Gidö et al. [100,101] showed that elicitation for several hours of cortical spreading depression in situations of induced hypoglycemia, hypoxia or combined hypoxia and ischemia to mimic the energy compromised penumbral conditions only resulted in very minor histopathological brain damage even though the tissue was challenged with a higher frequency or longer duration of depolarizations than what is usually observed in perifocal areas following rat MCAO. Furthermore, Kastrup et al. [146] reported on a magnetic resonance imaging study of rat thromboembolic stroke that repetitive perifarct depolarizations did not eventually lead to permanent reductions of the apparent
diffusion coefficient of water indicative of infarction and concluded that perinfarct depolarizations not necessarily contribute to the expansion of the ischemic lesion volume in their model. As reported in Paper V, we found that the free radical scavenger α-PBN, in spite of having a clear cut effect in reducing the number and duration of perinfarct depolarizations during the initial 3 hours following permanent MCAO in rats, did not diminish infarct volume one week later. In this context it should be mentioned that intriguing results have recently been obtained in studies where PIDs were recorded through a period of up to 72 hours after permanent and transient MCAO in rats [123,124]. These studies revealed a delayed secondary phase of PIDs that occurred approximately 8-20 hours after the initial phase of PIDs observed during the first two hours ischemia. In the intervening period between the two phases PID activity was not observed. Treatment with an NMDA receptor antagonist shortly before the secondary PID phase significantly reduced infarct volumes indicating a possible pathogenetic role of these secondary PIDs which may have been overlooked in previous studies.

**Reactive oxygen species**

Excessive formation of oxygen-derived free radicals, or reactive oxygen species (ROS), is an important event in brain damage caused by focal ischemia, especially if followed by reperfusion. Because of their high reactivity free radicals are extremely short-lived and hence very difficult to directly measure *in vivo*. In spite of this, several lines of evidence have supported the notion that accelerated formation of reactive oxygen species such as the superoxide anion (\(\text{O}_2^-\)), hydrogen peroxide (\(\text{H}_2\text{O}_2\)), nitric oxide (\(\text{NO}^+\)) and the extremely reactive hydroxyl radical (\(^\cdot\text{OH}\)) takes place in focal ischemia and reperfusion, and that these reactive oxygen species contribute to brain damage through detrimental reactions damaging membrane lipids, proteins, DNA and other macromolecules (reviewed in [75,186,291]). Endogenous scavengers of free radicals, i.e. antioxidants, such as α-tocopherol, reduced ascorbate, reduced ubiquinones and reduced glutathione are consumed in tissue within the ischemic region early after MCAO in rats [11,159,216]. Products of lipid peroxidation formed as a consequence of hydroxyl radical attack on polyunsaturated fatty acids in cell membranes accumulate in areas affected by focal ischemia [141,142,147,148,283]. The formation of carbonyl groups in proteins as a result of ROS mediated oxidation of certain amino acid side chains has been reported to increase during reperfusion after MCAO in rats [295]. It should, however, be mentioned that an earlier MCAO study in rats failed to demonstrate this [89]. Oxidative DNA damage in the ischemic focus and the penumbra is detectable very early during reperfusion following MCAO in rats [64]. Using an immunohistochemistry method Imai *et al.* in two studies found oxidative DNA damage in focal and perifocal areas 4 hours after induction of permanent MCAO and after 22 hours of reperfusion following 2 hours of transient MCAO [141,142]. In both instances DNA damage was ameliorated by treatment with the antioxidant ebselen.

Demonstration of ROS formation *in vivo* has been accomplished more directly by use of exogenously administered compounds that react with oxygen radicals formed in the brain tissue producing more stable adducts which can then be collected from the tissue, e.g. by microdialysis. In the most widely applied technique salicylate (2-hydroxybenzoic acid) is used as an *in vivo* trap of hydroxyl radicals [87,88]. Salicylate reacts with hydroxyl radicals to form the stable adducts 2,3- and 2,5 dihydroxybenzoic acid (DHBAs). The microdialysate content of these adducts can thus be taken as an index of \(^\cdot\text{OH}\) formation [60]. Using this technique a significant and sustained increase in hydroxyl radical formation has been observed in the cortical penumbra both during MCAO and during reperfusion [219,302]. In the cortical ischemic focus hydroxyl radical formation is decreased or
unchanged during MCAO but subsequently increases significantly upon recirculation [99,302]. In the striatal ischemic focus hydroxyl radical generation is elevated both during focal ischemia and upon reperfusion [172,188]. The mechanisms underlying this difference between the cortical focus and the striatal focus are not clear but regional differences in the relative contribution from the many possible sources of free oxygen radicals mentioned below may be responsible. Peters et al. employed a different and technically very demanding lucigening-enhanced chemiluminescence technique, which mainly detects superoxide radicals, to study the dynamic pattern of free radical production in the cortical penumbra in rats after permanent and reversible MCAO [257]. They observed a steady increase in ROS production during MCAO and burst-like pattern of enhanced ROS production early after start of reperfusion. Also nitric oxide, a gas radical synthesized by Ca$^{2+}$-dependent nitric oxide synthases (NOS), increases in the ischemic brain during focal ischemia and reperfusion [195,271,352]. NO$^\bullet$ may in turn react with superoxide to produce peroxynitrite which itself is a toxic oxidant that decomposes to yield hydroxyl radicals [26].

Taken together these studies demonstrate that enhanced generation of free oxygen radicals occurs in focally ischemic brain tissue and that reperfusion of ischemic tissue may lead to exaggerated ROS production. The studies also show that regional differences in ROS formation exist which may have important implications for a successful neuroprotective treatment with antioxidants in experimental and probably also clinical stroke.

Several possible intracellular sources of free oxygen radicals during ischemia/reperfusion have been proposed. These include the mitochondrial electron transport chain, autooxidation and monoamine oxidase metabolism of monoamines released during ischemia, xanthine oxidase mediated metabolism of breakdown products of the adenosine phosphates, phospholipase A$_2$ metabolism of arachidonic acid released from cell and mitochondrial membranes as well as nitric oxide synthases (reviewed in details in [291] and [171]). In addition, it has been suggested that the low intra-ischaemic intra- and extracellular pH could cause iron to become delocalized from the physiological protein bound form to a free catalytic form catalysing the so-called Haber-Weiss reaction which converts superoxide to more reactive hydroxyl radicals [291].

The pathogenetic importance of the increased ROS generation is underscored by a continuously growing number of studies showing that treatment with scavengers of free radicals and other antioxidant compounds that interfere with free radical mediated reactions ameliorate the brain damage incurred by focal ischemia. This has been firmly established in models of focal ischemia followed by reperfusion (see for example [13,275,325,339,347]). In models of permanent focal ischemia free radical scavengers have also been reported to reduce infarct size [25,48,143,178,199,247,255,307,315]. It is of note that in all but one of these studies [178] infarct volumes were measured between one and three days after MCAO. In our own study of the radical scavenger $\alpha$-PBN we measured infarct size one week after permanent MCAO and failed to demonstrate a beneficial effect of $\alpha$-PBN given as a single dose 1 hour after occlusion (paper V). This could indicate that free radical scavengers administered as a single dose close to the point of arterial occlusion postpone damage evolution rather than convey lasting neuroprotection in the setting of permanent focal ischemia. This notion is supported by the findings of a transient MCAO study in rats in which a single dose of the hydroxyl radical scavenger dimethylthiourea was administered prior to MCAO [161]. In that study infarct size was reduced at 24 hours but not seven days after occlusion. Considering that production of free radicals may continue for many hours following arterial occlusion [302] and the wide time window of therapeutic opportunity, which has been reported to be up to 12 hours after MCAO [48], it seems
crucial to devise optimal dosing regimens in these kinds of experiments.

**Dysfunction of mitochondria**

A steadily accumulating amount of evidence also suggests that mitochondrial dysfunction is an important pathogenetic factor in the complex cascade of cell death mechanisms which are involved in brain infarction. As intracellular energy producers providing ATP for anabolic biosynthetic reactions, maintenance of ion homeostasis and normal neurotransmission, mitochondria are critically important for normal cell function. In addition, it has become clear that mitochondria may act as intracellular switches that govern the mode of cell death, i.e. whether a cell dies by necrosis or apoptosis (for reviews see [239,293]). Mitochondrial ATP synthesis by oxidative phosphorylation is dependent on a sufficient oxygen and glucose delivery to brain tissue, and energy production is hence sensitive to the substrate deprivation imposed by ischemia. As stated previously ATP synthesis is markedly depressed in the ischemic focus during MCAO. In the penumbra ATP synthesis is also compromised but to a lesser degree than in the focus [90,91,279-282,331]. Mitochondrial respiratory rates are reduced during MCAO and, again, the changes are more pronounced in the ischemic focus than in the penumbra [10,169,170,226]. During recirculation following 2 hours of MCAO both ATP synthesis and respiratory rates recover partially but signs of a secondary deterioration after 4 hours of reperfusion were observed in some of the studies [91,169,226]. It is unlikely that a secondary compromise in microcirculation can account for the secondary deterioration of bioenergetic state and mitochondrial respiration since cerebral blood flow was fully restored upon reperfusion and remained stable at preischemic levels for the entire postsischemic observation period [226]. Collectively, the results of these studies therefore clearly indicate that mitochondrial function is impaired during focal ischemia and reperfusion. Obviously one possible cause of impaired mitochondrial ATP synthesis and reduced respiration during ischemia could be reduced substrate supply from the blood to ischemic brain regions. This is a likely mechanism in the ischemic focus immediately after onset of ischemia before cells become irreversibly damaged. In the focus blood flow is severely reduced to less than 15-20% of normal [33,237,318], glucose consumption is arrested [237,287], glucose tissue content is almost depleted [90,91,237,281,282] and interstitial oxygen tension approaches zero [189,226]. In the penumbra the matter is more complicated. Glucose consumption increases in the penumbra early after arterial occlusion [237,287], indicating that glucose transport from blood to the oligemic penumbra is not a limiting factor. Oxygen tension in the penumbra has been reported to fall to 75% of preischemic levels immediately after induction of MCAO after which a slight improvement to 84% of preischemic levels were observed. During passage of periinfarct depolarizations tissue oxygenation was further reduced to 68% of control [19]. Whether this degree of tissue hypoxia during MCAO per se is sufficient to substantially affect mitochondrial energy production is questionable. Acidosis and lactate accumulation in the penumbra intuitively indicate hypoxic conditions and a switch to anaerobic glycolysis but could also, in principle, be contributed to by damage to mitochondria. Apparently inhibition of the activity of mitochondrial electron transport complexes is not a prominent feature of mitochondrial dysfunction in focal ischemia as shown in paper VI and by Canevari et al. [47]. Therefore other possible mitochondrial mechanisms have been proposed (reviewed in [294] and [292]). Mitochondria are considered a major site of production of reactive oxygen species, such as the superoxide anion and hydrogen peroxide, as well as targets of detrimental free radical attack leading to oxidative modification of mitochondrial lipids and proteins (reviewed in [85,223,291]). Particular attention has
also been focused on the potential involvement of the mitochondrial permeability transition (MPT) which results from assembly and opening a large pore in the inner mitochondrial membrane that makes the mitochondria indiscriminately permeable to substances with a molecular weight less than approximately 1500 Dalton. Pore opening causes equilibration of ions and other solutes across the inner mitochondrial membrane, breakdown of the electrochemical potential across the inner membrane and consequently cessation of ATP synthesis. In addition, it is envisaged that the mitochondrial permeability transition leads to the production of ROS, swelling of mitochondria, outer membrane rupture and ensuing release of proapoptotic factors to the cytosol, e.g. cytochrome c. The released proapoptotic factors activate different types of cytosolic caspases that, in turn, activate endonucleases producing DNA fragmentation [83,292]. In this way, mitochondria may serve as points of convergence for death stimuli and may initiate cell death pathways through the activation of the mitochondrial permeability transition pore. In vitro the mitochondrial permeability transition is induced by calcium uptake from the cytosol into mitochondria and by oxidative stress. MPT pore opening is facilitated by low levels of ATP and high levels inorganic phosphate [63]. These conditions prevail in focal ischemia followed by reperfusion which gives reasons to believe that reperfusion of the brain after a focal ischemic insults creates an microenvironment that is conducive for opening of the MPT pore. Direct demonstration of the mitochondrial permeability transition in the setting of focal ischemia has, however, not yet been produced but intriguing indirect evidence has been obtained showing that the immunosuppressant cyclosporin A and derivatives of this compound, which are considered virtually specific blockers of the mitochondrial permeability transition pore, significantly reduce infarct volume in rat models of transient MCAO [201,324,344-346]. Most impressive are the findings by Yoshimoto et al. that cyclosporin A administered as an intracarotid infusion after 5 minutes of reperfusion following 2 hours of transient MCAO reduced infarct volume by up to 90%. When administration of the compound was delayed until 3 hours, but not 6 hours, after initiation of reperfusion, the neuroprotective effect was attenuated but still significant. Collectively, these studies implicate a role for the mitochondrial permeability transition in the brain damage incurred by transient MCAO but it should also considered that the immunosuppressant cyclosporin A has other effects. Cyclosporin A is an inhibitor of calcineurin, a calcium-calmodulin-dependent protein phosphatase, which has many different substrates that, among others, encompass nitric oxide synthases, nuclear factor of activated T cells and the NMDA receptor. Furthermore, calcineurin is involved in regulation of the anti-apoptotic members of the Bcl-2 family (reviewed and discussed in [220] and [346]). These effects of cyclosporin A on calcineurin may also contribute to protection.

In conclusion, mitochondria related pathogenetic events are undoubtedly both involved and important in infarct pathogenesis. The exact mechanisms are, however, at present only incompletely understood and other as yet unidentified mechanism are probably involved.

**Cooperating pathogenetic mechanisms**

Excitotoxic mechanisms, cytosolic calcium overload, excessive ROS formation and probably also mitochondrial dysfunction seem to be intimately connected, and it is likely that these factors cooperate in infarct pathogenesis. Extracellular glutamate levels during MCAO in rats correlate positively with ROS formation during reperfusion indicating an association between glutamate release and formation of free radicals [219]. Nitric oxide is generated as a direct consequence of NMDA receptor stimulation via calcium mediated activation of neuronal nitric oxide synthase. Hence, the NMDA receptor may constitute a direct link between
excitotoxicity and oxygen radical formation although this issue continues to be controversial [152]. Furthermore, many of the other proposed intracellular sources of oxygen free radicals, e.g. phospholipase A2, xanthine oxidase and the mitochondrial permeability transition, are activated by an increased intracellular calcium concentration. In an MCAO study in rats, treatment with different antioxidants inhibited ischemia-induced glutamate release in the ischemic focus, increased the activity of mitochondrial electron transport complexes I-III, enhanced mitochondrial ATP synthesis in the ischemic cortex and reduced infarct size [139]. In our own study periinfarct depolarizations, whose propagation into perifocal areas is dependent on glutamate receptor activation, were blocked by treatment with a free radical scavenger [paper V]. It is therefore likely that glutamate receptor stimulation, intracellular calcium and ROS formation act in concert as mutually reinforcing pathogenetic events in focal ischemia/reperfusion.

CONCLUDING REMARKS AND OUTLOOK

Specific conclusions
The main results and specific conclusions of the seven studies presented in this thesis have already been stated in Part Two. They can be briefly reiterated as follows:

Paper I
- Expression of Fos protein in perinfarct tissue is a molecular marker of salvageable penumbral tissue 2 hours after permanent MCAO in rats.
- Expression of Fos protein in the infarct borderzone 2 hours after permanent MCAO was inhibited by treatment with the NMDA receptor antagonist MK-801 but not by treatment with the AMPA receptor antagonist NBQX.

Paper II
- Extensive inhibition of protein synthesis occurred in focally ischemic rat brain tissue. In the third hour after induction of permanent MCAO protein synthesis was completely inhibited in the ischemic focus. In the surrounding penumbra protein synthesis rates were reduced by approximately 50 %.
- MK-801 treatment, but not treatment with NBQX, mitigated inhibition of protein synthesis in the penumbra in the third hour following permanent MCAO.

Paper III
- A double-tracer autoradiographic method employing a washout procedure with trichloroacetic acid was devised and was used to study glucose consumption and protein synthesis in parallel cryosections of individual rat brains. The method allowed direct spatial comparison of tissue with reduced protein synthesis and tissue with disturbed glucose metabolism.
- In the acute phase after MCAO inhibition of protein synthesis is spatially more extensive than disturbances of glucose consumption, i.e. the volume of cortex with reduced protein synthesis is larger than the volume of cortex with aberrant glucose consumption. It was therefore possible to define a metabolic penumbra whose volume could be quantitated by subtracting the tissue volume with reduced glucose consumption from the tissue volume with reduced protein synthesis. By definition the metabolic penumbra had normal or increased glucose consumption indicating continuing metabolic capacity and hence a potential for recovery.
- The volume of the metabolic penumbra was not significantly affected by treatment with MK-801 and NBQX but a tendency to reduction of the volume was observed with MK-801 treatment.
Paper IV
- The opioid receptor agonist and NMDA receptor antagonist ketobemidone neither ameliorated the acute perturbations of protein synthesis and glucose metabolism nor reduced infarct volume 1 day after permanent MCAO in rats. It was concluded that ketobemidone is too weak an NMDA receptor antagonist to effectively interfere with the pathogenetic mechanisms involved in infarction.

Paper V
- The free radical scavenger α-PBN significantly reduced the number of transient periinfarct depolarizations and the total depolarization time during the first 3 hours after MCAO in rats. Despite the attenuation of periinfarct depolarizations α-PBN did not reduce infarct volume measured 7 days after MCAO indicating that periinfarct depolarizations per se are not critical determinants of the final infarct size as previously suggested [135]. With longer survival periods, i.e. one week or more, the beneficial effect of attenuation of periinfarct depolarizations, which has been demonstrated after 1 or 2 days survival, may be overrun by later occurring pathogenetic events.

Paper VI
- The activities of mitochondrial electron transport complexes I, II and IV were not reduced in the ischemic penumbra 2 hours after permanent MCAO in rats. Reduced activity of the investigated electron transport complexes were restricted to the most central parts of ischemic focus which already had signs of irreversible morphological damage. These findings therefore do not support a critical pathogenetic role for inhibition of mitochondrial electron transport complexes in the evolution of experimental brain infarcts.

Paper VII
- Pinokalant (LOE 908 MS), a broad-spectrum blocker of several types of cation channels, given as an intravenous infusion of approximately 24 hours duration starting 30 min after MCAO in rats, significantly reduced infarct size measured one week after MCAO. Considering the multiple pathways by which detrimental increases in the intracellular sodium and calcium concentration may take place in the acute phase of focal cerebral ischemia, the study indicates that wide-spectrum inhibition of cation fluxes is a rational pharmacological principle to pursue in future neuroprotection studies.

General conclusions
The pathogenesis of brain damage incurred by experimental focal ischemia is multifactorial in the sense that many different molecular mechanisms and biochemical pathways are involved. Following arterial occlusion a highly complex sequence of pathophysiological and pathogenetic events is initiated eventually leading to irreversible brain damage. The different pathogenetic mechanisms involved seem to be interrelated in a characteristic temporal pattern as indicated in fig. 16. The work presented in this thesis has dealt with pathogenetic mechanisms which are important in the acute phase of focal ischemia, i.e. the first up to approximately 6 hours after arterial occlusion. In the penumbra disturbed calcium homeostasis subsequent to release of excitatory amino acids and waves of periinfarct depolarizations, oxidative stress, persistent inhibition of protein synthesis and specific gene expression all contribute to recruitment of the penumbra to infarct and hence to the extent and severity of the final brain damage.

As illustrated in fig. 16 the acute phase is followed by a secondary phase lasting from days to weeks in which inflammation and apoptosis are important for the development of delayed damage within the ischemic penumbra.
penumbra [75,355]. The early excitotoxic events, encompassing glutamate receptor activation, increased cytosolic calcium levels and formation of reactive oxygen species, activate intracellular signalling pathways which result in expression of a number of proinflammatory genes via activation of different transcription factors, e.g. nuclear transcription factor, kappa B (NFkB) (reviewed in [355]). This, in turn, leads to expression of mediators of inflammation such as adhesion molecules [328,349] and cyto- and chemokines (e.g. interleukin-1 (IL-1) and tumor necrosis factor (TNF-α)). In addition, inducible cyclo-oxygenase (COX-2) and inducible and neuronal nitric oxide synthases are expressed [133,193,241,343]. Activation of microglia during the inflammatory phase may contribute to the production neurotoxic substances, including NO and other free oxygen radicals [176,303]. Attenuation of the inflammatory response in different ways have in many studies resulted in amelioration of focal ischemic brain damage illustrating a deleterious role for inflammation in stroke models (see for example [27,46,67,117,190,222,351,353]). It is speculated that post-ischemic inflammation may contribute to ischemic damage through neuronal and glial production of toxic substances such as nitric oxide, TNF-α and superoxide generated via the COX-2 pathway [75,355]. Whereas cells in the ischemic focus predominantly die by necrosis in the acute phase, some cells in the penumbra may, especially if the ischemic insult is transient or mild, succumb by apoptosis, or programmed cell death, following a time course similar to that of inflammation [52,182]. Blocking apoptotic cell death pathways by e.g. pharmacological inhibition of caspases, a family of cysteine proteases centrally involved in execution of apoptosis, have resulted in reduction of infarct size in rodent models of focal ischemia [83,84,121,221].

**Outlook and unresolved questions**

Experimental models of focal cerebral ischemia have greatly augmented our knowledge of the pathophysiology and pathogenetic events in the ischemic penumbra. Ample evidence have now been obtained in PET scanning and magnetic resonance imaging studies that an ischemic penumbra with pathophysiological similarities to the penumbra in the experimental ischemia models also exists in humans suffering a stroke (for reviews see e.g. [21,116,128]). This observation is encouraging and should stimulate further basic and clinical research within the area of cerebral ischemia despite the disappointing fact that most compounds that reduce the stroke lesion in experimental studies have failed in clinical trials (examples given in
There are many reasons for this (discussed in [110] and [5]) but obviously one is the complexity of the many pathogenetic mechanisms involved and their temporal relationship which must also be taken into consideration. Therefore, in terms of future neuroprotective strategies, both in the experimental setting and predictably also in the clinical setting, the sequence of pathogenetic events outlined above warrants design of experimental protocols that combine several types of drugs that each interferes with the pathogenetic factors relevant at a given time following onset of ischemia. One could therefore predict a beneficial effect of administration of one or more antieexcitotoxic compounds immediately after onset of arterial occlusion. Such early antieexcitotoxic treatment with for example glutamate receptor antagonists, broad-spectrum cation blockers and/or scavengers of free radicals may be envisaged to extend the therapeutic time window for reperfusion. This is an important therapeutic goal in the clinical setting because thrombolysis with recombinant plasminogen activator, the only treatment which to date has been proven efficacious in human stroke, must be performed within a narrow 3 hours time window following first onset of symptoms in order to have a beneficial effect on stroke outcome [273]. A free radical scavenger may need to be administered as early as possible after arterial occlusion as well as at the time of reperfusion since formation of reactive oxygen species may particularly occur in tissue with incomplete ischemia such as the penumbra [219,302] and when tissue is reoxygenated following ischemia [60,302]. Subsequent administration of antiinflammatory and antiapoptotic compounds during the following hours to days after the initial insult may be foreseen to convey further neuroprotection. It should be emphasized, however, that experimental evidence for a beneficial effect of such a sequential multiple drug treatment strategy still remain to be produced. Several other issues remain unresolved. The pathogenetic significance of the hundreds of gene regulations at the transcriptional level observed using molecular biology techniques in rodent models of experimental focal ischemia is for example not known at present. The emerging research field of proteomics which allows study of multiple simultaneous protein regulations, i.e. gene expression at the translational level rather than gene expression at the mRNA level, may provide meaningful answers to this [155]. Furthermore, as mentioned earlier, mitochondrial dysfunction obviously plays a central pathogenetic role in ischemic cell death, but the exact mechanisms are presently poorly understood and need further characterization. The role of glial cells in the pathogenetic cascade leading to infarction has also only been sparsely investigated in animal models of focal ischemia. Astrocytes have critical roles in regulation of ionic homeostasis and extracellular glutamate levels. It has recently been shown in mice that astrocytes may participate in regulation of local cerebral blood flow and may be critically involved in coupling of metabolism and blood flow under physiological circumstances [314]. Astrocytes also participate in propagation of cortical spreading depression via astrocytic gap junctions [9,233], and may therefore also be involved in spread of perinfarct depolarizations in the ischemic penumbra. In addition, reactive astrocytes produce various growth factors and mediators of inflammation [264] and are responsible for the reactive gliosis that develops around a brain infarct (see fig. 6). At least for these reasons the role astrocytes in focal ischemic brain damage deserves more focus in future experimental studies. Lastly, it should also be mentioned that previous experimental studies have mainly concentrated on neuronal mechanisms of ischemic damage in gray matter. Very little is therefore known about the mechanisms involved in ischemic damage to white matter and oligodendrocytes.
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iskæmiske fokus og reduceret med ca. 50% i penumbra i den tredje time efter start af MCAO. Effekten på proteinsyntesen af blokade af ionotrope NMDA og AMPA glutamatreceptorer undersøges ved behandling med henholdsvis MK-801 og NBQX, og det vistes i et af studierne, at MK-801, men ikke NBQX, signifikant forbedrede proteinsyntesen i penumbra. En dobbelttracer autoradiografisk metode blev udviklet og benyttet i to arbejder til korrelerere forstyrrelserne i proteinsyntese og glukoseforbrug i nabolset fra samme rottehjerne. Det demonstreredes, at hæmning af proteinsyntesen i den iskæmiske hemisfære var spatialt mere udbredt end forstyrrelser i glukoseforbruget, idet det kunne vises, at det kortikale volumen, hvor proteinsyntesen var hæmmet, var signifikant større end det kortikale volumen med forstyrret glukoseforbrug. På grundlag af denne forskel kunne en metabolisk penumbra defineres og volumen af denne kunne kvantiteres. Der var ingen statistisk signifikant effekt af behandling med glutamatreceptorantagonisterne MK-801 og NBQX på volumen af den metaboliske penumbra omend MK-801 tenderede til at reducere denne. I det andet dobbelttracer autoradiografiske arbejde fandt vi, at behandling med ketobemidon, en opioid agonist med antagonistvirkning på NMDA-receptorer, ikke påvirkede den metaboliske penumbra eller det histologiske infarktvolumen sammenlignet med saltvandsbehandlede kontrolrotter. I et immunhistokemisk studie undersøges udtrykket af Fos protein, genproduktet af immediate-early genet c-fos som aktiveres af stigninger i den intracellulære calcium-koncentration, samt effekten af blokade af ionotrope NMDA og AMPA glutamatreceptorer ved behandling med henholdsvis MK-801 og NBQX. Det vistes, at Fos protein udtrykkes i penumbra to timer efter MCAO, og at Fos protein udtrykket kan hæmmes ved behandling med NMDA glutamatreceptorantagonisten MK-801 men ikke med AMPA receptorantagonisten NBQX. I et enzymhistokemisk arbejde undersøges aktiviteten af mitokondriernes elektrontransportkomplekser I, II og IV under og i de første timer efter 2 timers reversibel MCAO. Elektrontransportaktiviten var udelukkende nedsat i det iskæmiske fokus i væv med tidlige morologiske tegn på irreversibel skade, mens aktiviteten i penumbra var uændret. En selektiv hæmning af elektrontransportkomplekserne syntes således ikke at være årsag til mitokondrie-dysfunktion i penumbra. Transiente depolarisationer i penumbra menes at bidrage til infarktudvidelsen. Med mikroselektroder placeret i penumbra detekteredes disse transiente periinfarktdepolarisationer i de første timer efter MCAO, og det vistes, at behandling mediltradikal scavengeren α-PBN reducerede antallet og varigheden af periinfarktdepolarisationerne. På trods heraf var infarktvolumina ikke mindre hos behandlede rotter sammenlignet med kontrolrotter efter en uges overlevelse, hvilket tyder på, at periinfarktdepolarisationer ikke alene er bestemmende for den endelige infarktstørrelse. Endelig vistes det i det sidste arbejde, at behandling med pinokalant, en ny bredspektret blokker af flere typer kationkanaler, resulterede i en signifikant og vedvarende reduktion i de histologiske infarktvolumina udmålt en uge efter permanent MCAO. Det vistes at denne effekt ikke kunne tilskrives pinokalant-induceret hypotermi eller forskelle i systemiske arterielle blodtryk mellem pinokalantbehandlede rotter og kontrolrotter. I den sammenfattende redefgørelse diskuteres disse fund i relation til resultater opnået i andres studier.
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