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JOHANNA KUHMONEN

Neuroprotection in Experimental Acute Cerebral Ischaemia:

α_2-adrenoreceptor agonism, MAO-B inhibition, and enhancement of GABAergic neurotransmission as neuroprotective strategies

Doctoral dissertation

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Department of Neurology, University of Kuopio
Department of Neurology, Kuopio University Hospital

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Distributor: Department of Neurology
University of Kuopio
FIN-70211 KUOPIO
FINLAND
Tel. +358 17 162 682
Fax +358 17 162 048

Author's address: Department of Neurosurgery
Helsinki University Central Hospital
Topeliuksenkatu 5
FIN-00260 Helsinki
FINLAND
Tel. +358 50 427 0384
Fax +358 9 471 87719

Supervisors: Professor Juhani Sivenius, M.D., Ph.D.
Department of Neurology
University of Kuopio

Professor Risto Kauppinen, M.D., Ph.D.
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio

Reviewers: Professor Dale Corbett, M.D., Ph.D.
Basic Medical Sciences
Faculty of Medicine
Memorial University
CANADA

Docent Turgut Tatlisumak, M.D., Ph.D.
Deparment of Neurology
University of Helsinki

Opponent: Docent Aarne Ylinen, M.D., Ph.D.
Department of Neurology
University of Tampere

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ABSTRACT

Cerebral ischaemia is a major neurological problem that still lacks an effective treatment. The purpose of this series of studies was to investigate the neuroprotective efficacy of different pharmacological treatment options in experimental global and focal cerebral ischaemia. One of the initial responses in the central nervous system after cerebral ischaemia is the massive release of noradrenaline and other excitatory neurotransmitters. Although the role of catecholamines in ischaemic damage is still unclear, in some experimental models, α_2-adrenergic agonism has proved to be neuroprotective. We tested the efficacy of α_2-adrenoreceptor agonist, dexmedetomidine, in two ischaemia animal models the gerbil global and the rat transient and permanent focal cerebral ischaemia models. Another approach to neuroprotection was explored using the MAO-B inhibitor, selegiline, in the gerbil global cerebral ischaemia model. Selegiline has previously been shown to have also MAO-B inhibition independent potentially neuroprotective efficacy against ischaemia-induced injury. By using different doses and drug administration schedules, the possibilities of free radical scavenging, long-lasting MAO-B inhibition and acute free radical reduction, as well as neuronal rescuing as the underlying salvaging mechanisms of selegiline were explored. Finally, the possibility of counteracting the massive excitatory response to cerebral ischaemia was investigated in the rat transient focal cerebral ischaemia model. Inhibitory GABAergic activity was enhanced using diazepam.

The main results were as follows: (1) Dexmedetomidine, an α_2-adrenoreceptor agonist, may have neuroprotective efficacy in global cerebral ischaemia, since it ameliorated neuronal damage in the hippocampal CA3 region and hilus of the dentate gyrus when the administration was started before occlusion and extended up to 48 h postischaemia. (2) After transient and permanent focal cerebral ischaemia, dexmedetomidine did not reduce infarct volumes, after permanent MCAO it, on the contrary, increased the damage. Dexmedetomidine, according to previous studies, and the present data, exerts several symptomatic effects that might be desirable in the treatment of stroke patients, including, haemodynamic stabilation, attenuation of cerebral oedema via enhanced diuresis, and sedation. (3) Selegiline caused only a non-significant trend towards attenuated neuronal damage in global cerebral ischaemia in all treatment groups. (4) Diazepam failed to protect against transient focal cerebral ischaemia when normothermia was carefully maintained.

Taken together, these present studies provide new information concerning neuroprotection in experimental acute cerebral ischaemia. None of the tested agents was clearly protective, although with dexmedetomidine indications for neuroprotectivity in the global cerebral ischaemia, and with selegiline a non-significant trend towards attenuated neuronal damage were detected.

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Medical Subject Headings: brain ischemia; brain infarction; magnetic resonance imaging; cerebrovascular accident; ischemic attack, transient; hippocampus; neuroprotective agents; selegiline; adrenergic alpha-agonists; gamma-aminobutyric acid; animal; rats; gerbillinae

Alles Gescheite ist schon gedacht worden,

man muß nur versuchen es noch einmal zu denken.

Eigentlich weiß man nur wenn man wenig weiß;

mit dem Wissen wächst der Zweifel.

Johann Wolfgang Goethe

To My Parents

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Helsinki, June 2003

Johanna Kuhmonen

ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ATP	adenosine triphosphate
BBB	blood-brain barrier
CBF	cerebral blood flow
CCA	common carotid artery
CCAO	common carotid artery occlusion
CNS	central nervous system
D_{AV}	the trace of the diffusion tensor
DNA	deoxyribonucleic acid
EAA	excitatory amino acid
ECA	external carotid artery
FOV	field of view
GABA	γ-aminobutyric acid
ICA	internal carotid artery
ICAM-1	intercellular adhesion molecule-1
IEG	immediate early gene
IP_3	inositol 1,4,5-triphosphate
HSP	heat shock proteins
MAO	monoamine oxidase
MAP	mean arterial blood pressure
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MRI	magnetic resonance imaging
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
$PaCO_2/O_2$	partial arterial pressure of carbon dioxide/oxygen
ROS	reactive oxygen species
SOD	superoxide dismutase
SPSS/PC+	statistical package for social sciences/personal computer
T_2	spin-spin relaxation time
TE	echo time
TR	repetition time
TTC	2,3,5-triphenyltetrazolium chloride

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications that are referred to in the text by the Roman numerals I-IV.

I Kuhmonen J, Pokorný J, Miettinen R, Haapalinna A, Jolkkonen J, Riekkinen P Sr, Sivenius J: Neuroprotective effects of dexmedetomidine in the gerbil hippocampus after transient global ischemia. *Anesthesiology, 87: 371-377, 1997.*

II Kuhmonen J, Haapalinna A, Sivenius J: Effects of dexmedetomidine after transient and permanent occlusion of middle cerebral artery in the rat. *Journal of Neural Transmission, 108: 261-271, 2001.*

III Kuhmonen J, Jolkkonen J, Haapalinna A, Sivenius J: The neuroprotective effects of (-)deprenyl in the gerbil hippocampus following transient global ischemia. *Journal of Neural Transmission, 107: 779-786, 2000.*

IV Kuhmonen J, Lukkarinen J, Gröhn O, Jolkkonen J, Sivenius J: Diazepam does not reduce infarct size in rats subjected to transient occlusion of the middle cerebral artery when normothermia is maintained. *Journal of Pharmacy and Pharmacology, 54: 1565-1569, 2002.*

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1. INTRODUCTION

Stroke is a serious public health problem and one of the leading causes of death and disability worldwide. It is defined (WHO, 1988) as "the rapid development of clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 h or longer or which leads to death, with no apparent cause other than of vascular origin". The incidence of ischaemic stroke is approximately 75 - 80% of all stroke cases, the remaining 20 - 25% being haemorrhagic, i.e. subarachnoidal or intracerebral haemorrhage. In Finland, ischaemic stroke is the third most common cause of death (The World Bank, 1993). About 20% of the ischaemic stroke patients die, either during the acute phase or within a few weeks time. Furthermore, the mental and physical handicaps among the patients surviving stroke are major problems in terms of quality of life and socioeconomic costs. While considerable progress has been achieved in secondary prevention (antithrombotic treatment, carotid endarterectomy, management of risk factors such as arterial hypertension, diabetes, lipid disorders, etc.) acute stroke therapies are limited to thrombolysis with rTPA (recombinant tissue plasminogen activator) within 3 hours of symptom onset, and only a fraction of ischaemic stroke patients (10 - 15%) are suitable for thrombolytic therapy. Futhermore, despite decades of intensive research and investigation of numerous different approaches, an adequate, effective, and safe neuroprotective therapy remains to be discovered.

Clinically ischaemic stroke is an extensively variable state regarding location, severity, symptoms, etiology, infarct size, and confounding factors. Although experimental ischaemia models usually do not reflect this state of affairs, models are neccessary if we are to explore various therapeutic strategies. Cerebral ischaemia models are commonly categorized into global and focal models, simulating cardiac arrest or severe hypotension and cerebral infarction, respectively. Global ischaemia results in degeneration of only selectively susceptible neuron populations (Brierley, 1976; Pulsinelli et al., 1982a), and the damage develops, after a latent period, slowly over 2 to 4 days (delayed neuronal death) (Kirino, 1982; Pulsinelli et al., 1982). Focal cerebral ischaemia, on the other hand, is characterized by a spatially heterogenous reduction of cerebral blood flow (Nagasawa and Kogure, 1989) resulting in an ischaemic core where the neuronal damage is most dense and pan-necrosis takes place, and a penumbral area where neurons are compromised but still viable (Symon et al., 1977). As in the selectively vulnerable areas of global ischaemia, also in the penumbra

neuronal damage develops slowly in few days of time (Pulsinelli et al., 1982a; Hossmann, 1994).

The fact that ischaemia-induced neuronal degeneration is partly delayed, and evidently a result of a complex process rather than a simple instantaneous incident, offers a window of opportunity for therapeutical interventions in both types of cerebral ischaemia. The initial event in the neuronal damage process in severe ischaemia is the rapid exhaustion of high-energy phosphate compounds followed by impaired intracellular homeostasis, especially accumulation of calcium ions intracellularly (Siesjö and Bengtsson, 1989), and a massive release of excitatory neurotransmitters, such as glutamate (Benveniste et al., 1984). Ionic influx is accompanied by cellular swelling and cytotoxic oedema (Baethmann, 1978). In conjunction with reperfusion, further potentially damaging processes follow, including oxygen free radical formation (Siesjö et al., 1989), the inflammatory response with vasogenic oedema and production of cytotoxic nitric oxide (Nowak et al., 1990; Kato and Kogure, 1999), activation of glial cells (Wood, 1995), and altered protein synthesis and gene expression (Thilmann et al., 1986; Abe et al., 1991; Li et al., 1992; 1994; Liu et al., 1993).

To date, the most promising neuroprotective agents in different experimental ischaemia studies, glutamate antagonists, have been somewhat disappointing in clinical trials because of their severe side-effects (Grotta, 1995, Albers et al., 1995; Turrini, 1996; Hickenbottom and Grotta, 1998). The purpose of this thesis was to explore neuroprotective efficacy of pharmacologically well defined and clinically usable agents. In the present work, three different pharmacological approaches to neuroprotection were investigated. We tested the efficacy of the α_2-adrenoreceptor agonist, dexmedetomidine, in the global as well as transient and permanent focal cerebral ischaemia models; MAO-B inhibitor, selegiline, in the global ischaemia model; and GABAergic neurotransmission enhancer, diazepam, in the transient focal cerebral ischaemia model. They all have been previously demonstrated to have neuroprotective efficacy against ischaemia-induced injury in some experimental models, but at the same time, also clearly contradictory results have been reported.

2. REVIEW OF LITERATURE

2.1. Cerebral ischaemia

2.1.1. Forms and models of cerebral ischaemia

Cerebral ischaemia is clinically an extremely variable state. The cause, location, severity and reversibility of stroke vary, and the patients are primarily middle-aged or older people who often have other pre-existing diseases. Animal models of cerebral ischaemia, in contrast, try to eliminate or control all of these variables, and aim to produce a relatively constant neuronal damage in young, and usually otherwise healthy, animals. Although clinical stroke can not completely be simulated in animal models, these experimental models are still the best existing means available with which to evaluate various therapeutic strategies.

Global cerebral ischaemia. Cerebral ischaemia models are commonly categorized into global and focal models. Global cerebral ischaemia is considered to mimic cardiac arrest or severe hypotension, situations where the whole brain, or a large proportion of it (usually the forebrain), is affected by ischaemia and only certain selectively susceptible neuron populations are destroyed, including hippocampal pyramidal cells, neocortical neurons in layers 3, 5 and 6, the medium-sized striatal neurons, and cerebellar Purkinje cells (Brierley, 1976; Pulsinelli et al., 1982a). In the rodent dorsal hippocampus, a transient global ischaemia of a short period produces a well-described pattern of selective vulnerability with damage to the CA1 region and to polymorphic cells of CA4/hilus region, while sparing the CA3 and the dentate gyrus (Kirino, 1982; Jorgensen and Diemer, 1982; Kirino and Sano, 1984a; Smith et al., 1984a; Blomqvist and Wieloch, 1985)(Figure 1). This kind of ischaemic injury develops slowly after a latent period, and is therefore known as the "maturation phenomenon" (Ito et al., 1975) or "delayed neuronal death" (Kirino,1982; Pulsinelli et al., 1982). Morphological signs of irreversible cell damage can not be detected until 2 - 4 days postischaemia (Kirino and Sano, 1984b), but ultrastructural defects (polyribosome disaggregation, proliferation of smooth endoplasmic reticulum and deposits of dense material close to the cell surface membrane) are evident after 24 h of reperfusion (Kirino and Sano, 1984a). Futhermore, the postischaemic histopathology observed in the rodent hippocampus is not just a characteristic of animal models, similar delayed (Petito et al., 1987) changes have been detected in the human hippocampal CA1 after cardiac arrest (Zola-Morgan et al., 1986; Petito et al., 1987).

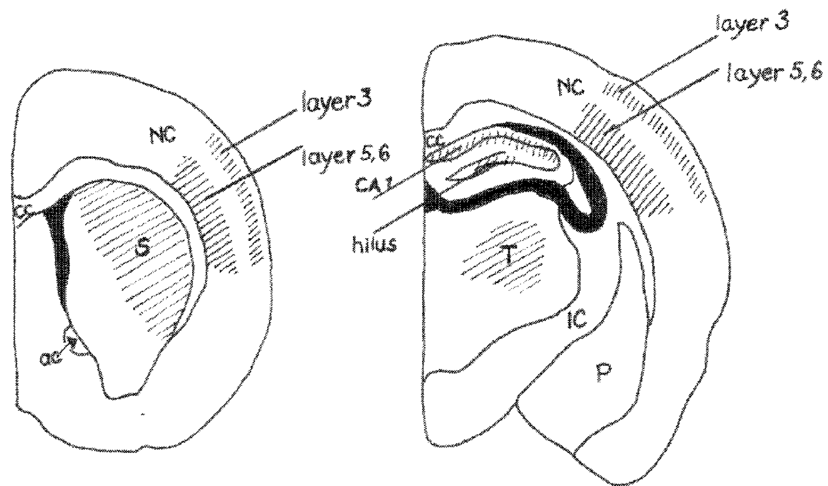


Figure 1. The selectively vulnerable brain regions after brief global cerebral ischaemia. Coronal sections of the rat brain at the level of striatum (caudate nucleus) and anterior hippocampus. NC = neocortex; S = striatum; P = putamen; T = thalamus; IC = internal capsule; cc = corpus callosum; ac = anterior commissure. Hatched areas represent regions with ischaemic neuronal damage (the cortical layers 3, 5 and 6; and the hippocampal CA1 and hilus regions), and black areas the lateral ventricles.

Global ischaemia in experimental models is produced by occluding the arteries supplying the brain, i.e. common carotid (CCA) and vertebral arteries, and in some models, occlusion is combined with hypotension. The gerbil (*Meriones unguiculatus*) bilateral common carotid artery occlusion (CCAO) model (Ito et al., 1975) is perhaps the most widely used global ischaemia model because of the simplicity of surgery involved. Gerbils have no communication between carotid and vertebral arteries (an incomplete circle of Willis), and therefore, forebrain ischaemia can be achieved by mere CCAO. The hippocampus is supplied by the first division of the internal carotid artery, anterior choroid artery, both in the rat as well as the gerbil brain. The rat models, including two (Smith et al., 1984b) and four vessel occlusion (Pulsinelli and Brierley, 1979) models, are more complex, the former requiring hypotension and the latter involving two-staged surgery. The main problem with all these global models is their significant variability in outcome (Pulsinelli and Brierley, 1979; Pulsinelli et al., 1982a; 1982b; Blomqvist et al., 1984; Smith et al., 1984; Crain et al., 1988;

Schmidt-Kastner and Hossmann, 1988; Schmidt-Kastner et al., 1989), mostly due to variations in the cerebral circulation.

Focal cerebral ischaemia. Focal cerebral ischaemia results from a complete occlusion of a cerebral artery. Embolic occlusion of proximal middle cerebral artery (MCA) is clinically one of the most common forms of stroke (Mohr et al., 1986; Karpiak et al., 1989), and also the majority of focal models simulate it. Both transient and permanent MCA occlusion (MCAO) models are widely used. Transient MCAO is probably closer to clinical stroke than permanent MCAO since spontaneous reperfusion has been demonstrated with repeated angiography in up to 50% of stroke patients (Saito et al., 1987). On the other hand, both MCAO models are valuable tools in studying different aspects of the neurodegenerative process: a permanent model to study the consequences of ischaemic ischaemia, and a transient model to study the consequences of ischaemia together with reperfusion.

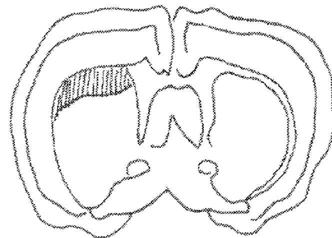
MCAO can be induced by techniques which include cauterization, clips and intraluminal threads, or using thromboembolic methods: photochemically induced lesion, injection of microspheres or autologous or human clotted blood (see Ginsberg and Busto, 1989 for review). All of these methods have certain disadvantages. Producing MCAO by cauterization or using a clip, requires craniectomy, which in turn may influence intracranial pressure, blood-brain barrier permeability and brain temperature (Hudgins et al., 1970; Olessen, 1987). The intraluminal thread method (Koizumi et al., 1986; Longa et al., 1989) is minimally invasive but subarachnoid haemorrhage, insufficient MCAO, thrombus formation, and intra and postischaemic hyperthermia may cause interanimal variability (Longa et al., 1989; Nagasawa and Kogure, 1989; Bederson et al., 1995; Memezawa et al., 1992; 1995; Zhao et al., 1994a; Schmid-Elsaesser et al., 1998). In photochemically induced infarction (Watson et al., 1985), damage develops rapidly and lacks completely the penumbra. Furthermore, collateral blood flow as well as reperfusion are not possible in this model. Other thromboembolic methods produce infarctions which do not control the size or the site of the lesion (Kogure et al., 1974; Kudo et al., 1982).

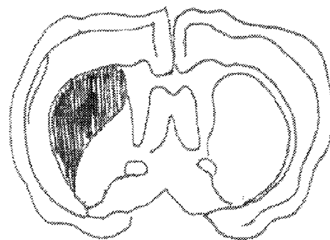
Many animal species have been used in stroke studies, but rats have been most popular reflecting costs and simplicity of experimentation. Also, the circulation has been suggested to be similar in rat and human cranium, especially, when compared to gerbil, dog and cat

cranium. On the other hand, variations in the collateral blood supply may be a problem when using rats (Yamori et al., 1976).

Focal cerebral ischaemia is characterised by spatially heterogenous reduction of cerebral blood flow; gradually reduced from the periphery to the core region of the affected vascular region (Nagasawa and Kogure, 1989). The neuronal damage is most intense in the ischaemic core where pan-necrosis, i.e. damage to neurons, glia and often endothelial cells, takes place. In the moderately perfused region between the core and normally perfused areas, often referred to as the penumbra, the neurons are compromised but still viable (Symon et al., 1977). This penumbral tissue is at risk because of the depolarization waves (spreading depression) emanating from the ischemic core and the depletion of ATP by increasing the activity of $Na^{+}K^{+}$- ATPase. In a tissue where blood flow is reduced, depletion of ATP crucially weakens the survival chances of the neurons. In the rat MCAO model, the ischaemic core is located in the basal ganglia and the penumbra in the parietal cortex (Nagasawa and Kogure, 1989). Figure 2 shows ischaemic core and penumbra in the rat brain resulting from various durations of MCAO.

In focal cerebral ischaemia due to MCAO, perfusion of penumbral tissue is dependent on the degree of collateral circulation from the anterior and the posterior cerebral arteries. Critical threshold values of cerebral blood flow (CBF) have been proposed for the break-down of different brain functions (Hossmann, 1994). Normal CBF rate in the human brain is about 50 ml/100 g/min (at normal cerebral perfusion pressures of 70 - 100 mmHg). Synaptic function ceases when CBF declines below 16 - 20 ml/100 g/min (electrical failure). This was first observed in connection with carotid endarterectomy, but similar CBF rates have been detected, for instance, in the baboon and the cat MCAO. Although silent electrically, the cerebral tissue in the penumbra remains viable and the ion pump mechanism functional until CBF falls below 6 - 10 ml/100 g/ min (membrane failure) (see Astrup, 1982 for review).





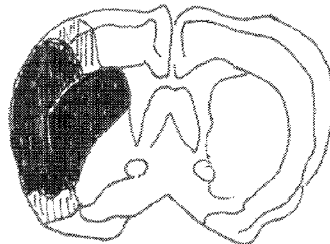


Figure 2. Schematic drawing of the coronal sections of the rat brain after different durations of middle cerebral artery occlusion. Hatched area represents the penumbra and black area the ischaemic core.

2.1.2. Pathogenesis of cerebral ischaemia

The severity of brain damage and the rate of its progression depend on the density and duration of ischaemia. A brief, transient ischaemia causes only selective neuronal damage, long, severe ischaemia, on the other hand, evokes pan-necrosis and infarction. In contrast to the almost immediate necrosis occurring in the ischemic core of focal ischaemia, in penumbra as well as in selectively vulnerable areas of global ischaemia, neuronal damage develops slowly in a few days time (Pulsinelli et al., 1982a; Hossmann 1994). Apoptosis has been proposed to be the probable mechanism for this delayed neuronal damage (Choi, 1996).

2.1.2.1. Intraischaemic changes

In severe ischaemia, when the tissues are deprived of oxygen and glucose, the initial event in neuronal damage process is the rapid exhaustion of high-energy phosphate compounds, first ATP and, soon later, its storage form, phosphocreatine. Anaerobic glucolysis leads to increased lactate production and acidosis. Furthermore, the lack of ATP crucially perturbs the maintenance of intracellular homeostasis. ATP-dependent ion pumps (Na^+K^+-ATPase) are inhibited and this compromises the transmembrane concentration gradients, resulting in membrane depolarization. Intracellular potassium leaks out of cells and extracellular sodium accumulates intracellularly (Hossmann, 1976). Depolarization, in turn, causes activation of voltage-sensitive calcium channels and Ca^{2+} influx (Siesjö and Bengtsson, 1989). Additionally, ionic influx is accompanied by water influx, resulting in intracellular swelling of neurons, astrocytes and endothelial cells, a phenomenon called cytotoxic oedema (Baethmann, 1978).

Another key event in the ischaemic cascade, along with the calcium influx, is the excessive release of excitatory amino acids (EAAs), especially glutamate (Benveniste et al., 1984). The intracellular accumulation of Ca^{2+} stimulates glutamate release from presynaptic terminals and astroglial storage pools, while at the same time, the ATP-dependent glutamate uptake mechanisms and clearance from the extracellular space are disturbed (Mitani et al., 1994). Glutamate activates ionotropic N-methyl-D-aspartate (NMDA) and non-NMDA receptors as well as metabotropic receptors. First, Na^+ influx through non-NMDA channels depolarizes the cell and relieves the voltage-dependent Mg^{2+} block from the NMDA channels resulting in a

massive Ca^{2+} influx (Siesjö and Bengtsson, 1989). Activation of metabotropic receptor, linked by G proteins to phospholipase C, leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate into two second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 binds to its receptor on the endoplasmic reticulum promoting intracellular Ca^{2+} mobilization and a further increase in the intracellular calcium concentration (Schoepp and Conn, 1993). Excitable cells, such as neurons undergo also an additional intracellular Ca^{2+} release via activation of ryanodine receptors, which are located apparently in a different location from the IP_3 receptors and which have a different temporal release profile (Walton et al., 1991; Sharp et al., 1993). Accumulation of intracellular calcium results in activation of several Ca^{2+}-stimulated enzymes, i.e. proteases, lipases, nucleases etc., which are subsequently responsible for the destruction of cellular integrity.

2.1.2.2. Postischaemic changes

Early reperfusion can restore energy levels almost completely (Pulsinelli and Duffy, 1983) and salvage penumbral tissue (Memezawa et al., 1992; Aronowski et al., 1994). On the other hand, reperfusion has been proposed to provoke additional damage by excessive generation of free radicals, neutrophil and monocyte/macrophage accumulation and microvascular plugging (Zhang et al., 1994; Matsuo et al., 1995). An ischaemic episode is also followed by activation of glial cells and changes in protein synthesis and gene expression.

Oxygen free radicals. Reactive oxygen species (ROS), i.e. the superoxide and peroxynitrite anions, hydrogen peroxide and the hydroxyl radical are highly reactive molecules that are produced under physiologic conditions but also have an important role in the ischaemia-induced damage process causing peroxidation of membrane lipids and damage to DNA and proteins (Siesjö et al., 1989). During reperfusion, the antioxidative defense mechanisms (e.g. superoxide dismutase; SOD, glutathione peroxidase and catalase) are likely to be overwhelmed because of overproduction of ROS, impairment of detoxification systems and consumption of antioxidants (Siesjö et al., 1989; Chan, 1998). In particular, the massive release of glutamate, impairs glutathione production, and thus free radical inactivation, by neurons (Choi, 1990).

The superoxide radical, which is the most important and most abundant radical in ischaemic tissue, has several sources. These include arachidonic acid release by activated phospholipases and its subsequent metabolism, oxidation of amine neurotransmitters (enzymatic and auto-oxidation), conversion of xanthine dehydrogenase to xanthine oxidase, as well as mitochondrial production and leakage, both of which are caused by excessive intracellular Ca^{2+}, and activation of microglia and infiltrating neutrophils and macrophages (Hall, 1997). Superoxide undergoes spontaneous dismutation to hydrogen peroxide, a reaction strongly accelerated by SOD. During hypoxia/ischaemia, progressive intracellular acidosis causes release of the organic bound iron. Free iron in turn catalyzes the conversion of hydrogen peroxide to the hydroxyl radical (Fenton's reaction), the most powerful ROS (Gutteridge, 1994). Another pathway to the hydroxyl radical formation is combination of superoxide and nitric oxide (NO) into the peroxynitrite radical (Ischiropoulos et al., 1992). Hydrogen peroxide production, on the other hand, may be contributed by ischaemia-induced release of dopamine and its metabolism by monoamine oxidase B (MAO-B) (Ter Horst and Postige, 1997).

Inflammatory response. Inflammation with vasogenic oedema, attributed to endothelial cell damage by ROS and subsequent blood-brain barrier (BBB) breakdown, is also associated with impaired reperfusion (Nowak et al., 1990). Exogenous inflammatory response includes infiltration and accumulation of neutrophils (polymorphonuclear leucocytes) and monocytes/macrophages from the blood circulation into the ischaemic tissue. Neutrophils arrive in the first wave, 12 - 24 h after the insult, and monocytes/macrophages 2 -3 days after the insult (Kochanek and Hallenbeck, 1992). Migration of inflammatory cells is not only a repair process but may also induce secondary damage in the potentially viable tissue by capillary plugging or vasoconstrictor release, as well as via release of protease, free radicals and lipid-derived mediators. Additionally, neutrophils induce nitric oxide synthase (NOS) and this evokes an increased production of cytotoxic NO (Kato and Kogure, 1999).

A critical step in the neutrophil migration is adhesion to endothelial cells (Furie et al., 1987). It is regulated partially by interactions between intercellular adhesion molecule-1 (ICAM-1) on endothelial cells and a group of CD11/CD18 glycoproteins on leucocytes (Smith et al., 1989). ICAM-1 expression in endothelial cells is upregulated after focal cerebral ischaemia (Okada et al., 1994). This is thought to be caused by inflammation mediators, cytokines,

produced in the brain, such as tumor necrosis factor-α, interleukin-1b and interleukin-8 - all powerful upregulators of adhesion molecules.

Activation of glial cells. Glial cells are the most abundant cells in the CNS and their interactions with neurons are thought to influence the outcome of the ischaemic insult. Under physiologic conditions, astrocytes have an important role in maintaining the local microenvironment, including rapid uptake and inactivation of released neurotransmitters, for example glutamate, contribution to the upkeep of the BBB and maintenance of water and ionic homeostasis (Abbott et al., 1992). After ischaemia, astrocytes acutely disappear from the core of the infarct. However, in the penumbra their number increases and these cells become activated. It is thought that in addition to their many direct actions maintaining the microenvironment, ischaemia-induced reactive astrocytes act beneficially by upregulating the expression of nerve growth factor (Hsu et al., 1993).

At the time as one detects penumbral reactive astrocytosis, microglial activation and infiltration appear in the core region. Activated microglial cells (ameboid microglia) remove the neuronal debris, but in so doing, may increase the injury via formation of cytotoxic cytokines, free radicals and proteases, as well as via secretion of EAAs and NO (Wood, 1995). Reactive astrocytes counteract these actions, and are therefore crucial for neuronal viability and survival.

Altered protein synthesis and gene expression. Under physiologic conditions, the neurons use approximately 1/3 of their energy supplies to the protein synthesis. Thus, general protein synthesis is severely and rapidly suppressed in the cells subjected to ischemia (Thilmann et al., 1986). Protein synthesis never recovers in these cells destined to die, in the sublethally injured cells recovery still takes several hours, much longer than the recovery of energy metabolism (Hossmann, 1993). An environmental stress restricts protein synthesis to stress proteins (or heat shock proteins; HSP) (Lindquist and Craig, 1988) and induction of altered specific gene expression including expression of immediate early genes (IEG) (Abe et al., 1991; An et al., 1993), genes encoding stress proteins (Li et al., 1992; Kato et al., 1995), amyloid precursor protein (Abe et al., 1991), neurotrophic substances (Lindvall et al.,1992), a variety of cytokines and adhesion molecules (Liu et al., 1993; 1994; Okada et al., 1994), as well as apoptosis-related genes (Li et al., 1994; Chen et al., 1995).

The earliest detectable response after ischaemia, occuring within minutes, is the expression of IEGs: the fos (c-fos, fosB) and jun (c-jun, junB, junD) families and zinc finger genes (NGFI-A, krox 20) (Abe et al., 1991; An et al., 1993). Expressions of IEGs mediate long-term cellular changes that possibly cause upregulation of other genes in response to external stimuli (third messengers of the ischaemic cascade) (Sheng and Greenberg, 1990). In contrast to HSP70, which is restricted to ischaemic territory, IEG products have also been detected in areas remote from the ischaemic region (An et al., 1993). This distribution is possibly caused by spreading depression and influx of calcium (Kato and Kogure, 1999).

Expression of IEGs is followed by elevated expression of HSPs. There are several families of HSPs and they are categorized by their molecular weight. A major ischaemia-inducible HSP, the 70-kDa HSP, is expressed within hours postischaemia (Abe et al., 1992). There is also a specific hierarchy of HSP70 expression in different cell types and anatomic sites reflecting the pattern of cellular and anatomic susceptibility to ischaemia (Li et al., 1992; 1993). Furthermore, it has been suggested that dying neurons are unable to express HSP70, since after 30 min of MCAO (sublethal ischaemia) HSP70 is induced in cortical neurons of MCA region and after 90 min of MCAO, neurons expressing HSP70 have been detected only outside of the ischaemic core (possibly a marker of the penumbra) (Li et al., 1992; 1993).

The induction of HSP synthesis is likely to be an adaptation attempt of the cells to the disturbed environment, and thus, a vital response for neuronal survival. HSPs control conformation, stabilization and transport of normal and partially denatured proteins, and HSP70 is needed in the restoration of ribosomal function after heat shock (Beckmann et al., 1990), and probably also in postischaemic brain cells (Kato and Kogure, 1999).

2.1.2.3. Delayed neuronal death and apoptosis

A distinctive type of cell destruction, apoptosis, is considered as a possible mechanism for the delayed neuronal death seen in selectively vulnerable areas of hippocampus after global ischaemia as well as in the penumbra after focal ischaemia (Choi, 1996). Apoptosis is a physiologically essential mechanism during normal development, and also a defense system, that removes unwanted and potentially dangerous cells (Steller, 1995). In contrast to necrosis, apoptosis requires activation of the cellular self-destruction program that leads to membrane

folding, cell shrinking, chromosome condensation followed by DNA fragmentation and subsequent membrane enveloping of cellular fragments that are cleared away without inflammation (Searle et al., 1982). These events are initiated by irreversible proteolysis carried out by a family of cysteine proteases, caspases (the human homologues of the cell death gene product of *Caenorhabditis elegans*, CED-3) (Thornberry and Lazebnik, 1998).

DNA fragmentation and, especially its typical pattern (laddering), have been demonstrated after transient (Li et al., 1995) and permanent MCAO (Linnik et al., 1993; Tominaga et al., 1993) as well as after global ischaemia (Heron et al., 1993; MacManus et al., 1993), supporting the theory of apoptosis being the mechanism of ischaemia-induced delayed neuronal death. The temporal and anatomic profile of the apoptotic process depends on the experimental model used (MacManus et al., 1993; Tominaga et al., 1993; Li et al., 1995).

However, also contradictory results concerning the mechanism and temporal progression of delayed neuronal death after global ischaemia have been obtained. Recently, Colbourne and coworkers (1999) reported that, a brief forebrain ischaemia resulted in the ongoing neuronal death in CA1 long after the 72 hours that has been considered to be the maturation time of delayed neuronal death (Kirino, 1982; Pulsinelli and Brierley, 1982a). Furthermore, they detected no definitive morphological signs of apoptosis in the CA1, instead the neuronal degeneration was typical to necrosis. The discrepancy between morphological and biochemical findings might be possible because TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) staining used to identify apoptotic cells, also labels necrotic and autolytic cells (van Lookeren Campagne et al., 1996), and because apoptotic-like DNA fragmentation is also present in morphologically necrotic cells (Collins et al., 1992). Furthermore, glial cells may undergo apoptosis after global ischaemia and this may account for DNA laddering (Petito et al., 1998). On the other hand, these findings do not totally rule out the possibility that ischaemia-induced CA1 neuronal death has an apoptotic component.

The process of programmed cell death requires induction of IEGs as well as expression of proteins promoting cell death (such as p53) and/or absence of inhibitory proteins (such as bcl-2) (Li et al., 1994; Chen et al., 1995). Moreover, in focal ischaemia models, p53 knock-out mice (Crumrine et al., 1994) and human bcl-2 overexpressing mice (Martinou et al., 1994) have shown reduced neuronal susceptibility.

The original trigger of the postischaemic apoptotic process is under debate, but several potential candidates have been proposed including accumulation of intracellular calcium (Kiyota et al., 1993), excessive glutamate release (Coyle and Puttfarcken, 1993), free radicals (Linnik et al., 1993; Steller, 1995) and absence of nerve growth factor (Yao and Cooper, 1995). The fact that apoptotic cells have been detected in the penumbral region suggests microcirculatory perturbations as a possible trigger for apoptosis. Moreover, Vexler and coworkers (1997) have shown that the prevalence of apoptosis is related with the severity of perfusion deficits during transient focal ischaemia.

2.2. Pharmacological approaches to neuroprotection

The fact that hypoxia/ischaemia-induced neuronal death is partly delayed, and evidently a result of a complex process rather than a simple instantaneous event, offers a window of opportunity for neuroprotection. Each step of the ischemic cascade provides a potential target for pharmacological interventions. Numerous previous experimental studies have shown various agents to be neuroprotective at different steps along this cascade. Some of them have also been tested in clinical trials but the results so far have not been unambiguously positive. One possible reason for the discrepancy between preclinical and clinical results may be the fact that treatment should be started within a few minutes to a few hours after the onset of ischaemia, and clinically this has been difficult to realize (Hickenbottom and Grotta, 1998).

2.2.1. Calcium antagonists

Perturbation of normally tightly regulated neuronal calcium homeostasis is one of the first and major consequences of cerebral ischaemia. Massive calcium influx is thought to be the key trigger of disruptive ischaemic cascade, and thus, it also provides a target for therapeutic strategies. The calcium channel antagonists are among the longest and most extensively tested neuroprotectant candidates.

Calcium influx could be attenuated by antagonizing the voltage-operated channels that are activated by membrane depolarization or the ligand-operated channels (for example, the NMDA receptor, discussed in the next paragraph). Five types of voltage-operated channels

have been identified in the brain: N, L, T, P, and R, and of which the L-type is the best characterized and the most extensively studied in regard to neuroprotection. The N-type channel is located presynaptically and during ischaemia its activation is required for glutamate release. The L-type is a high-threshold channel that responds to strong depolarizations and becomes very slowly inactivated (McCleskey, 1994; Triggle, 1994). It can be blocked by several agents including the dihydropyridine compounds, such as nimodipine. Nimodipine has undergone several investigations in different preclinical and clinical settings (Grotta, 1991), and although it is now standard prophylactic therapy after subarachnoid hemorrhage to prevent vasospasms, in ischaemic stroke its efficacy has not yet been unambiguously demonstrated (Hickenbottom and Grotta, 1998).

Calcium release from intracellular stores is another event that might be prevented. Stimulation of metabotropic glutamate receptor results in formation of IP_3 and activation of the IP_3 receptor initiating Ca^{2+} release from the endoplasmic reticulum (Schoepp and Conn, 1993). There are several receptor types in addition to metabotropic glutamate receptors that, when stimulated, can influence the IP_3 signaling pathway leading to ameliorated cytosolic Ca^{2+} release (adrenergic, cholinergic, histaminergic, serotonergic receptors, for example) (Luiten et al., 1997), all of which can potentially be antagonized. Formation of IP_3 can also be mediated via neurotrophins and activation of tyrosine kinase-linked receptors (Maness et al., 1994). Activation of ryanodine receptors contributes to the intracellular release of calcium (Walton et al., 1991; Sharp et al., 1993), and moreover, inhibition of this receptor has provided neuroprotection in a global model of cerebral ischaemia (Zhang et al., 1993).

In addition to proteins that become activated after binding to Ca^{2+} (for example, IP_3) there are proteins that bind Ca^{2+}, acting like buffers, and thereby modulating and limiting the rise in the intracellular free Ca^{2+}concentration. These include parvalbumin, calbindin and calretinin. In the CNS, their distribution is complex but an interesting finding is the coexistence of, for instance, parvalbumin with GABAergic neurons and calbindin, at least mostly, with voltage-gated calcium channels (Baimbridge et al., 1992). Due to their capacity to cushion the elevation in intracellular free Ca^{2+}, these proteins might also protect neurons against ischaemia. Some promising results have already been reported (Mattson, 1991; Luiten et al., 1997).

2.2.2. Glutamate antagonists

Glutamate is the main excitatory neurotransmitter in the mammalian CNS. The neuronal death subsequent to ischaemia-induced excessive glutamate release and excitation, often referred to excitotoxicity, results from at least two mechanisms: rapid cell swelling caused by sodium and water influx and, even more damaging, intracellular calcium accumulation triggering the proteases, phospholipases and protein kinases. Glutamatergic effects are mediated via ionotropic (NMDA, AMPA and kainate) and metabotropic receptors. Ionotropic channels are permeable to Na^+ and K^+, and NMDA channels have a very high permeability to Ca^{2+}, which makes them especially interesting in regard to neuroprotection. NMDA receptors have binding sites for glutamate, glycine, polyamines, magnesium (Mg^{2+}), zinc (Zn^{2+}) and phencyclidine (PCP). In addition to glutamate, the receptor is activated by glycine and polyamine binding, whereas Mg^{2+}, Zn^{2+} and PCP are able to block the channel noncompetitively. Competitive NMDA antagonists bind directly to the glutamate binding site, non-competitive antagonists, on the other hand, block the associated ion channel, and there are also compounds antagonizing the separate binding sites (see Choi, 1990 for review).

Glutamate antagonists have been extensively studied in several different experimental stroke models, and furthermore, NMDA antagonists were the first class of acute stroke therapy agents proceeding to clinical trials. Early experimental studies of competitive and non-competitive NMDA antagonists suggested that they effectively protect selectively vulnerable areas in global ischaemia as well as penumbral region in focal ischaemia. In more recent studies however, it has been proved that neuroprotection of the glutamate receptor antagonists (both NMDA and AMPA) is mainly due to their hypothermic effects (Buchan and Pulsinelli, 1990; Corbett et al., 1990; Memezawa et al., 1995; Nurse and Corbett, 1996). Glutamate antagonists have also caused neuronal vacuolization distal from the injury area (Olney et al., 1991). Furthermore, the results attained so far in clinical studies have been rather disappointing. The competitive antagonist, Selfotel (CGS19755), and the non-competitive antagonists dextromethorphan and aptiganel hydrochloride (CNS1102), have evoked such severe adverse effects that clinical trials had to be terminated. The PCP-like neuropsychiatric side effects are likely to have limited the dose which could be given to stroke patients and this could well be one reason why therapeutic levels were not achieved and no neuroprotection detected (Grotta, 1995; Albers et al., 1995; Turrini, 1996; Hickenbottom and Grotta, 1998).

In order to avoid the adverse effects of direct NMDA receptor antagonism, other strategies of glutamate inhibition have been investigated, for instance, various glycine site antagonists (Yenari et al., 1997), magnesium sulphate (Tsuda et al., 1991; Marinov et al., 1996), AMPA receptor antagonists, such as NBQX (Gill et al., 1992), and the glutamate release inhibitor, lamotrigine (Crumrine et al., 1997). Polyamine site antagonists have been neuroprotective after global and focal ischaemia (Muszynski, et al., 1993) but it must be stated that polyamine treatment was also claimed to be beneficial (Gilad and Gilad, 1991).

To date, data from several studies indicate, that the glutamate receptor antagonists are not effective neuroprotectants. The reason why these drugs appeared protective in early experimental studies, in addition to their hypothermic effects, was the short survival time of the animals, typically 2 - 7 days after stroke. If the survival time is extended beyond a week or two, protective effect disappeares. Thus, these drugs seem to only delay but not prevent cell death (Valtysson et al., 1994; Colbourne et al., 1999).

2.2.3. Free radical scavengers

Free radical scavengers are among the most frequently studied neuroprotective strategies to block later events of the ischaemic cascade. The brain contains large amounts of unsaturated fatty acids and catecholamines which are thought to be targets for peroxidation by ROS. Hypoxia-ischaemia and reperfusion-reoxygenation, in particular, cause perturbations in the balance between free radical production and antioxidant defense systems (Siesjö, 1989). The free radicals can act independently or interact together like, for instance, NO reacts with superoxide anion to produce the highly reactive peroxynitrite (Ischiropoulos et al., 1992). For these reasons, scavenging free radicals by antioxidants (Xue et al., 1992), free radical spin traps (Zhao et al., 1994b) and NO synthesis inhibitors (Margail et al., 1997) have been tried in different experimental stroke models to enhance neuronal survival. The results show beneficial effects after both global and focal cerebral ischaemia with very little toxicity. Some of the free radical scavengers have already reached clinical trials, for example, tirilizad mesylate, a 21-amino steroid, has been well tolerated but it also failed to show any improvement in neurologic outcome (Hickenbottom and Grotta, 1998).

In addition to ROS production in the mitochondrial respiratory chain, also neutrophils traveling into reperfused regions interact with endothelium and release cytotoxic products including oxygen free radicals (Granger, 1988; Matsuo et al., 1995). It has also been reported that neutrophil depletion or inhibition of neutrophil function has a protective effect after ischaemia-reperfusion (Matsuo et al., 1995).

2.2.4. Selegiline

The first attempts to use selegiline, also named (-)deprenyl, which selectively and irreversibly inhibits MAO-B in the therapy of Parkinson's disease, were originally aimed at enhancing the bioavailability of levodopa, later also selegiline monotherapy was found to slow down the progression of the disability in the early stages of disease (Parkinson Study Group, 1993). Additionally, selegiline has been claimed to slow the cognitive decline in mild to moderate Alzheimer disease (Sano et al., 1997). Its apparent beneficial effect in these neurodegenerative diseases has made selegiline also an interesting candidate for neuroprotective or neurorescuing agent in cerebral ischaemia. To date, the results from different ischaemia models have been in part conflicting: mostly ameliorated neuronal survival (Matsui and Kumagae, 1991; Knollema et al., 1995; Semkowa et al., 1996; Lahtinen et al., 1997; Paterson et al., 1997; Erdö et al., 2000) but also no significant effects (Ballabriga et al., 1997; Speiser et al., 1999) have been reported. On the other hand, selegiline has shown protection against neuronal injury induced by a variety of neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Wu et al., 1995), N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) (Zhang et al., 1996) and 6-hydroxydopamine (6-OHDA) (Salonen et al., 1996). Moreover, selegiline has been previously reported to enhance the survival of facial motoneurons subsequent to axotomy (Salo and Tatton, 1992; Ansari et al., 1993).

The mechanisms underlying neuronal survival ameliorating effects of selegiline have been the focus of intensive research. The slowing of neurodegeneration detected in Parkinsonian and Alzheimer patients has been attributed to MAO-B inhibition resulting simply in a symptomatic improvement due to increased dopaminergic neurotransmission or, alternately, reduced free radical production of inhibited dopamine catabolism (Tatton et al., 1996). Increased dopamine levels could alternatively result from MAO-A inhibition, since prolonged

selegiline treatment inhibits MAO-A, even at doses lower those than required for MAO-B inhibition (Tatton et al., 1996). The substrate for MAO-B, 2-phenylethylamine, can possibly also down-regulate β-adrenergic receptors that normally enhance the excitatory effects of glutamate (Nicoll et al., 1987; Paetsch and Greenshaw, 1993). However, previous studies have suggested that selegiline also exerts several MAO-B independent, potentially neuroprotective or neurorescuing effects, since, for example, not all MAO-B inhibitors are neuroprotective (Ansari et al., 1993), concentrations of selegiline lower than those required for MAO-B inhibition have resulted in improved neuronal survival (Tatton et al., 1993) and selegiline can protect against DSP-4 toxicity which does not involve MAO-B (Finnegan et al., 1990).

The precise mechanism of MAO-B independent protection is unknown but selegiline has been demonstrated to alter neuronal and glial gene expression and protein synthesis. For example, levels of neurotrophic substances, such as basic fibroblast growth factor in activated astrocytes (Biagini et al., 1994), ciliary neurotrophic factor (Seniuk et al., 1994) and nerve growth factor (NGF) (Semkova et al., 1996) as well as neurotrophin receptor (trk C) mRNA (Ekblom et al., 1993) have been shown to become altered by selegiline. Hypoxic brain injury is known to induce a clear increase in NGF synthesis in the cortex and hippocampus (Lorez et al., 1989) and several studies have demonstrated that NGF can have a protective effect on hippocampal neurons (Cheng and Mattson, 1991; Pechan et al., 1995). Accumulation of NGF seems to be crucial in order to achieve neuroprotection with selegiline (Semkova et al.,1996).

The neuronal death in most of the models in which selegiline has ameliorated neuronal survival is thought to involve apoptosis. The altered gene expression and protein synthesis after selegiline administration includes an increase in inhibitory bcl-2 formation and a reduction in the levels of cell death promoting bax onco-proteins resulting in attenuated DNA fragmentation characteristic to apoptosis (see Tatton et al., 1997, for review). Another finding in favour of antiapoptotic mechanisms is the evidence that the enzyme accumulating in the nucleus and resulting in ultimate cell death, glyceraldehyde-3-phosphate dehydrogenase (Ishitani et al., 1998), is the putative target of the antiapoptotic propargylamine, CGP 3466, a compound which is not a MAO inhibitor, as well as a target for selegiline (Kragten et al., 1998).

Selegiline has been shown to reduce the levels of oxidative radicals also at concentrations too low to inhibit MAO-B (Chiueh et al., 1994). Moreover, free radical scavengers Cu/Zn (SOD1) and Mn (SOD2) superoxide dismutases as well as catalase are among the proteins whose synthesis is altered by selegiline (Carrillo et al., 1994). A further possible protective mechanism might be the maintenance of mitochondrial membrane potential and a decrease in intramitochondrial calcium levels detected following the transcriptional changes induced by selegiline (Tatton et al., 1997). In a recent *in vitro* study selegiline has also been demonstrated to induce an increase in interleukin-1b and a decrease in tumor necrosis factor production (Muller et al., 1998). Hence, also an immunologically protective effect by selegiline could be possible.

2.2.5. α_2-adrenoreceptor agonists

Sedation, analgesia and muscle relaxation as well as anaesthetic reduction are the effects resulting from α_2-adrenoreceptor activation that were first utilized, since the early 1970s, in veterinary medicine (xylazine and medetomidine). Next, it was observed that the α_2-agonist, clonidine, could prevent hypertensive crisis in perioperative patients, and subsequently, that α_2-agonism, in general, results in a variety of further beneficial effects (anxiolysis, antiemesis, antisialogogition, reduced endocrine stress response and protection against myocardial ischaemia, in the absence of respiratory depression) for surgical and critically ill intensive care unit patients. These findings of α_2-agonism led to the development of the more potent and specific α_2-agonist, dexmedetomidine (more than a sevenfold greater α_2:α_1 specificity than clonidine).

In the CNS, noradrenaline is a widely distributed neurotransmitter which is involved in several physiological processes. Noradrenergic cell bodies are clustered in the caudal pontine nucleus, locus coeruleus, from which axons project to cerebral and cerebellar cortices, thalamus, striatum, hypothalamus, olfactory bulbs, mesencephalon and the spinal cord (Cooper et al., 1991). The actions of catecholamines are mediated through α (α_{1A-D}, α_{2A-C}) and β (β_1, β_2, β_3) receptors. α_2-receptors are found in the CNS, peripheral nerves and autonomic ganglia. In the rat brain, α_{2C}-receptors have been detected, for example, in cerebral cortex, striatum and hippocampus, and α_{2A}-receptors in neurons in layer six of the cerebral

cortex (Nicholas et al., 1996). In the hippocampus, noradrenergic afferents from locus coeruleus form a dense network within the hilus and in the stratum lucidum of the CA3, while in the CA1, the input is less extensive and terminates mainly in the stratum lacunosum moleculare (Swanson et al., 1987). In human brain, α_2-receptors are found at high densities in the cerebral cortex, claustrum and leptomeninges, and at lower densities in the basal ganglia, thalamus, pons, substantia nigra, cerebellum and medulla oblongata (De Vos et al., 1991).

In the CNS, activation of postsynaptic α_2-receptors leads to inhibition of sympathetic activity, decrease in blood pressure and heart rate, sedation and anxiolysis. In the spinal cord, α_2-agonism produces analgesia and in the peripheral blood vessels vasoconstriction. Furthermore, α_2-adrenoreceptor activation can evoke diuresis by increasing sodium and water excretion (Hayashi and Maze, 1993), an increase in blood glucose concentrations via inhibition of insulin release (Katada, 1980), and promotes platelet function by enhancing the hyperaggregability induced by true agonists (e.g. thrombin) (Steen et al., 1993).

The catecholamines are also likely to have a role in pathological conditions characterized by deteriorations in the control of neuronal excitation, such as cerebral hypoxia-ischaemia and epilectic seizures. The α_2-agonists in general, and lately especially dexmedetomidine, have been widely investigated in several cerebral ischaemia models, and the results indicate that in some models they partially reduce ischaemic damage. Neuroprotection has been demonstrated with ganglionic blockade in rat incomplete ischaemia (Werner et al., 1990), with clonidine in rat incomplete ischaemia (Hoffman et al., 1991), with dexmedetomidine in rat incomplete ischaemia (Hoffman et al., 1991), and in rabbit focal ischaemia (Maier et al., 1993). In contrast, dexmedetomidine has failed to protect against severe forebrain ischaemia in rats (Karlsson et al., 1995). However, dexmedetomidine has provided protection against kainic acid-induced convulsions and neuronal damage in the rat (Halonen et al., 1995).

The neuroprotective mechanism of α_2-agonism is still unclear but several possible actions have been suggested. Cerebral ischaemia results in central sympathetic activation with increased noradrenaline release, most prominently in the hippocampus (Globus et al., 1989). Moreover, the release of glutamate and other excitatory neurotransmitters can be influenced by changes in the balance between different adrenoreceptor activities: α_1 (and β_1) receptors increase neuronal excitation (Madison and Nicoll, 1986), whereas α_2-receptors hyperpolarize

neurons (Dodt et al., 1991) and attenuate depolarization-mediated excitatory neurotransmitter release (Kamisaki et al., 1992) as well as glutamate receptor-mediated intracellular calcium accumulation (Bickler and Hansen, 1996), although dexmedetomidine *in vitro* has no effect at the postsynaptic NMDA receptor-mediated changes in the intracellular calcium concentrations (Talke and Bickler, 1996). Another possible neuroprotective mechanism has been suggested in a recent *in vitro* study where it was demonstrated that dexmedetomidine could stimulate glutamine oxidation in the astrocytes and thereby enhance glutamine disposal (Huang et al., 2000). A decrease in the glutamine concentration has been previously shown to protect against anoxic death of cultured neurons (Huang et al., 1997). A further possible protective mechanism, especially during reperfusion, might be reduction of oxidative stress and free radical formation by inhibition of enzymatic deamination of catecholamines (Simonson et al., 1993; Suzuki et al., 1995).

2.2.6. Enhancement of GABAergic neurotransmission

γ-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the mammalian CNS and, thus the main counterbalancer of the excitatory and excitotoxic effects of glutamate (Roberts, 1974). Antagonizing the detrimental effects of excessively released glutamate has been a widely studied neuroprotective strategy but the glutamate antagonists used have caused problematic adverse effects. Therefore, GABA agonists have been chosen as an alternative method to block ischaemia-induced excitotoxicity. Many of the GABA agonists have been used clinically for decades (for example, benzodiazepines as anticonvulsants, anxiolytics, sedatives/hypnotics, and muscle relaxants, and chlormethiazole as an anticonvulsant), and are pharmacologically well defined without evoking any major toxic side-effects (see Lyden, 1997 for review).

The GABA receptor is an ionophore complex with binding sites for GABA agonists and distinct binding sites for modulatory substances, such as benzodiazepines and barbiturates. GABAergic actions are mediated through two receptor subtypes, A and B. The $GABA_A$ receptor is a ligand-gated chloride channel that mediates a fast inhibitory response. Stimulation of $GABA_B$, a second messenger G-protein-linked receptor, on the other hand, has a more long-lasting effect by reducing presynaptic release of several neurotransmitters and inhibiting postsynaptic potential via a potassium channel (Macdonald and Olsen, 1994). The

distribution of GABA receptors is ubiquitous and heterogeneous; relatively higher densities in the striatum and cerebellum and moderate densities in the hippocampus and cortex. Interestingly, comparing the GABA receptor densities with glutamate receptors, the ratio is highest in the hippocampus, favouring glutamate by 4.3 times (Zilles, 1992).

Most of the neuroprotective effects following GABAergic stimulation are related to the properties of the $GABA_A$ receptor, since $GABA_B$ agonism has not been shown to unambiguously protect against ischaemic injury. These potentially neuroprotective effects include membrane hyperpolarization via increased chloride influx, and subsequent prevention of calcium influx through voltage-gated glutamate channels (Olsen et al., 1984), inhibition of cytosolic free radical formation via phospholipase A_2 activation (Schwartz et al., 1992), promotion of ATP recovery and prevention of cytochrome *c* release from mitochondria (Galeffi et al., 2000), reduction of cerebral metabolic rate for glucose (Kelly and McCullogh, 1983), and vasodilatation of cerebral blood vessels (Edvinsson and Krause, 1979).

In several previous studies, ischaemia-induced neuronal damage has been shown to be attenuated by a variety of $GABA_A$ agonists and other GABAergic neurotransmission enhancing agents. These agents include, phenobarbital which opens chloride channels and enhances GABA binding (Yoshida et al., 1983; Sternau, et al., 1989), the GABA transaminase inhibitor valproic acid (Sternau et al., 1989), the GABA uptake inhibitor No-328 (Johansen and Diemer, 1991), the benzodiazepine receptor full agonist diazepam (Sternau et al., 1989; Voll and Auer, 1991; Huff and Schwartz, 1991; Schwartz et al., 1994; 1995), and partial agonists PNU-101017 (Hall et al., 1997) and imidazenil (Schwartz-Bloom et al., 1998), the $GABA_A$ receptor agonists muscimol (Sternau et al., 1989; Lyden, 1997) and chlormethiazole (Cross et al., 1991; Shuaib et al., 1995; Sydserff et al., 1995a; 1995b). Most of these results have been achieved in different global cerebral ischaemia models, and only $GABA_A$ agonists have proved to be protective against transient (Lyden, 1997; Sydserff et al., 1995b) and permanent (Sydserff et al., 1995a) focal ischaemia.

Cerebral ischaemia results in evident changes in GABA biochemistry and function. Both permanent (Hillered et al., 1989; Graham et al., 1990; Butcher et al., 1990) and transient (Uchiyama-Tsuyuki et al., 1994) cerebral ischaemia cause a dramatic increase in the extracellular GABA concentration. Moreover, the GABA increase is not exclusively a characteristic of animal models, since it has been detected in neurosurgical patients

experiencing an ischaemic episode (Kanthan et al., 1995). The extracellular GABA levels decline back close to baseline values after 20 min and completely to baseline values after1 h of reperfusion (Uchiyama-Tsuyuki et al., 1994). Furthermore, it is possible that after the initial massive ischaemia-induced GABA release there might exist a feedback inhibition of both synthesis and release of GABA for a longer period, such as occurs after an epileptic convulsion (Green et al., 1987). Indeed, in the mouse permanent MCAO, it was demonstrated that immediately after the induction of ischaemia, 70% of GABA synthesis was inhibited (Green et al., 1992).

Additionally, several factors that are evidently connected with cerebral ischaemia are able to change $GABA_A$ receptor function. These include reduced levels of ATP (Gyenes et al., 1988; Stelzer et al., 1988; Duchen, 1989; Chen et al., 1990), increased cytosolic calcium levels (Mouginot et al., 1991; Schwartz et al., 1992), and the formation of oxygen free radicals (Schwartz et al., 1992). In global cerebral ischaemia studies, $GABA_A$ receptor function has been shown to become impaired transiently during the early reperfusion period (Li et al., 1993; Verheul et al., 1993; Alicke and Schwartz-Bloom, 1995). Expression of two $GABA_A$ receptor subunit mRNAs have been demonstrated to be affected by ischaemia in the hippocampus: initially the expression declined in all areas, later normalized in all but the selectively vulnerable parts (Li et al., 1993). Furthermore, permanent MCAO has been reported to cause long-term down-regulation of [^{3}H]muscimol binding sites of the GABA receptors both in the ipsilateral and the contralateral neocortex (Qü et al., 1998).

3. AIMS OF THE STUDY

Although the search for an effective neuroprotective therapy for ischaemia-induced injury has been the focus of intensive research for the last decades, no totally convincing results have yet been achieved. Experimental studies have pointed to several potential candidates for neuroprotective agents, such as calcium and glutamate antagonists. Nonetheless, these potentially neuroprotective agents have mainly proved either clinically ineffective, have had unrealistic therapeutic time-windows, or they have evoked severe harmful side effects. Therefore, the present series of studies sought a clinically usable neuroprotective agent. The drugs tested here have been in clinical use (diazepam and selegiline) or are in an advanced stage of clinical trials (dexmedetomidine), and are pharmacologically well defined. Furthermore, in previous studies these agents have revealed some tendency towards neuroprotection. The aims were:

1. to investigate whether the α_2-adrenergic agonist, dexmedetomidine, is neuroprotective in a model of global cerebral ischaemia,

2. to study whether the α_2-adrenergic agonist, dexmedetomidine, is neuroprotective in models of transient and permanent focal cerebral ischaemia,

3. to study whether the MAO-B inhibitor, selegiline, is neuroprotective in a model of global cerebral ischaemia,

4. and to investigate whether the GABAergic neurotransmission enhancer, diazepam, is neuroprotective in a model of transient focal cerebral ischaemia.

4. MATERIALS AND METHODS

4.1. Animals

Adult female Mongolian gerbils (*Meriones unguiculatus*) weighing 60 - 85 g and obtained from Mollegaard Breeding Centre Ltd (Copenhagen, Denmark) were used in the global cerebral ischaemia studies (**I** and **III**; n = 50 and n = 49, respectively). In the focal cerebral ischaemia studies (**II** and **IV**; n = 62 and n = 12, respectively) adult male Hannover origin Wistar rats weighing 220 - 325 g, were obtained from the National Animal Centre (University of Kuopio, Kuopio, Finland). The animals were housed individually in stainless steel cages which were placed in a temperature (20 ± 1 °C), humidity (55 ± 10%) and light period (12 h light/12 h dark cycle) controlled environment. Food and water were available *ad libitum* before and after the surgical interventions. These studies were approved by the Provincial Government of Kuopio (approval number Zd 45, 1994 and 1997).

4.2. Drug treatment

The methods for drug testing are described in detail in the individual publications (**I** - **IV**).

In publications **I** and **II**, the effects of dexmedetomidine (Orion Corporation, Orion Pharma, Turku, Finland), a highly potent and selective α_2-adrenoreceptor agonist, were tested in both global and focal cerebral ischaemia models. Dexmedetomidine was dissolved in 0.9% NaCl. In publication **I**, the bilaterally CCA occluded gerbils received 3 or 30 µg/kg dexmedetomidine either 30 min **before and thereafter** at 3, 12, 24 and 48 h after occlusion, or at 3, 12, 24 and 48 h **after** occlusion. Ischaemic control animals received only 0.9% NaCl. All injections were made subcutaneously in a volume of 0.01 ml/g body weight. The selection of doses was based on a previous study that showed a U-shaped dose-response curve against kainic acid-induced convulsions (Halonen et al., 1995). In publication **II**, the intravenous infusion was started immediately after the MCAO. Dexmedetomidine was administered as a bolus of 3 µg/kg in 5 min followed by a 120 min infusion either at a dose of 3 µg/kg/h or 6 µg/kg/h (total doses 9 µg/kg and 15 µg/kg, respectively). The dose selection was based on the

previous study of Halonen et al. (1995) and the present global ischaemia study with dexmedetomidine.

In publication **III**, the effects of (-)deprenyl (selegiline), a selective irreversible MAO-B inhibitor that also possibly exerts free radical scavenger and rescue-type anti-apoptotic actions, were investigated in the gerbil global cerebral ischaemia model. The first group of CCA occluded animals received 0.25 mg/kg (-)deprenyl once a day for two weeks before occlusion to test if increased activity of free radical scavenger enzymes could protect selectively vulnerable neurons in hippocampal CA1. The second group received a single dose of 5 mg/kg (-)deprenyl 3 h after occlusion to test the effect of long-lasting MAO-B inhibition and acute free radical reduction on neuronal protection. The third and fourth groups received 0.25 mg/kg (-)deprenyl once a day, starting 3 h after occlusion, for one week or two weeks, respectively, in order to test the possible rescue-type anti-apoptotic effect on neuronal protection. The control animals received 0.9% NaCl. (-)Deprenyl HCl (Orion Corporation, Orion Pharma, Turku, Finland) was dissolved in 0.9% NaCl. The injection volume was 0.01 ml/g body weight. All injections were given subcutaneously. The selection of doses was based on previous studies (Matsui and Kumagae, 1991; Knollema et al., 1995; Lahtinen et al., 1997; Ballabriga et al., 1997).

In publication **IV**, the effect of the benzodiazepine receptor full agonist and GABA enhancer, diazepam (Diapam®, Orion Pharma, Finland) was tested after transient MCAO in rats. After 30 and 90 min of reperfusion the animals received an intraperitoneal injection of either diazepam (10 mg/kg) or vehicle (33% polyethylene glycol in 0.9% NaCl). The dose selection was based on previous studies (Sternau et al., 1989; Johansen and Diemer, 1991: Schwartz et al., 1994; 1995; Hall et al., 1998).

4.3. Surgery

4.3.1. Global cerebral ischaemia (I, III)

The global cerebral ischaemia model used in publications **I** and **III** is essentially the same as described by Ito et al. (1975). Each gerbil was anaesthetized using diethyl aether inhalation via a mask. The CCAs were exposed through an anterior midline cervical incision and

separated carefully from the surrounding vagus nerve fibres. Before the induction of ischemia, the anaesthesia was discontinued, and subsequently the CCAs were occluded with atraumatic aneurysmal clips for 5 min. Both cessation of the circulation and recirculation were visually confirmed with a stereomicroscope. Sham-operated animals were only anaesthetized and their CCAs exposed.

4.3.2. Focal cerebral ischaemia (II, IV)

Focal cerebral ischaemia in publications **II** and **IV** was produced using the intraluminal thread method (Koizumi et al., 1986; Longa et al., 1989). Anaesthesia was initiated in an induction box with 4 - 5% halothane (in 70% nitrous oxide and 30% oxygen) and maintained during the operation with 0.5 - 1% using a face mask. Under a stereomicroscope, the right CCA and the bifurcation area were exposed through a midline anterior cervical incision, and carefully separated from the surrounding vagus nerve fibres. First, the ECA was sutured (6 - 0 silk) beyond the branch of the superior thyroidal artery, a loose suture was placed around the ICA, and CCA and ICA were occluded with atraumatic aneurysmal clips. The ECA was dissected and the distal stump electrocoagulated. A nylon suture (0.28 mm diameter), its tip rounded, was then introduced into the lumen of ECA, and advanced into the ICA. The suture around the ICA was tightend, the clip on the ICA removed and the suture advanced gently for a distance of 22 mm from the bifurcation in order to occlude the origin of the MCA (Longa et al., 1989)(Figure 3). In the **transient** MCAO (**II** and **IV**), the suture was removed after 90 min, the ECA stump electrocoagulated, and the CCA clip removed to allow reperfusion in the MCA territory. In the **permanent** MCAO (**II**), the nylon suture was left in the ICA, the CCA was sutured, and the clip removed.

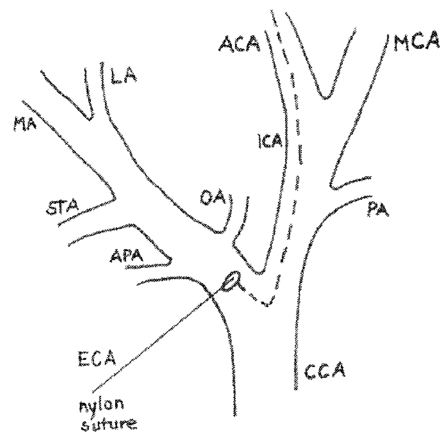


Figure 3. A schematic diagram of middle cerebral artery occlusion of the rat using the intraluminal thread method. CCA = common carotid artery, ECA = external carotid artery, ICA = internal carotid artery, MCA = middle cerebral artery, ACA = anterior cerebral artery, OA = occipital artery, APA = ascending pharyngeal artery, STA = superior thyroid artery, MA = maxillary artery, LA = lingual artery, PA = pterygopalatine artery.

4.4. Physiological parameters

In all studies, the animals were kept normothermic (37°C) during the operation and occlusion with a thermoregulatory heating unit (Harvard Apparatus, South Natick, MA) connected to a rectal probe. In publication **IV**, the animals were also closely monitored for several hours after diazepam administration and kept normothermic using the same procedure. In publications **I** and **II**, an additional probe was inserted into the temporalis muscle to monitor head temperature (Omega, Model 680, Stamford, CT) during the operation and occlusion.

In order to monitor blood pressure (CardioCap II, Datex, Finland), and to obtain arterial blood samples for blood gas analysis, $PaCO_2$, PaO_2 and blood pH (ABL5, Radiometer, Denmark), and for blood glucose concentrations (OneTouch II, Lifescan, USA), the right femoral artery (**II** and **IV**) or the left external iliac artery (**I**) was cannulated. The specific time points of measurements are indicated in the individual publications (**I, II** and **IV**).

4.5. Histology

4.5.1. Tissue preparations (I - III)

After the treatments were completed (one week, **I**; one/two weeks, **III**), gerbils were deeply anaesthetized (chlornembutal 3.0 ml/kg, intraperitoneally), and their brains perfused transcardially, first 10 min with saline and then 60 min with perfusion fixative (4% paraformaldehyde, 0.05% glutaraldehyde, 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4). The brains were removed from the skull (and postfixed with the same fixative if required) and the dorsal hippocampus was sectioned with a vibratome coronally into 60 μm thick slices. (**I, III**) In publication **II**, the rats were anaesthetized with carbon dioxide and decapitated 24 h after the MCAO. Brains were removed from the skull and placed in ice-cold saline for 10 min. Coronal slices, 2 mm thick, were sectioned using a dissecting matrix.

4.5.2. Silver impregnation (I, III)

To evaluate the severity of neuronal damage in the CA1 area of gerbil hippocampus after 5 min of global cerebral ischaemia the slices were stained using the silver impregnation technique (Gallyas et al., 1980). The following procedure was used: 2 x 5 min in the pretreating solution (2% NaOH and 2.5% NH_4OH), 10 min in the impregnating solution (0 - 0.8% NaOH, 2.5% NH_4OH, 0.5% $AgNO_3$), 3 x 5 min in washing solution (0.5% Na_2CO_3 and 0.01% NH_4NO_3 in 30% ethanol), 1 min in developing solution (0.4 - 0.6% formaldehyde and 0.1% citric acid in 10% ethanol, pH 5.0 - 5.5) and finally, 3 x 10 min wash in 0.5% acetic acid. Slices were mounted on gelatin coated slides, dehydrated and covered with Depex (BDH, Poole, England).

Microscopic evaluation of delayed neuronal death of hippocampal pyramidal cells in the CA1 sector was done in a blind manner on coded sections. Both hippocampi were evaluated using 4 - 8 sections from each animal and the mean score was used for statistical analysis. In publication **III**, the damage was graded into four groups: (0) no degenerated neurons; (1) <10% of the neurons dead; (2) 10 - 50% of the neurons dead; (3) >50% of the neurons. Only the shrunken argyrophilic neurons were considered to be irreversibly degenerated (Crain et al., 1988). In publication **I**, the CA1 and CA3 pyramidal cells were counted using an ocular

micrometer grid limiting a circular area of 0.08 mm^2 under the microscope. In the hilus of the dentate the entire area confined by the dorsal and ventral blades of the granule cell layer was analysed. The mean number of damaged cells was calculated for each of the three hippocampal regions.

4.5.3. TTC-staining (II)

To measure infarct volume after transient and permanent MCAO (**II**), the brain sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in 0.1 M phosphate buffer at 37° C for 10 min. Sections were stored in neutral buffered 10% formalin (Bedersen et al., 1986). An estimation of the infarcted area in the striatum and cortex was performed using MCID image processing software (Imaging Research Inc., Ontario, Canada) and a DAGE MTI CCD-72 series camera (DAGE.MTI). In order to minimize the possible error introduced by brain oedema, the infarcted area was determined according to the indirect method of Swanson et al. (1990) where areas of intact grey matter with optical density above the threshold values were automatically recognised and measured separately for each hemisphere. The difference between the size of an intact area in the contralateral hemisphere and the respective residual area in the ipsilateral hemisphere was then taken as the infarcted area. Total infarct volume was calculated by multiplying the infarct area by the thickness of a slice and by summing together the infarct volumes of each slice.

4.6. MRI methods (IV)

MRI was performed using a s.m.i.s microimaging console (Surrey Medical Imaging Systems, Guilford, UK) interfaced to a horizontal 4.7 T magnet (Magnex Scientific, Abdington, UK) equipped with actively shielded gradient coils (Magnex Scientific, Abdington, UK). A single loop surface coil was used for signal transmission and reception. The existence of an ischaemic episode was verified by measuring the trace of diffusion tensor ($D_{AV} = 1/3\ Trace\ \bar{D}$) and the region of hypoperfusion during MCAO. D_{AV}-maps were calculated from images acquired using a spin-echo sequence (TR 2000 ms, TE 60 ms, matrix 128 x 64, slice thickness 1 mm) with four bipolar gradients along all three orthogonal directions using b-values of 38, 611 and 1200 s/mm^2 (Mori and van Zijl, 1995). Perfusion imaging was

performed using bolus tracking with a gradient-echo method (TR 9.5 ms, TE 4.5 ms, matrix 128 x 64, slice thickness 1.5 mm). Gadodiamide (Omniscan, Nycomed, Oslo, Norway) 0.20 mmol/kg was rapidly injected into the right femoral vein during uninterrupted data acquisition of 35 s.

Total infarct volume after 48 h of reperfusion was quantified from the multislice images acquired by measuring the area of the bright T_2-enhanced region in each of the slices (parameters TR 2500 ms, TE 60 ms, matrix 256 x 128, 15 slices of 1 mm thickness). The severity of oedema at the same time was assessed from T_2-maps calculated based on images acquired using a spin-echo sequence (TR 2000 ms, TEs 20, 50, 80 ms, slice thickness 1 mm) and D_{AV} -maps calculated as described above.

4.7. Statistical analysis

Statistical analyses were made by using SPSS/PC+ programs. The neuroprotective efficacy (**I**, **III**) and differences in infarct volumes (**IV**) were statistically analysed with Kruskall-Wallis test followed by Mann-Whitney U-test. Infarct volumes (**II**) and MRI parameters (**IV**) were compared using General Linear Model Univariate Analysis of Variance. Physiological parameters (**I, II, IV**) were analysed with General Linear Model Analysis of Variance for repeated measures.

5. RESULTS

5.1. Effect of the α_2-adrenergic agonist, dexmedetomidine, in global cerebral ischaemia (I)

The neuroprotective efficacy of the α_2-adrenergic agonist, dexmedetomidine, was first tested in a gerbil model of global cerebral ischaemia. Ischaemia was induced by bilateral CCAO for 5 min in normothermic animals. Dexmedetomidine was administered subcutaneously at doses of 3 or 30 µg/kg, either 30 min before and thereafter at 3, 12, 24 and 48 h after occlusion, or at 3, 12, 24 and 48 h after occlusion. Control animals received 0.9 % NaCl. Neuronal damage was evaluated microscopically one week after the insult in the hippocampal CA1, CA3 and in the hilus of the dentate gyrus.

The neuronal damage showed a consistent pattern in the saline-treated animals. In the CA1 region dexmedetomidine, at a dose of 3 µg/kg given before and then subsequent to the occlusion, showed only a statistically unsignificant tendency towards reduced neuronal damage mean of 571 ± 362 compared with 1125 ± 305 degenerated neurons/mm^2 in the control group ($p = 0.125$). However, neuronal damage in the CA3 and in the dentate hilus was significantly reduced. The dose of 3 µg/kg, given before and after ischaemia induction showed neuroprotective efficacy in the CA3 ($p < 0.01$). In the hilus, both the doses of 3 µg/kg given before and after ischaemia induction and 30 µg/kg given only after the ischaemia provided neuroprotection ($p < 0.01$ and $p < 0.05$, respectively)(Figure 4).

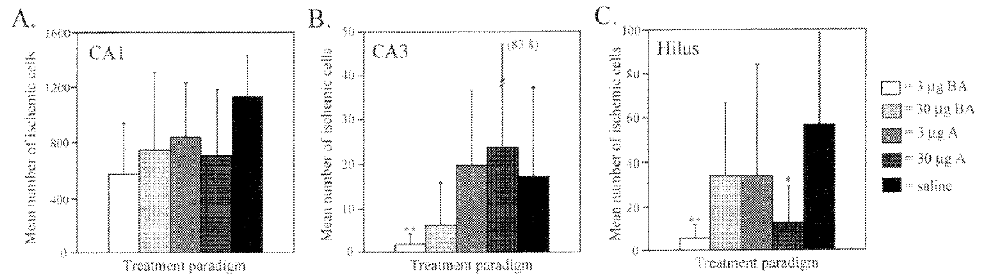


Figure 4. The effects of dexmedetomidine on pyramidal cells in hippocampal CA1 (A) and CA3 subfields (B) and on the neurons of dentate hilus (C) after 5 min of global cerebral ischaemia in gerbils. The bars represent the mean ± SD of degenerated cells in each treatment paradigm. BA = the drug given 30 min before the induction of ischaemia and thereafter at 3, 12, 24, and 48 h postischaemia. A = the drug given at 3, 12, 24, and 48 h postischaemia. Statistical significance: *$P < 0.05$; **$P < 0.01$ (compared with saline-treated ischaemic gerbils, Kruskall-Wallis test followed by Mann-Whitney U-test).

Additionally, the effects of dexmedetomidine (3 µg/kg) on various physiological parameters in ischaemic animals were investigated. No differences between groups were found in PaO_2, $PaCO_2$, blood pH, mean arterial blood pressure (MAP) or head and rectal temperatures. However, blood glucose concentrations increased by 68 - 83 % ($p < 0.001$) after dexmedetomidine administration (Table 1).

Table 1. Physiological parameters in supplementary gerbils given 3 μg/kg of dexmedetomidine 30 min before CCAO (I).

	Baseline	30 min after treatment	During occlusion	30 min after reperfusion	*p*
Head temperature (°C)					
Saline (n = 5)	35.8 ± 0.4	36.5 ± 0.2	36.1 ± 0.3	36.1 ± 0.4	NS
Dexmedetomidine (n = 5)	36.0 ± 0.5	36.4 ± 0.3	35.9 ± 0.4	36.2 ± 0.2	
PCO_2 (mmHg)					
Saline (n = 5)	34.8 ± 6.5	27.0 ± 6.1	26.2 ± 7.7	23.8 ± 8.5	NS
Dexmedetomidine (n = 5)	32.2 ± 5.9	33.0 ± 5.9	27.2 ± 6.7	25.2 ± 3.0	
PO_2 (mmHg)					
Saline (n = 5)	128 ± 25	151 ± 17	107 ± 47	156 ± 21	NS
Dexmedetomidine (n = 5)	132 ± 15	127 ± 18	114 ± 14	153 ± 15	
pH					
Saline (n = 5)	7.33 ± 0.33	7.32 ± 0.03	7.31 ± 0.04	7.29 ± 0.06	NS
Dexmedetomidine (n = 5)	7.35 ± 0.03	7.30 ± 0.04	7.29 ± 0.03	7.26 ± 0.07	
B-Gluc (mM)					
Saline (n = 5)	4.9 ± 0.6	4.2 ± 0.4	4.9 ± 1.4	4.8 ± 0.6	<0.001
Dexmedetomidine (n = 5)	5.3 ± 0.7	7.7 ± 1.6**	8.4 ± 1.9*	8.1 ± 1.5**	
MAP (mmHg)					
Saline (n = 5)	69 ± 5	62 ± 14	86 ± 8	56 ± 13	NS
Dexmedetomidine (n = 5)	66 ± 7	54 ± 7	76 ± 18	55 ± 15	

Values are mean ± SD. The number of animals is in parentheses. Statistical significance between saline and dexmedetomidine groups (ANOVA), NS = not significant; * $p < 0.05$; and ** $p < 0.01$ *versus* saline (Student's test).

5.2. Effect of the α_2-adrenergic agonist, dexmedetomidine, in transient and permanent focal cerebral ischaemia (II)

Given the promising results in the global cerebral ischaemia model (**I**), the neuroprotective efficacy of dexmedetomidine was also tested in rat models of transient (ischaemia/reperfusion) and permanent (ischaemic ischaemia) focal cerebral ischaemia. The MCAO was produced using an intraluminal thread that was either removed after 90 min (transient) or left in place (permanent). Intravenous administration of dexmedetomidine was started immediately after the MCAO as a bolus of 3 µg/kg in 5 min followed by a 120 min infusion either at a dose of 3 µg/kg/h or 6 µg/kg/h (total doses of 9 and 15 µg/kg, respectively). Control rats received 0.9% NaCl. The infarct volume was evaluated 24 h after the MCAO.

A consistent pattern of ischaemic brain damage was detected in the control animals both after transient and permanent MCAO. The infarct volumes of whole hemisphere, striatum and cortex were significantly greater after permanent MCAO compared to that of after transient MCAO ($p<0.05$). At a dose of 15 µg/kg, dexmedetomidine administration showed only a statistically unsignificant trend towards reduced infarct volume after transient MCAO (in the hemisphere -29.4%, in the cortex -30.9% and in the striatum -20.3%, compared with the control group)(Figure 5).

Dexmedetomidine treatment did not decrease head or rectal temperatures. There were also no differences in PaO_2, $PaCO_2$ or blood pH between the groups. On the other hand, MAP was significantly decreased after dexmedetomidine administration (both doses) up to 45 min after MCAO, and blood glucose concentrations were increased after the higher dose, in both the transient (Table 2) and permanent (Table 3) MCAO groups.

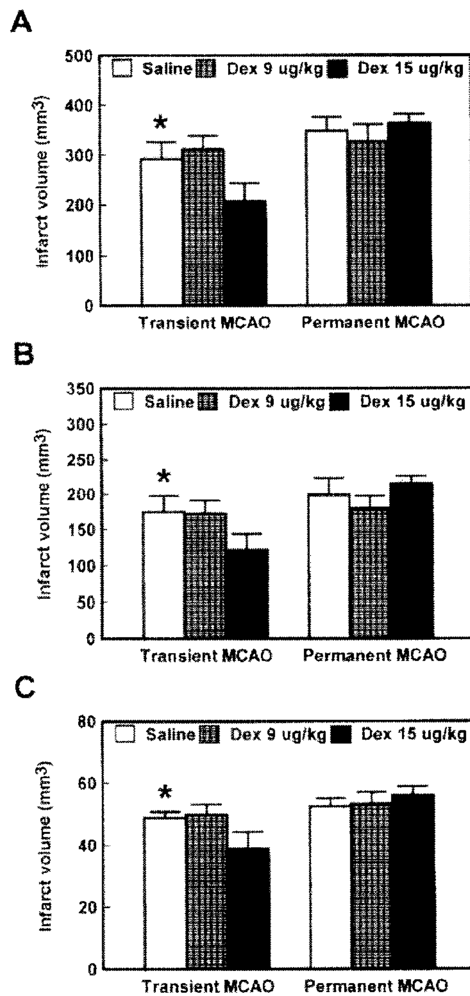


Figure 5. Comparison of infarct volumes after transient and permanent middle cerebral artery occlusion. Treatment groups in both models were saline or dexmedetomidine 9 μg/kg and 15 μg/kg. Infarct volumes (mm^3) (A) whole hemisphere, (B) cortex, and (C) striatum, are shown. *$P < 0.05$, General Linear Model Univariate Analysis of Variance comparing transient and permanent focal cerebral ischaemia models. All values are presented as mean ± SEM. The difference between treatment groups was not statistically significant.

Table 2. Physiological parameters in the transient occlusion groups of publication II.

Group	Baseline	10 min postischaemia	45 min postischaemia	90 min postischaemia	20 min reperfusion
Head temp (°C)					
Saline	36.9 ± 0.4	37.5 ± 0.6	37.6 ± 0.6	37.5 ± 0.6	37.3 ± 0.4
Dex 9	36.7 ± 0.3	36.6 ± 0.1	37.2 ± 0.2	36.9 ± 0.4	36.9 ± 0.3
Dex 15	36.7 ± 0.4	36.7 ± 0.2	36.8 ± 0.3	36.8 ± 0.3	37.4 ± 0.8
Rectal temp (°C)					
Saline	37.0 ± 0.4	37.3 ± 0.4	37.8 ± 0.4	37.6 ± 0.4	37.2 ± 0.4
Dex 9	37.4 ± 0.3	36.7 ± 0.2	37.5 ± 0.4	37.1 ± 0.5	37.1 ± 0.6
Dex 15	36.9 ± 0.4	36.7 ± 0.2	36.9 ± 0.4	36.8 ± 0.3	36.9 ± 0.2
MAP (mmHg)					
Saline	94 ± 12	110 ± 13	107 ± 16	88 ± 21	101 ± 8
Dex 9	101 ± 10	85 ± 14*	99 ± 10	86 ± 10	85 ± 7
Dex 15	100 ± 9	74 ± 10**	85 ± 14*	88 ± 9	99 ± 20
B-glucose (mM)					
Saline	5.7 ± 0.4		5.1 ± 0.8		5.4 ± 0.5
Dex 9	5.3 ± 0.4		6.4 ± 0.6		6.7 ± 0.9
Dex 15	6.2 ± 0.9		9.5 ± 2.5**		11.4 ± 2.8**
$PaCO_2$ (mmHg)					
Saline	40 ± 6		38 ± 6		40 ± 7
Dex 9	43 ± 7		34 ± 11		35 ± 4
Dex 15	45 ± 5		44 ± 5		45 ± 3
PaO_2 (mmHg)					
Saline	125 ± 25		100 ± 29		106 ± 30
Dex 9	143 ± 16		138 ± 11		122 ± 20
Dex 15	134 ± 19		130 ± 11		111 ± 14
pH					
Saline	7.40 ± 0.03		7.38 ± 0.03		7.35 ± 0.04
Dex 9	7.38 ± 0.06		7.40 ± 0.01		7.36 ± 0.01
Dex 15	7.37 ± 0.03		7.36 ± 0.01		7.33 ± 0.03

Dex 9 and 15 indicate dexmedetomidine at doses of 9 and 15 µg/kg, respectively; temp, temperature; MAP, mean arterial blood pressure; $PaCO_2$, arterial carbon dioxide pressure; PaO_2, arterial oxygen pressure. All values are represented as mean ± SD. * $p < 0.05$, ** $p < 0.001$ vs. saline controls by General Linear Model Analysis of Variance for repeated measures.

Table 3. Physiological parameters in the permanent occlusion groups of publication II.

Group	Baseline	10 min postischaemia	45 min postischaemia	90 min postischaemia	110 min postischaemia
Head temp (°C)					
Saline	36.8 ± 0.6	37.1 ± 0.4	37.9 ± 0.3	38.1 ± 0.7	38.2 ± 0.4
Dex 9	37.8 ± 0.7	37.3 ± 0.5	38.2 ± 0.6	38.2 ± 0.5	38.1 ± 0.3
Dex 15	37.4 ± 0.6	37.2 ± 0.6	37.5 ± 0.7	37.6 ± 0.5	37.5 ± 0.5
Rectal temp (°C)					
Saline	37.3 ± 0.5	37.2 ± 0.3	38.0 ± 0.4	38.3 ± 0.4	38.1 ± 0.4
Dex 9	37.0 ± 0.3	36.6 ± 0.2	37.5 ± 0.5	37.2 ± 0.5	37.0 ± 0.3
Dex 15	37.0 ± 0.4	36.6 ± 0.4	37.3 ± 0.4	37.1 ± 0.4	37.0 ± 0.3
MAP (mmHg)					
Saline	100 ± 9	115 ± 12	115 ± 15	106 ± 10	105 ± 11
Dex 9	108 ± 5	75 ± 8**	84 ± 14*	93 ± 6	92 ± 9
Dex 15	98 ± 6	74 ± 4**	88 ± 13*	98 ± 15	99 ± 18
B-glucose (mM)					
Saline	5.5 ± 0.9		4.8 ± 0.6		4.6 ± 0.6
Dex 9	5.3 ± 0.5		7.1 ± 1.9		7.4 ± 2.2
Dex 15	6.6 ± 0.8		9.3 ± 2.1**		10.1 ± 3.8**
$PaCO_2$ (mmHg)					
Saline	47 ± 3		36 ± 6		41 ± 7
Dex 9	43 ± 3		42 ± 6		45 ± 5
Dex 15	43 ± 5		47 ± 4		44 ± 4
PaO_2 (mmHg)					
Saline	129 ± 21		135 ± 14		127 ± 24
Dex 9	151 ± 16		120 ± 26		112 ± 18
Dex 15	146 ± 19		122 ± 13		126 ± 14
pH					
Saline	7.36 ± 0.02		7.38 ± 0.03		7.36 ± 0.04
Dex 9	7.38 ± 0.03		7.37 ± 0.05		7.33 ± 0.03
Dex 15	7.35 ± 0.03		7.32 ± 0.02		7.32 ± 0.03

Dex 9 and 15 indicate dexmedetomidine at doses of 9 and 15 μg/kg, respectively; temp, temperature; MAP, mean arterial blood pressure; $PaCO_2$, arterial carbon dioxide pressure; PaO_2, arterial oxygen pressure. All values are represented as mean ± SD. * $p < 0.05$, ** $p < 0.001$ vs. saline controls by General Linear Model Analysis of Variance for repeated measures.

5.3. Effect of the MAO-B inhibitor, selegiline, in global cerebral ischaemia (III)

The neuroprotective potential of selegiline ((-)deprenyl), a selective irreversible MAO-B inhibitor that also exerts several independent effects of MAO-B inhibition, was investigated in the gerbil model of bilateral CCAO for 5 min. Selegiline was given subcutaneously (1) at a dose of 0.25 mg/kg once a day for two weeks before occlusion to test whether the increased free radical scavenger enzyme activity is protective. (2) A single dose of 5 mg/kg 3 h after CCAO to test if acute free radical reduction is crucial. (3) A dose of 0.25 mg/kg daily for one or (4) two weeks starting 3 h after CCAO to test whether rescue-type actions are involved. The severity of neuronal damage in the hippocampal CA1 was assessed at one of the four grades.

Complete forebrain ischaemia of 5 min resulted in a consistent neuronal damage in the CA1 region. Selegiline treatment, however, only resulted in a non-significant trend towards attenuated neuronal damage. At a dose of 0.25 mg/kg for two weeks before CCAO, neuronal damage was reduced by 20%, at a single dose of 5 mg/kg, the reduction was 32%, and at a dose of 0.25 mg/kg for one week and two weeks after CCAO, the reductions were 22% and 30%, respectively (Figure 6).

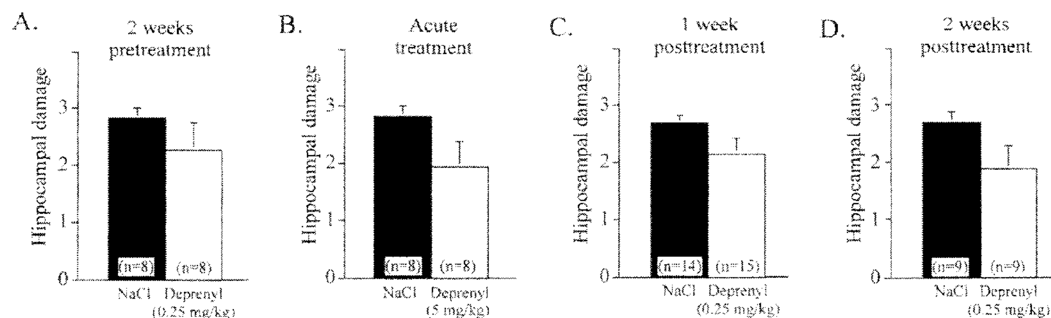


Figure 6. The effects of four different protocols of selegiline treatment on damage to hippocampal CA1 pyramidal cells after 5 min of global forebrain ischaemia in gerbils. Selegiline was given daily (0.25 mg/kg) two weeks prior to occlusion (A), as a high single dose (5mg/kg) 3h after occlusion (B), or daily starting 3 h after occlusion (0.25 mg/kg) for one week (C) or for two weeks (D). The bars represent the damage score (mean ± SEM). There were no statistically significant differences between selegiline-treated animals compared to saline-treated (Mann-Whitney U-test).

5.4. Effect of the GABAergic neurotransmission enhancer, diazepam, in transient focal cerebral ischaemia (IV)

Diazepam, a full agonist of benzodiazepine receptor and an enhancer of the GABA activity, which has been shown to be neuroprotective in several previous global cerebral ischaemia studies, was tested in a rat model of transient focal cerebral ischaemia. Diazepam at a dose of 10 mg/kg (or vehicle) was administered intraperitoneally. The evaluation of ischaemia was conducted using MRI.

Diazepam caused drowsiness and inactivity in the rats lasting up to 6 h after the MCAO. The core body temperature was controlled up to 7 h, and there were no significant differences between experimental groups (Table 4). Further, other physiological parameters monitored were similar between the groups (Table 5). Diffusion-weighted and perfusion imaging after 30 min of MCAO demonstrated an ongoing acute ischaemic episode without differences in the size of hypoperfusion regions between the two groups (vehicle: 38.7 ± 1.0 mm^2, diazepam: 37.7 ± 5.8 mm^2; $p = .74$). Additionally, there were no statistical differences in D_{AV} values, either in the acute phase or with 48 h postischaemia.

Diazepam treatment did not cause any significant reduction in the infarct volumes (Table 6) or any changes in the T_2 relaxation times (in the ipsilateral or contralateral hemispheres) when compared with the control group. In the ipsilateral hemisphere, the T_2 relaxation times were 86.2 ± 5.2 (vehicle) and 88.2 ± 5.3 (diazepam), and in the contralateral hemisphere, the values were 53.9 ± 1.8 (vehicle) and 54.4 ± 1.5 (diazepam).

Table 4. Core temperature (°C) in rats treated with vehicle or diazepam after transient occlusion of the middle cerebral artery (IV).

Time after administration	Vehicle (n=5)	Diazepam (n=5)
0	37.2 ± 0.5	37.4 ± 0.3
1 h	37.3 ± 0.6	37.5 ± 0.9
2 h	37.4 ± 0.5	37.1 ± 0.2
3 h	37.2 ± 0.3	37.0 ± 0.1
4 h	37.2 ± 0.3	37.1 ± 0.2
5 h	37.1 ± 0.2	37.0 ± 0.2
6 h	37.0 ± 0.2	37.0 ± 0.2
7 h	37.1 ± 0.1	37.1 ± 0.1
24 h	38.0 ± 0.3	37.9 ± 0.3
48 h	37.6 ± 0.5	37.4 ± 0.3

Vehicle or drug was administered 30 min and 90 min after the onset of occlusion. All values are represented as mean ± SD. The number of rats is given in parentheses. There were no statistically significant differences between groups when analysed by analysis of variance (ANOVA) for repeated measures.

Table 5. Physiological variables during transient occlusion of the middle cerebral artery in rats in the publication IV.

Variable	Before occlusion	30 min after occlusion	80 min after occlusion
PaO_2 (mmHg)			
Vehicle	137 ± 22	108 ± 12	102 ± 14
Diazepam	147 ± 13	127 ± 12	112 ± 18
$PaCO_2$ (mmHg)			
Vehicle	40 ± 3	41 ± 9	46 ± 11
Diazepam	43 ± 3	39 ± 5	44 ± 12
pH			
Vehicle	7.40 ± 0.01	7.38 ± 0.05	7.33 ± 0.03
Diazepam	7.40 ± 0.03	7.37 ± 0.04	7.34 ± 0.05
MAP (mmHg)			
Vehicle	93 ± 6	103 ± 9	92 ± 9
Diazepam	96 ± 5	100 ± 8	93 ± 5
B-glucose (mM)			
Vehicle	6.5 ± 0.3	5.6 ± 0.5	6.1 ± 0.7
Diazepam	6.2 ± 0.3	5.7 ± 0.4	6.3 ± 0.6

PaO_2 indicates partial arterial oxygen pressure, *$PaCO_2$* partial arterial carbon dioxide pressure, *MAP* mean arterial blood pressure. All values are presented as mean ± SD. There were no statistically significant differences between groups when analysed by analysis of variance (ANOVA) for repeated measures.

Table 6. Infarct volumes (mm^3) quantified from T_2-weighted MRI 48 h after transient occlusion of the middle cerebral artery in rats (IV).

	Hemisphere	Cortex
Vehicle (n=5)	260.9 ± 81.3	132.1 ± 97.2
Diazepam (n=5)	294.7 ± 114.5	126.9 ± 59.2

Values are expressed as mean ± SD. The number of rats is given in parentheses. There were no statistically significant differences between groups.

6. DISCUSSION

6.1. Methodological aspects

6.1.1. Global cerebral ischaemia (I, III)

The global cerebral ischaemia model used in these present experiments (**I, III**) was the gerbil bilateral common carotid artery occlusion (CCAO) for 5 min. It is an extensively used model because of its simple technical procedures and reproducibility. The gerbil CCAO model also provides a relatively constant and quantifiable neuropathological change, i.e. a typical pattern of selective neuronal death, which makes possible, for example, the comparison of potentially therapeutic agents. The neuroprotective effect is often evaluated as the degeneration degree of the hippocampal CA1 pyramidal cells (0, no degenerated neurons; 1, <10% of neurons degenerated; 2, 10-50% of neurons degenerated; >50% of neurons degenerated). However, this kind of evaluation system might prove too indiscriminate in cases where the differences are small. Therefore, we evaluated the protective efficacy of dexmedetomidine (**I**) by counting the degenerated neurons in a grid (0.08 mm^2) in the CA1 and CA3, and all degenerated neurons in the hilus. In the selegiline study (**III**), on the other hand, this was not possible because of the different survival times (from one week to two weeks), and thus the damage was assessed in degrees.

One critical physiological variable in this model is the brain temperature, which has been reported to fall 2-4°C during 5 min ischaemia without heating (Mitani and Kataoka, 1991). Additionally, a 2°C reduction in the intraischaemic brain temperature has been shown to provide significant protection against ischaemic injury (Busto et al., 1987; 1989; Mitani and Kataoka, 1991), though the postischaemic hypothermia fails to protect (Welsh and Harris, 1991), and further, after CCAO gerbils show a mild prolonged hyperthermia for hours (Colbourne and Corbett, 1995). In the present experiments during the CCAO, the core temperature of the animals was maintained at 37°C with a thermoregulatory heating unit. In the dexmedetomidine study (**I**) also head temperatures were controlled in an additional animal group. Even when the physiological parameters have been strictly controlled, some variability in the outcome has still been reported in all of the global models (Pulsinelli and Brierley, 1979; Pulsinelli et al., 1982a; 1982b; Blomqvist et al., 1984; Smith et al., 1984a; Crain et al.,

1988; Schmidt-Kastner and Hossmann, 1988; Schmidt-Kastner et al., 1989). This discrepancy might be due to interanimal anatomical and physiological differences, i.e. variations in the cerebral circulation.

Although delayed neuronal death after a brief forebrain ischaemia has been found to occur over 24 to 72 hours, a one week (or two weeks in one of the selegiline study groups) survival time was chosen in order to assure that the possible therapeutic effect would not simply be a postponed maturation of neuronal damage. It is possible that even longer survival times might be required, since it has recently been reported that in several studies well documented neuroprotective agents (the NMDA receptor antagonist, dizocilpine, the AMPA receptor antagonist, NBQX, and the calcium N-type channel antagonist SNX-111) merely slow down the degenerative process in the CA1, such that after 28 days there are no differences when compared to saline treatment (Valtysson et al., 1994; Colbourne et al., 1999).

6.1.2. Focal cerebral ischaemia (II, IV)

The intraluminal thread method of rat middle cerebral artery occlusion (MCAO) (Koizumi et al, 1986; Longa et al., 1989), used in the present experiments (**II, IV**), has become a widely accepted model to study the pathophysiology and therapeutic approaches in transient and permanent focal cerebral ischaemia because of the accurate induction of reperfusion and the simplicity of the technical procedure that leaves cranium intact and therefore ensures minimal disturbance to the intracranial pressure, blood-brain barrier permeability and brain temperature. In the rat MCAO, the ischaemic core is located in the basal ganglia and the actual target for therapeutic interventions, the penumbra, in the parietal cortex (Nagasawa and Kogure, 1989).

The proximal MCA is also clinically one of the most common places where thromboembolic occlusions occur (Mohr et al., 1986; Karpiak et al., 1989). Further, transient MCAO is probably the closest to the clinical situation since early reperfusion has been demonstrated in up to 50% of stroke patients with repeated angiography (Saito et al., 1987). On the other hand, both permanent and transient options are needed to investigate different aspects of the neurodegenerative process, i.e. ischaemia-induced and reperfusion-induced damage. The difference in outcome between transient and permanent MCAO was evident in our study (**II**).

Larger infarct volumes of the entire hemisphere, striatum as well as cortex were detected following permanent occlusion compared with transient MCAO of 90 min. These findings are consistent with the previous study of Zhang et al. (1994) where transient 2 h and permanent MCAO were compared and transient occlusion produced a smaller cortical infarct size up to 48 hours.

Although widely used, the rat MCAO model has also been suggested to result in undesirable interanimal variability in the severity of ischemic injury caused by intra and postischaemic hyperthermia, inadvertent subarachnoid haemorrhage, intraluminal thrombus formation, inaccurate filament insertion and subsequent inadequate MCAO (Longa et al., 1989; Nagasawa and Kogure, 1989; Memezawa et al., 1992; 1995; Bederson et al., 1995; Zhao et al., 1994; Schmid-Elsaesser, 1998). These methodological problems were investigated in a recent study and the results showed that subarachnoid haemorrhage and inadequate occlusion were two significant problems (Schmid-Elsaesser, 1998). In our dexmedetomidine study (**II**), we observed subarachnoid haemorrhage in three animals and excluded them from the study. The thread was advanced carefully until a clear resistance was felt, or at 22 mm from the CCA bifurcation, resulting in relatively constant infarction volumes in the saline-treated animals. In the transient MCAO model, spontaneus postischaemic hyperthermia, probably due to hypothalamic damage and increasing neuronal destruction, has been reported up to 48 hours resulting in clearly larger infarctions (Li et al., 1999; Reglodi et al., 2000). In the present MCAO studies, head and/or body core temperatures were controlled during the occlusion (**II, IV**) and up to 7 hours postischaemia (**IV**), and additionally at 24 and 48 hours postischaemia. The control animals were normothermic during this period of time.

In addition to methodological problems, also variations in collateral blood supply might contribute to the inconsistencies in the extent of the infarction in rats. There is no documentation of anatomic MCA variations in the Wistar rats but it is likely that they do exist, since Sprague-Dawley rats have been reported to have evident variations (Rubino et al., 1988; Fox et al., 1993).

6.2. Effect of α_2-adrenergic agonism in cerebral ischaemia (I, II)

The present data indicate that the α_2-adrenoreceptor agonist, dexmedetomidine, attenuates delayed neuronal damage in the gerbil global ischaemia model but does not reduce infarction size in the transient or permanent focal ischaemia models of the rat. Dexmedetomidine at a dose of 3 µg/kg given subcutaneously 30 min before and 3, 12, 24, and 48 after CCAO significantly protected the neurons of hippocampal CA3 and dentate hilus. Drug administration starting only after CCAO did not prove to be beneficial, and the higher dose, 30 µg/kg pre and postischaemia, resulted in a decrease of neuronal death only in the hilus. In the transient focal ischaemia model, dexmedetomidine at a total dose of 15 µg/kg given intravenously at the beginning of MCAO resulted in only a slight non-significant trend towards reduced infarct volumes. In the permanent MCAO model, the same dose caused even worse damage compared to the control animals.

In the gerbil model of global ischaemia, neuronal damage is usually evaluated in the CA1 region since the pyramidal cells of that region are the most vulnerable neurons to a brief global ischaemic period (Brierley, 1976; Pulsinelli et al., 1982a). However, we detected no reduction in neuronal death of the CA1 region. This could be explained by the differences in anatomic connections of neurons in various hippocampal subfields. The noradrenergic afferents arising from the locus coeruleus form a dense network within the hilus of the dentate gyrus and in the stratum lucidum of the CA3 area (Swanson et al., 1987). On the other hand, the CA1 region receives clearly less extensive, noradrenergic input mainly in the stratum lacunosum moleculare. Thus, any therapeutic treatment targeting the noradrenergic system is likely to have more efficacy in the hilus and in the CA3 subfield.

Previously, dexmedetomidine has attenuated neuronal damage in the rat incomplete (Hoffman et al., 1991) and rabbit focal (Maier et al., 1993) ischaemia models, and now in the present work with gerbil global ischaemia model. The fact that also contradictory results have been obtained, in severe forebrain ischaemia of the rat (Karlsson et al., 1995), and now in our transient and permanent MCAO models, suggests that dexmedetomidine might be neuroprotective only in certain experimental models and under specific conditions. For instance, in the rabbit focal ischaemia model, infarct volumes were evaluated after only 6 hours of reperfusion (Maier et al., 1993) in contrast to 48 hours in our present study. Furthermore, there is no clear evidence that the reduction of ischaemic damage by

dexmedetomidine is a direct cause of reduced cerebral sympathetic activity. Systemic administration of α_2-agonist results in complex changes in a variety of physiological parameters, some of them are beneficial and some damaging to an ischaemic brain. The protective effect/lack of protection might, therefore, be caused by these changes; or as a combination with direct cytoprotection by reduced sympathetic tone.

Earlier studies with dexmedetomidine have suggested that inhibition of cerebral catecholaminergic neurotransmission and subsequent reduction of sympathetic activity of ischaemic neurons are likely to be the neuroprotective mechanisms. One of the first responses to cerebral ischaemia is a massive release of excitatory neurotransmitters, including noradrenaline, in addition to glutamate (Globus et al., 1988; 1989). Dexmedetomidine attenuates this ischaemia-induced elevation of noradrenaline release (Matsumoto et al., 1993), and this could at least partly explain the protective effect, assuming that the increased noradrenaline release essentially contributes to ischaemic damage. A further potentially protective effect by α_2-agonists is direct reduction of hypoxia-evoked glutamate release, which was demonstrated *in vitro* (Bickler and Hansen, 1996; Talke and Bickler, 1996). On the other hand, dexmedetomidine did not alter changes in calcium levels mediated by glutamate receptor stimulation under hypoxic conditions (Talke and Bickler, 1996). Finally, inhibition of oxidative deamination of catecholamines can reduce free radical formation (Simonson et al., 1993; Suzuki et al., 1995), which might subsequently attenuate reperfusion injury after transient MCAO.

Dexmedetomidine systemically administered has symptomatic effects that might also provide desirable protection in cerebral ischaemia. In fact, it has lately been tested in numerous clinical trials where its various symptomatic effects have proved to be advantageous for surgical patients and for critically ill patients in the intensive care unit (Duke et al., 1998). In our present focal ischaemia work, dexmedetomidine clearly produced per and postischaemic haemodynamic stability, i.e. it decreased blood pressure during the occlusion without producing hypotension which is known to enhance the extent of ischaemic damage (Zhu and Auer, 1995). It also prevented the fluctuation of blood pressure which normally occurs at the beginning of reperfusion in the transiently occluded animals compared to ischaemic control animals. The decrease in blood pressure was most marked after the dexmedetomidine bolus at the beginning of occlusion. At the same time point in the ischaemic controls, blood pressure typically started to increase resulting in persistent hypertension until the MCA suture was

removed. An additional potentially protective effect of dexmedetomidine could be the reduction of ischaemia-induced brain oedema and elevated intracranial pressure via ameliorated diuresis. It has dose-dependent diuretic, natriuretic and kaliuretic effects in rats at doses higher than 10 μg/kg s.c. (Virtanen, 1988). We detected an apparent increase in diuresis after the dose of 15 μg/kg. Furthermore, sedation (Aantaa et al., 1990), anxiolysis (Aho et al., 1992; Scheinin et al., 1993) and analgesia (Aho et al., 1991) produced by dexmedetomidine are also desirable effects in a stroke patient.

On the other hand, dexmedetomidine has some less beneficial symptomatic effects in cerebral ischaemia, that could explain why it has not proved to be unambiguosly neuroprotective in all experimental models. Hyperglycemia preceding ischaemia (Wass and Lanier, 1996) as well as ischaemic hyperglycemia (Kawai et al., 1997) have been reported to enhance ischaemic damage, and dexmedetomidine has previously been shown to increase the blood glucose concentration (Hoffman et al., 1991). Our results support this finding: the glucose concentration was significantly increased during the occlusion after the higher dose (15 μg/kg) in both transient and permanent MCAO models, as well as in the global ischaemia model after administration of 3 μg/kg dexmedetomidine.

Additionally, one explanation why dexmedetomidine failed to protect especially in the permanent occlusion model might be the fact that platelets possess α_2-receptors (Anfossi et al., 1996). Although α_2-agonism alone does not increase platelet hyperaggregability, it enhances the stimulation induced by true agonists (e.g., thrombin) (Steen et al., 1993), and in this model introducing a nylon suture into the lumen of artery might quite possibly cause activation of these platelet activators, especialy when the suture is left in the lumen for 24 hours. Another problem in this ischaemia model is halothane anesthesia and its combined effects with dexmedetomidine treatment. Halothane is known to increase intracranial pressure in neurosurgical patients (Jorgensen and Misfeldt, 1975) as well as in different experimental animals, especially after acute brain injury (Kaieda et al., 1989; Artru, 1983). This effect might weaken the possible anti-oedematic influence of dexmedetomidine via enhanced diuresis. Also, halothane itself decreases sympathetic tone. Perhaps the combination of halothane with α_2-agonism meant that the sympathtetic tone was too low to provide protection to the ischaemic brain.

6.3. Effect of selegiline in cerebral ischaemia (III)

In our present study, the MAO-B inhibitor, selegiline did not significantly reduce neuronal death in the gerbil hippocampal CA1 area when administered either repeatedly before the ischaemic insult, acutely after, or repeatedly after a short period of global ischaemia. However, a modest reduction of neuronal damage was detected in all treatment groups. Previously, selegiline has been shown to have neuroprotective and neuronal rescue effects against neurotoxins in various *in vitro* and *in vivo* models (Wu et al., 1995; Zhang et al., 1996; Salonen et al., 1996). It has also enhanced the survival of neurons subsequent to axotomy, nerve crush, or global cerebral ischaemia (Salo and Tatton, 1992; Ansari et al., 1993; Lahtinen et al., 1997). On the other hand, there are also conflicting results in ischaemia models. Selegiline decreased ischaemia-induced necrosis in the striatum and thalamus, but not in the cortex or hippocampus, when administered before the insult (Matsui and Kumagae, 1991; Knollema et al., 1995). Ballabriga and co-workers (1997) reported that selegiline given intraperitoneously at a dose of 10 mg/kg had no neuroprotective effects in a gerbil global ischaemia model when administered either 1 h after or 2 h before the insult with administration continued for 4 days thereafter.

Previous experimental studies with selegiline have suggested that the possible neuroprotective mechanism is not related to MAO-B inhibition since other MAO-B inhibitors do not enhance neuronal survival after axotomy (Ansari et al., 1993). Furthermore, selegiline has protected against neurotoxin-induced apoptosis in a dopaminergic cell line that did not contain MAO-B (Le et al., 1997).

The prophylactic administration of selegiline has previously resulted in neuroprotection in the rat thalamus and striatum, but not in the hippocampus or cortex (Matsui and Kumagae, 1991; Knollema et al., 1995). One possible explanation for the lack of protection in the hippocampus might be due to regional differences in free radical scavenger enzymes, such as SOD and catalase (Carrillo et al., 1992). Also, the doses of selegiline in the studies showing thalamic and striatal protection (Matsui and Kumagae, 1991; Knollema et al., 1995) were 8 to 40 times higher than those used in our present study, suggesting that higher concentrations enlist additional mechanisms to help provide neuroprotection, such as down-regulation of β-adrenoreceptors, which are receptors that normally enhance the excitatory effects of glutamate (Nicoll et al., 1987; Paetsch and Greenshaw, 1993).

The relatively low MAO-B activity in the hippocampus (Youdim and Finberg, 1994) might explain why long-lasting MAO-B inhibition using a single dose of selegiline did not provide significant protection in the hippocampus. Perhaps a reduction in the free radical production resulting from a decline in dopamine degradation by MAO-B inhibition more adequately explains the neuroprotective effects of selegiline in the other brain regions. Additionally, peroxyl-radical (Thomas et al., 1997) and ·OH-radical (Wu et al., 1993) trapping by selegiline might not reduce oxidative stress to such an extent that significant protection could be observed in the hippocampus.

Postischaemic selegiline treatment did not significantly reduce neuronal damage in the hippocampus. This result is not consistent with the claim that selegiline rescues neurons through induction of synthesis of trophic factors (Biagini et al., 1994; Ekblom et al., 1994; Semkova et al., 1996). After hypoxic brain injury, there is a major increase in NGF synthesis in the cerebral cortex and hippocampus (Lorez et al., 1989), and several studies have shown that NGF has protective actions on hippocampal neurons (Cheng and Mattson, 1991; Pechan et al., 1995). It is not clear why postischaemic selegiline treatment in the present study did not significantly prevent neuronal death of CA1 pyramidal cells. On the other hand, it is not unambiguously certain that ischaemia-induced delayed neuronal death in the CA1 region is purely apoptotic in nature (Collins et al., 1992; Héron et al., 1993, MacManus et al., 1993; Choi et al., 1996; van Lookeren Campagne et al., 1996; Colbourne et al., 1999). Perhaps both prophylactic and postischaemic treatments are required to achieve significant protection. Indeed, Semkova et al. (1996) demonstrated that the neuroprotective activity of selegiline requires an accumulation of NGF.

The present data showed merely a trend towards reduced neuronal damage, which taken in conjunction with previous partly conflicting results, suggests that selegiline may have a role in neuroprotection against ischaemia-induced delayed neuronal death of gerbil hippocampal CA1 pyramidal cells. Since the reduction in neuronal damage was similar after prophylactic and postischaemic treatments, it is probable that several mechanisms might contribute to the neuroprotective effects.

6.4. Effect of enhanced $GABA_A$ receptor activity after transient MCAO (IV)

The present data show that postischaemic administration of the benzodiazepine, diazepam (2 x 10 mg/kg) does not provide neuroprotection against focal cerebral ischaemia in rats. Diazepam potentiates GABAergic neurotransmission acting through a specific benzodiazepine binding site located on the GABA ionophore receptor complex. The majority of GABAergic, potentially neuroprotective effects are mediated through $GABA_A$ receptors, resulting in chloride channel opening and subsequent membrane hyperpolarization (Olsen et al., 1984), inhibition of cytosolic free radical formation (Schwartz et al., 1992), promotion of ATP recovery and prevention of cytochrome *c* release from mitochondria (Galeffi et al., 2000), reduction of cerebral metabolic rate for glucose (Kelly and McCullogh, 1983), and vasodilatation of cerebral blood vessels (Edvinsson and Krause, 1979).

Previous studies have suggested that agents enhancing GABAergic activity might reduce neuronal damage following global forebrain ischaemia. These agents include pentobarbital, valproic acid, No-328, diazepam, PNU-101017, imidazenil, muscimol, and chlormethiazole (Sternau et al., 1989; Johansen and Diemer, 1991; Shuaib et al., 1993; 1995; Schwartz et al., 1994; 1995; Hall et al., 1997; Schwartz-Bloom et al., 1998). Also, after transient (Sydserff et al., 1995b; Lyden, 1997) and permanent (Sydseff et al., 1995a) MCAO in the rat, $GABA_A$ receptor agonists have been shown to be able to reduce infarct volumes.

We did not detect any neuroprotective effect with diazepam in ischaemia with reperfusion damage, although one would have anticipated that enhancement of GABA effects might limit the spread of neuronal damage in the penumbra. This is because the damage mechanism in penumbra may be similar (Hossmann, 1994) as in the selectively vulnerable areas of global ischaemia (Pulsinelli et al., 1982), where diazepam has been shown to be neuroprotective (Huff and Schwartz, 1991; Schwartz et al., 1994; 1995; Sternau et al., 1989). GABA is thought to be the main factor which can counteract the excitatory effects (Roberts, 1974), a process with a well established role in the cell death pathway (Rothman, 1983; Rothman et al., 1987). There is additional evidence for impaired $GABA_A$ receptor function in the hippocampus after global ischaemia (Verheul et al., 1993) and bilaterally in the neocortex after permanent MCAO (Qü et al., 1998). Furthermore, after the initial massive GABA release, which has been well documented in several studies (Hillered et al., 1989; Kanthan et al., 1995; Uchiyama-Tsuyuki et al., 1994), there may ensue some feedback inhibition and

down-regulation of the GABA system (Green et al., 1987). Decreased GABA synthesis (Green et al., 1992) and release may also explain why neuroprotection was not detected in the present study. Interestingly, in contrast to $GABA_A$ receptor agonists, benzodiazepines are able to enhance GABA function only in the presence of endogenous GABA (Tallman et al., 1980).

Another reason why diazepam may be neuroprotective in global but not in focal brain ischaemia, is that GABAergic interneurons in the hippocampus are unaffected by transient forebrain ischaemia (Francis and Pulsinelli, 1982; Nitsch et al., 1989). In contrast, striatal GABAergic neurons are damaged following transient ischaemia (Francis and Pulsinelli, 1982). Inglefield et al. (1997) showed that CA1 hippocampal interneurons express structural abnormalities after transient cerebral ischaemia despite their long-term survival, and that the ischaemic injury of dendrites can be prevented by diazepam. It may well be that this kind of neuroprotection by diazepam involves only the hippocampus, and not other brain structures.

One possible explanation for previously demonstrated neuroprotection might be the alteration in body temperature occurring after diazepam administration. Diazepam (10 - 20 mg/kg) is known to cause substantial hypothermia (2 - 3 °C) (Elliot and White, 2001), and this may provide secondary protection of neurons from ischaemic damage (Busto et al., 1987). In the transient MCAO model, postischaemic body temperature may be elevated for up to 48 hours, possibly due to hypothalamic damage and ongoing neuronal destruction (Li et al., 1999; Reglodi et al., 2000). If not controlled, diazepam might counteract the hyperthermia and in this way reduce neuronal damage. A recent work by Dowden and co-workers (1999) supports this theory. They demonstrated in a global ischaemia model that when the postischaemic temperature was regulated, histologic as well as behavioral protection by diazepam was abolished. On the other hand, hypothermia as a sole neuroprotective mechanism remains to be confirmed, since neuroprotection has been reported even after direct infusion of diazepam into the hippocampus (Schwartz et al., 1995) as well as in hippocampal slices (Galeffi et al., 2000).

The search for a convenient and safe neuroprotectant in acute brain infarct is still ongoing. Benzodiazepines have been clinically used for decades and are pharmacologically well defined and do not evoke any severe adverse effects (Tallman et al., 1980). They might therefore offer a safer alternative to glutamate antagonists, which have so far all been rather toxic (Grotta, 1995, Albers et al., 1995; Turrini, 1996; Hickenbottom and Grotta, 1998).

Presently, diazepam is also being tested in a multicenter clinical trial, Early GABA-ergic Activation Study In Stroke (EGASIS) (Lodder, 2002). A feasibility study of 104 stroke patients showed that 10 mg of diazepam twice daily for 3 days was well-tolerated (Lodder et al., 2000). Regretably, the contribution of possible hypothermia to these results will remain unknown since body temperature can not be monitored or controlled in a clinical setting with severely ill patients (Lodder personal communication).

7. CONCLUSIONS

1. Dexmedetomidine, an α_2-adrenoreceptor agonist, may have neuroprotective efficacy in global cerebral ischaemia, since it ameliorates neuronal damage in the hippocampal CA3 region and hilus of the dentate gyrus when its administration is started before occlusion and extended up to 48 hours postischaemia (**I**).

2. After transient and permanent focal ischaemia, dexmedetomidine does not reduce infarct volumes, after permanent MCAO, on the contrary, it increased the damage. On the other hand, dexmedetomidine has several symptomatic effects that might be desirable in the treatment of stroke patients. These include haemodynamic stabilization, attenuation of cerebral oedema via enhanced diuresis, sedation, anxiolysis, and analgesia (**II**).

3. The MAO-B inhibitor, selegiline, which also clearly possesses several potentially neuroprotective MAO-B unrelated effects, does not improve neuronal survival in the global cerebral ischaemia. By using different doses and drug administration schedules, the possibilities of free radical scavenging, long-lasting MAO-B inhibition and acute free radical reduction, as well as neuronal rescuing as underlying salvaging mechanisms of selegiline were explored (**III**).

4. GABAergic activity enhancement by diazepam does not reduce neuronal damage after transient focal cerebral ischaemia when normothermia is maintained (**IV**).

In summary, these present studies provide new information concerning neuroprotection in experimental acute cerebral ischaemia. None of the tested agents was clearly protective, although with dexmedetomidine indications for neuroprotectivity in the global cerebral ischaemia, and with selegiline a non-significant trend towards attenuated neuronal damage were detected. On the other hand, diazepam, a drug which previously in several global cerebral ischaemia studies has been shown to offer neuroprotection, clearly failed to protect after transient focal cerebral ischaemia when drug-induced hypothermia was prevented.

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