Ischemic Cell Death in the CNS - applications of a new in vitro model

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Ischemic Cell Death in the CNS

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We have used the organotypic tissue culture from mouse hippocampus to establish a new model of *in vitro* ischemia (IVI). Similar to previous models we combine anoxia and aglycemia but in addition we apply a combination of ions similar to what is found in the brain extracellular fluid during ischemia. We found that the combination of a high potassium level (70mM), a low calcium level (0.3mM) and acidosis (pH 6.8) during IVI made the pattern of cell death more similar to what is found following global ischemia *in vivo* in that became more delayed and selective. In addition, a high level of glucose was found to increase cell death in this new model in contrast to what had previously been found in other cell culture models of ischemia but in similarity to what is found *in vivo*. We next characterized the models of IVI and hyperglycaemic IVI on some important aspects.

While cell death following IVI could be completely prevented by the withdrawal of extracellular calcium during the insult or antagonists of glutamatergic NMDA-receptors, no effect of either was found in the hyperglycaemic IVI paradigm. On the other hand, intracellular calcium chelation prevented against cell death following hyperglycaemic IVI but not IVI. Inhibition of free radicals was ineffective in both paradigms. These findings in combination with other recent findings using the same models, illustrates that IVI and hyperglycaemic IVI induces two different patterns of cell death both of which may be important during ischemia *in vivo*.

In a separate study we showed that the pyramidal neurons in the hippocampal CA3-region were significantly more protected against IVI than those in CA1. Acidosis inhibited NMDA-receptors in both regions but they recovered significantly slower in the CA3-region. This prolonged inhibition could contribute to the sparing of the CA3-neurons following IVI and global ischemia.

The neuromodulator adenosine inhibits glutamate release by the activation of presynaptic A_1-receptors. We used transgenic A_1-receptor knock-out mice to study the importance of this receptor for the development of cell death following IVI and global ischemia *in vivo*. No effect of the knock-out was found in any of the two paradigms. The A_1-receptor antagonist, 8-CPT increased damage *in vivo* but had no effect *in vitro*. This discrepancy between the models could be explained by a less importance of vesicular glutamate release *in vitro* or an undiscovered systemic side-effect of 8-CPT *in vivo*.

The described models of IVI and hyperglycaemic IVI are well suited for further studies on the pathophysiology of cerebral ischemia using transgenic, pharmacological, electrophysiological and imaging techniques.

Key words: Brain ischemia, cell culture, acidosis, hyperglycaemia, calcium, free radicals, NMDA, adenosine, neuroprotection

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Ischemic Cell Death in the CNS

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To Carin

One never notices what has been done;
one can only see what remains to be done.

Marie Curie

Contents

Original Articles

This thesis is based on the following papers, which are referred to in the text by their respective roman numerals (I-V).

I. Rytter A*, Cronberg T*, Asztély F, Nemali S, Wieloch T. (2003) Mouse hippocampal organotypic tissue cultures exposed to *in vitro* "ischemia" show selective and delayed CA1 damage that is aggravated by glucose. *J Cereb Blood Flow Metab* 23:23-33

II. Cronberg T*, Rytter A*, Asztély F, Söder A, Wieloch T. (2004) Glucose but not lactate in combination with acidosis aggravates ischemic neuronal death *in vitro*. *Stroke* 35:753-757

III. Cronberg T, Wieloch T. Calcium, but not nitric oxide or free radicals, affects cell death after ischemia and hyperglycaemic ischemia in murine hippocampal slice cultures. *Manuscript*

IV. Cronberg T, Jensen K, Wieloch T. Selective vulnerability to ischemia in hippocampal slice cultures is a pH-dependent process. *Manuscript*

V. Olsson T, Cronberg T, Rytter A, Asztély F, Fredholm B, Smith ML, Wieloch T. Deletion of the Adenosine A_1 receptor gene does not alter neuronal damage following ischemia *in vivo* or *in vitro*. *Manuscript*

* Equal contribution

List of Abbreviations

8-CPT	8-cyclopentyl-theophylline
A_1R	adenosine 1 receptor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASA	acetyl salicylic acid
ATP	adenosine triphosphate
CA1-3	cornu ammonis 1-3, neuronal regions of the hippocampus
CNS	central nervous system
aCSF	artificial cerebrospinal fluid
iCSF	"ischemic"cerebrospinal fluid
AMP	adenosine monophosphate
APV	D-2-amino-5-phosphonopentanoic-acid
BAPTA-AM	1,2-bis(2-amino-5-fluorophenoxy)ethane-N,N,N´,N´-tetraacetic acid tetrakis (acetoxymethyl) ester
DG	dentate gyrus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EPSC	excitatory postsynaptic currents
EPSP	excitatory postsynaptic potentials
GABA	γ-aminobutyric acid
GluR 1-4	subunits of AMPA-receptors
HBSS	Hank´s balanced salt solution
IVI	*in vitro* ischemia
L-NAME	N-nitro-L-arginine methyl ester hydrochloride
MEM	minimal essential medium
MCA	the middle cerebral artery
MFI	mean fluorescence intensity
MPT	mitochondrial permeability transition
NAC	N-acetyl-L-cysteine
NBQX	2,3-dihydro-6-nitro-7-sulphamoyl-benzo(F)quinoxaline'
NMDA	N-methyl-D-aspartate
NR1-NR3A	subunits of NMDA-receptors
OGD	oxygen and glucose deprivation
PBN	N-tert-butyl-α-phenylnitrone
PCR	polymerase chain reaction
PI	propidium iodide
SOD	superoxide dismutase
rTPA	recombinant tissue plasminogen activator
TGA	transient global amnesia
VGCC	voltage-gated calcium channels

Summary

Ischemic brain damage is a common cause of death and disability in the western world. A global ischemic insult is usually the result of a transient cardiac arrest while the occlusion of a cerebral blood vessels leads to a focal ischemic lesion, commonly termed stroke. During the last decades our knowledge about the metabolic and cellular events leading to cell death following ischemia has expanded mainly due to experimental studies *in vivo* and *in vitro*. Important findings concerning the relevance of body temperature and blood glucose levels have been confirmed in humans. However, a vast number of pharmacological agents with protective effects in animal models of ischemia have failed in subsequent clinical trials. This illustrates that our knowledge of the mechanisms of ischemic cell death is still incomplete and that we need to question the models we use to mimic the human disorders.

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The described models of IVI and hyperglycaemic IVI are well suited for further studies on the pathophysiology of cerebral ischemia using transgenic, pharmacological, electrophysiological and imaging techniques.

Background

Ischemic cell death in the brain – a clinical perspective

The term ischemia (Gr. *ischein* to suppress + *haima* blood) means a lack of blood supply. Ischemic injury to the brain can occur in two, principally different, modes.

A complete cessation of blood flow to the brain is termed ***global ischemia*** and is most commonly the result of a cardiac arrest and the subsequent circulatory failure. Cardiac disease is responsible for approximately 2/3 of all out-of-hospital sudden deaths (Holmberg et al. 1999). In spite of modern ambulance health care with trained personal and defibrillators, the prognosis after out-of-hospital cardiac arrest is still grave with a one-month survival of only 5% (Holmberg et al. 2000). Even if a return of spontaneous circulation is accomplished, a majority of patients will either succumb due to the subsequent development of ischemic brain injury or suffer from a persisting neurological disability (Jørgensen and Holm 1998). As the first neuroprotective treatment for global ischemic brain damage, induced hypothermia has a proven effect, diminishing disability and death (Bernard et al. 2002; The Hypothermia After Cardiac Arrest Study Goup 2002), and is now being implemented in the clinic.

Stroke is the clinical term for a sudden onset of neurological symptoms caused by a vascular incident. In the majority of patients, the underlying cause is a vascular occlusion in the arterial cerebral circulation with subsequent ***focal ischemia*** in the territory supplied by the particular blood vessel. The cause of a stroke could also be an intracerebral or subarachnoid haemorrhage. However, ischemia due to compression of the surrounding tissue or vasospasm is frequently a complicating factor. Stroke is a common disease, second only to cancer and cardiac disease as a cause of death and disability in the industrialized world (1989). The incidence of stroke varies between countries but is roughly between 100 and 180 per 100 000 age-adjusted person-years (Johansson et al. 2000; Wolfe et al. 2000) in Europe. Stroke is mainly a disease of the elderly and the incidence more than ten-folds in the population older than 75 years (Johansson et al. 2000; Wolfe et al. 2000).

The introduction of thrombolysis by intravenously administered recombinant tissue plasminogen activator, rTPA, is currently changing the view of stroke as a disease without a treatment to an emergency diagnosis with the highest priority. Given within 3 hours of symptom onset rTPA reduces death and disability by a restoration of blood flow to the ischemic tissue.

Unfortunately, only a small part of stroke patients will be eligible for treatment within three hours but trials are ongoing to investigate whether the therapeutic window for thrombolysis can be expanded. Acetyl salicylic acid (ASA) is an anti-platelet agent and a standard treatment for the prevention of recurrent stroke. Interestingly, ASA has a neuroprotective action through the inhibition of glutamate release in rats (De Cristobal et al. 2002). Such an effect has also been indicated in clinical trials (Chen et al. 2000; Wilterdink et al. 2001).

The neuroprotective effect of post-ischemic hypothermia clearly shows that neuroprotection is a valid concept that needs to be refined and further explored.

In spite of the great number of clinical trials no neuroprotective agent has yet been shown to be effective in the treatment of stroke (Fisher and Brott 2003; Kidwell et al. 2001). This costly failure has incited a debate on the relevance of animal models of stroke (Richard Green et al. 2003) and an academic industry roundtable consensus on recommendations for pre-clinical drug development (Stroke Therapy Academic Industry Roundtable 1999). In general, the shortcomings of clinical trials could, at least in part, be attributed to unrealistic extrapolation of pre-clinical results and/or deficiencies in the setup of the trials (Green 2002). As an example, the NMDA-receptor antagonists have a short therapeutic window in experimental studies while in the clinical studies invariably a longer window for inclusion has been used (Dirnagl et al. 1999).

Experimental models of cerebral ischemia

The understanding of the pathogenic mechanisms of ischemic cell death in the brain has in large been derived from experimental systems where whole animals or cell cultures are exposed to conditions simulating a stroke or a cardiac arrest. During the last few decades our understanding of the pathophysiology of brain ischemic cell death has increased tremendously and principally important pathophysiological findings in animals have been confirmed in humans. Examples are the selective and delayed CA1-cell death after global ischemia, the aggravating effect of pre-ischemic hyperglycaemia in ischemia with reperfusion and the therapeutic window for reperfusion (see below).

Whole-animal (In vivo) models of cerebral ischemia

The *in vivo* models of cerebral ischemia could be divided into the global ischemia models designed to mimic a circulatory arrest and the focal ischemia models designed to mimic an ischemic stroke. The pathogenesis of the

tissue damage in these two models differs in many fundamental aspects but comparisons between models are often fruitful.

Global ischemia models have been developed for several animal species including monkey, dog and cat but the rodent models are the most widely used. In the rat models the blood-supply of the brain is reduced by occlusion of the common carotid arteries in combination with a reduction in blood pressure (Smith et al. 1984) or an occlusion of the vertebral arteries (Pulsinelli et al. 1982a). Due to the lack of posterior communicating arteries global ischemia can be induced in the gerbil by an isolated occlusion of the common carotid arteries (Ito et al. 1975). In the mouse, variability in the presence of the posterior communicating artery necessitates precautions such as monitoring blood flow during experiment to ensure reproducible damage (Olsson et al. 2003).

A complete cessation of blood flow to the brain can only be tolerated for a brief period. A characteristic of the global ischemia models is therefore the short interval between the period of ischemia after which the animals recover fully without morphological signs of brain damage and one that produces widespread cell death in the brain and is incompatible with survival (Olsson et al. 2003; Pulsinelli et al. 1982a; Smith et al. 1984). This is in accord with the findings in humans where the minority of patients that survive a cardiac arrest in general have only minor or no neurological deficits (Stiell et al. 2003).

A characteristic feature of the global brain ischemia models is the selective neuronal vulnerability of certain brain regions such as the neocortical layers III and IV and the CA1-region of the hippocampus (Pulsinelli et al. 1982a; Smith et al. 1984). The fact that the CA1-neurons degenerate while the neighbouring and morphologically similar CA3-neurons survive has stimulated a number of comparative studies but as yet the reason is unclear. Another important feature of the cell death in the CA1-region is the delayed fashion by which it occurs. Thus, in gerbils and rats, neurons in the CA1-region are morphologically intact during the first 24-36 hours after the insult but then degenerate (Kirino 1982; Pulsinelli et al. 1982a; Smith et al. 1988). In the mouse this delay seems to be somewhat shorter with cell death appearing after 12 hours and maturing at 48 hours (Olsson et al. 2003). Also in humans a selective and delayed cell death in the CA1-region has been documented after cardiac arrest (Petito et al. 1987). Theoretically, an extensive therapeutic window should therefore exist and this has been confirmed by the protective effect of post-ischemic hypothermia instituted as late as 12 hours after the insult in rodents (Coimbra and Wieloch 1994).

The rodent experimental models of focal ischemia commonly involve the middle cerebral artery (MCA). An important distinguishing difference

between the models of focal ischemia is whether the occlusion of the vessel is instituted in a permanent or a transient fashion and whether the central or the peripheral portion of the vessel is occluded. Thus, the MCA can be occluded at its origin from the internal carotid artery by a filament inserted through the common carotid artery (Longa et al. 1989; Memezawa et al. 1992a) or through a craniotomy where the peripheral portion of the MCA is obstructed with a clip or a suture (Buchan et al. 1992; Chen et al. 1986). Compared to the global ischemia models, the reduction in cerebral blood flow due to MCA occlusion is less severe but usually much more sustained (Siesjö 1992a). A characteristic feature of a focal ischemia is the distinction between a central part, the core, where blood flow is severely reduced and the tissue destined to succumb, and a peri-focal area with less severe ischemia, the penumbra, where the tissue could be rescued not only by reperfusion but also by neuroprotective agents and by hypothermia. In contrast to the selective neuronal cell death pattern seen following a brief global ischemia, focal ischemia engage neurons, glia and endothelial cells in a pan-necrotic lesion.

The time during which a restoration of blood flow will decrease the final infarct size differs between species and is approximately 2 hours in rats (Memezawa et al. 1992b) and 4-8 hours in primates (Crowell et al. 1981).

Cell culture (in vitro) models of cerebral ischemia

In the *in vitro* systems pathophysiological mechanisms of ischemic cell death can be studied without the influence of the cerebral circulation. Lacking the complexity of the intact organism, the *in vitro* systems instead offers the possibility to control external factors such as temperature, oxygen and nutrients. Drugs can be easily administered and the influence of different factors that affect the outcome following ischemia, such as calcium or glucose, can be investigated in isolation.

The preparations used in the *in vitro* models of cerebral ischemia can be divided into three main groups, the acute slice preparation, the dissociated cell culture and the organotypic cell culture (see (Lipton 1999)for review).

Acute slices from brain regions, frequently hippocampus, are submerged in an oxygenated physiological salt solution and are routinely accessible for electro-physiological applications. The major advantages with this preparation is that the slices can be prepared from adult animals and that major adaptive changes will not take place during the short survival time, approximately 10 hours. However, only acute phase events can be studied and the system can be regarded as slowly decaying with a continuous low-grade anoxia in the central portion of the slice.

When dissociated cell cultures are prepared from embryonic animals,

the neurons will differentiate and form synapses. Neurons can survive in culture for months and are easily studied with imaging or electrophysiological techniques. Despite these advantages, this system is particularly remote from the conditions *in vivo*.

Organotypic tissue cultures are brain slices that are grown on a cover slip in the roller-tube technique described by Gähwiler (Gähwiler 1981) or on a porous membrane according to Stoppini (Stoppini *et al*. 1991). With the roller-tube technique a monolayer of cells is formed that can easily be inspected through the glass cover slip. The Stoppini method is generally considered easier to perform. The slices grown on membranes can be inspected repeatedly during experiments and are easy to handle. Since the do not flatten to the same extent as roller-tube grown cultures they retain a three-dimensional architecture but are, for the same reason, less accessible for imaging. The increasing use of organotypic tissue cultures (Gähwiler et al. 1997) can be attributed to the preserved interaction between neuronal and non-neuronal cells and preserved neuronal wiring.

A true "ischemia" can never be accomplished *in vitro* since there is no blood in the first place. The terminology "*in vitro* ischemia" and "hyperglycaemic *in vitro* ischemia" therefore might seem somewhat misplaced. We still consider it justified using these terms to stress the couplings to the clinical conditions that we are modelling and to simplify the phraseology.

The common method to mimic ischemia *in vitro* is by the mere withdrawal of oxygen and glucose (OGD). This method was first applied in dissociated neuronal cultures by Goldberg and Choi (Goldberg and Choi 1993) but has since been widely used in acute slices and organotypic cultures. In general, oxygen is replaced by nitrogen in a glucose-free aCSF and sometimes 2-deoxy-glucose is added to inhibit glycolysis. To ensure anoxia during the insult the cultures have to be enclosed in an airtight chamber or an anaerobic incubator. To by-pass the technical difficulties maintaining anoxia, chemical hypoxia can be instituted by the blockade of the mitochondrial respiration by cyanide (Bernaudin *et al*. 1998) but this is obviously a less physiological approach.

In organotypic tissue cultures of the rat hippocampus, an selective sensitivity of the CA1-neurons to oxygen and glucose deprivation (Laake *et al*. 1999; Newell *et al*. 1995; Pringle *et al*. 1997; Strasser and Fischer 1995) and to chemical anoxia/aglycemia (Vornov *et al*. 1994) has previously been reported but the selectivity has generally been less pronounced and the insult durations much longer than *in vivo*.

Similar results with chemical anoxia/aglycemia have been obtained in murine hippocampal tissue cultures (Bernaudin *et al*. 1998).

The hippocampus

The hippocampus (sea horse) is a cortical structure and a part of the limbic system located in the central portion of the temporal lobe in humans. In rats it is relatively larger and, much like the horn of a ram (Cornu Ammonis), it lies in the medial part of the hemisphere, lateral to the caudatoputamen. The central role of hippocampus for memory consolidation is exemplified by the clinical syndrome of transient global amnesia (TGA) or the classical case report of H.M. who had a bilateral temporal lobectomy to treat an intractable epilepsy and whose ability to store any new memories was completely abolished (Eichenbaum 2003).

The neuronal wiring of the hippocampus is very complex (Amaral and Witter 1995). For practical purposes a simplified model based on the three-synaptic circuit described by Andersen (Andersen 1975) can be used to illustrate the neuronal regions relevant for the development of damage and electrophysiological measurements in this thesis. In this model, based on a transverse section of the hippocampus, only the two-dimensional and unidirectional pathways are outlined.

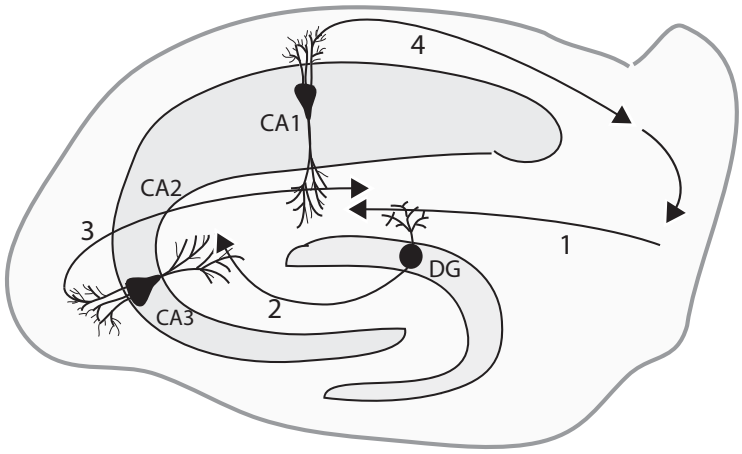

Figure 1. A simplified model of the hippocampal intrinsic circuitry
The neuronal regions CA1-CA3 (from *Cornu Ammonis*, the ram´s horn) constitute the hippocampus proper. The major projection area to the hippocampus is the entorhinal cortex from which the perforant path (1) carries impulses to the granule cells of the dentate gyrus (DG). The granule cells in turn send their axons through the mossy fiber pathway (2) to the pyramidal cells of the CA3-region. From the CA3-region the Schaffer collaterals (3) project to the pyramidal neurons in the CA1-region. The CA2-region differs from the CA3-region in that it does not receive any input via the mossy fibers. The CA1-neurons project further via the subiculum back to the entorhinal cortex. This entire loop is called the intrinsic pathway.

The synapses of the intrinsic pathway use glutamate as the transmittor substance (Storm-Mathisen 1981). The glutamate receptors of NMDA, AMPA and kainate sub-type have distinct distribution within the hippocampus (Monaghan and Cotman 1986; Pickard *et al*. 2000). The highest concentrations of NMDA-receptors are found in the CA1-area and moderate concentrations in the CA3-area and the dentate gyrus. The distribution of AMPA-receptors is similar to that of the NMDA-receptors but the kainate receptors have an inverse distribution and are mainly found in the CA3 and dentate gyrus.

The traffic through the intrinsic pathway is modulated by cholinergic septo-hippocampal fibers, serotonergic fibers from the *raphe nucleus* and noradrenergic fibers from *locus ceruleus* (Brodal 1998).

The structural organisation of the hippocampus and the fact that a complete intrinsic circuit can be obtained in a thin (200-400μm) slice, cut perpendicular to its surface, has made it a preferred brain region for electrophysiological and morphological investigations.

The hippocampal organotypic tissue culture

The neuronal subpopulations, field organization and intrinsic connections of the hippocampus *in situ* are maintained in the organotypic tissue cultures (Gähwiler 1984; Stoppini et al. 1991). In accordance, the distribution of the neuroactive amino acids GABA, glutamate, glutamine and taurine is retained (Torp *et al*. 1992). As an inevitable consequence of the dissection procedure all extrinsic connections will be lost together with the intrinsic connections projecting transverse to the axis of the cut and leading to a degree of synaptic reorganization (Stoppini et al. 1991; Zimmer and Gähwiler 1984). Buchs *et al* reported that the number of synaptic contacts in the CA1-region increase during the first month of culture similar to what is observed *in vivo* but to a slightly lower final level (Buchs *et al*. 1993). De Simoni *et al* has shown that the absolute frequence of glutamatergic post-synaptic currents are increased to about four or five-fold in organotypic slices compared to *in vivo* at three weeks of age and related this to an increased branching of dendritic spines. (De Simoni *et al*. 2003) An increasing spontaneous epileptic activity of cultures has also been reported after 28 days *in vitro* (Bergold and Casaccia-Bonnefil 1997).

The interface method leads to less thinning and a more retained three-dimensional organization compared to the roller tube technique. Moreover, the neuronal band of the CA1-3 and the dentate gyrus is not as compact as *in vivo* and a glia layer is formed close to the porous membrane (Buchs *et al*. 1993).

Pathophysiology of cerebral ischemia

The pathophysiology of cerebral ischemic cell death is very complex and it cannot simply be characterized as apoptotic and necrotic. There are also major differences between the global and focal *in vivo* models and between the *in vitro* models. Since only some aspects of major interest for this thesis will be related, the reader is referred to extensive reviews in the literature (Lipton 1999; Wieloch 2002) for details.

Depletion of energy stores

The high metabolic rate and low energy stores makes the brain critically dependent on a continuous supply of glucose and oxygen through the circulation. With a complete failure of blood supply, as in global ischemia, stores of high-energy phosphates, glycogen and glucose will only last for approximately 1 minute (Siesjö 1992a; Welsh 1998). In focal ischemia the blood supply in the lesion is dependent on the collateral circulation. In the core of the lesion the energy levels will fall rapidly as stores are consumed while in the surrounding, penumbra area, a continuous but inadequate supply will be present and increase the survival time (Nowicki *et al*. 1988).

Loss of ion homeostasis

The maintenance of ionic gradients over the plasma membrane is a prerequisite for the electrical function of the brain and a highly energy-consuming process. A direct consequence of a 80-90 % fall in blood supply is therefore the loss of ion homeostasis (Hossmann 1994). The membrane potential is maintained by the sodium/potassium ATP'ase extruding sodium in exchange for potassium. A cessation of its activity will lead to an equilibration of sodium and potassium concentrations over the plasma membrane and a depolarization of the membrane. Chloride and water will follow sodium passively into the cell and cause swelling of the cytosol and a reduction of the extracellular space.

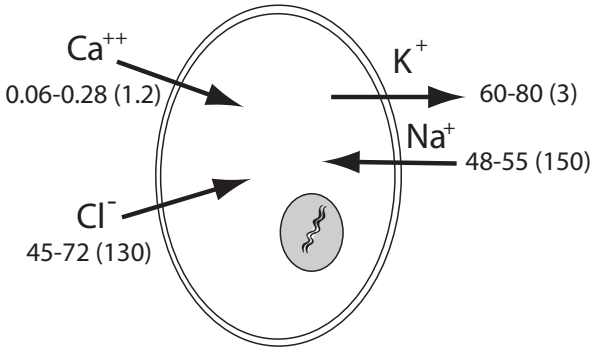

Figure 2 illustrates the major movements of ions as a result of the anoxic depolarisation. The resulting extracellular values are given in mM and based on recordings with ion-sensitive electrodes (Hansen 1998). Physiological levels are given within brackets.

Acidosis

Lack of oxygen rapidly inhibits the extraction of energy from pyruvate through the mitochondrial citric acid cycle and electron transport chain. Instead anaerobic glycolysis ensues through which pyruvate is reduced to lactate. Lactate is an acid and an acidification of the extracellular space occurs concomitantly with the production of lactate. The amount of lactate that is produced and thus the change in pH is a direct consequence of the pre-ischemic blood glucose level (Li *et al*. 1995). Hyperglycaemia leads to a more profound acidosis and also a more severe injury following global and transient focal ischemia (de Courten-Myers et al. 1989; Myers and Yamaguchi 1977; Nedergaard 1987; Pulsinelli et al. 1982b; Rehncrona et al. 1981; Siemkowicz and Hansen 1978). During ischemia, the pH of the extracellular space decrease to 6.8-6.1 (Erecinska and Silver 1994). A high blood glucose level has also been shown to correlate with a worse outcome after stroke in the clinic (Parsons *et al*. 2002; Pulsinelli *et al*. 1983; Weir *et al*. 1997; Woo *et al*. 1988). Aggravation of acidosis increases the production of free radical species and this has been suggested to mediate the toxic effect of hyperglycaemia (Li and Siesjö 1997). It has also been proposed that a compartmentalized and further aggravated intracellular acidosis in astrocytes could be responsible (Lascola and Kraig 1997).

That the toxic effect of hyperglycaemia is mediated by an aggravation of tissue acidosis has been questioned by contradictory findings both *in vivo* and *in vitro*. Acidosis inhibits NMDA-receptors and protects against OGD-induced damage *in vitro* (Giffard *et al*. 1990b; Tombaugh and Sapolsky 1990). Aggravated acidosis by hypercarbia has been shown to increase damage following global ischemia (Katsura *et al*. 1994), decrease damage following permanent focal ischemia (Simon *et al*. 1993) and be without effect on damage following transient focal ischemia (Gisselsson *et al*. 1999).

Calcium

The calcium ion is a ubiquitous regulator of neuronal processes and a tight control of the intracellular concentration within the 0.1-1μM range is essential for survival. Since the extracellular calcium concentration is approximately 10.000 fold, efficient extruding mechanism such as the Na^{+}/Ca^{++}-antiporter and the Ca^{++}ATPase are necessary to maintain intracellular levels (Wieloch 2002). During ischemia these energy-dependent extrusion mechanisms will fail.

In addition, an increase in the intracellular calcium concentration during ischemia can occur through the opening of voltage-gated calcium channels and agonist operated calcium channels or through the release of calcium from intracellular compartments, mainly the mitochondria and the endoplasmatic

reticulum (Kristian and Siesjö 1998). A simplified model is presented below.

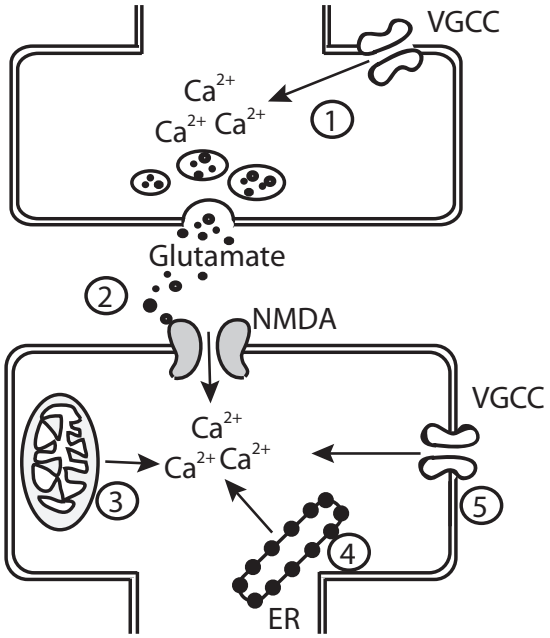

Figure 3. Mechanisms for intracellular calcium-increase during ischemia. Presynaptic entry of calcium through the voltage-gated calcium channels (VGCC) of N, P or Q type induces vesicular release of glutamate (1). Glutamate binds to the NMDA-receptor and opens a calcium-permeable channel (2). Mitochondria acts as a calcium sink but this energy-dependent function can reverse as a consequence of calcium-overload and calcium will then be released through the mitochondria permeability transition pore (3). Calcium can also be taken up by the endoplasmatic reticulum (4) and released after activation of metabotropic receptors or enter the postsynaptic neuron through VGCC (5).

Measurements of extracellular calcium levels during ischemia have revealed a decrease from 1.2 mM to approximately 0.06-0.28 (Erecinska and Silver 1994; Hansen 1985; Harris and Symon 1984; Harris *et al*. 1981) and a simultaneous increase in intracellular concentrations from 0.1μM to 0.03mM (Erecinska and Silver 1994).

Glutamate

Glutamate is the principal excitatory neurotransmitter in the brain and an increased glutamatergic transmission has been implied in the pathogenesis of several neurological disorders including cerebral ischemia (Meldrum 2000). This "excitotoxic hypothesis" was initiated by the discovery of a neurotoxic effect of glutamate in developing animals (Olney and Sharpe 1969) and the *in vitro* findings that synaptic release of glutamate mediated hypoxia-induced injury (Rothman 1984). It has since been strengthened by findings of elevated levels of glutamate during ischemia *in vivo* (Benveniste *et al*. 1984), protection against ischemic injury by a lesion of the glutamatergic pathways (Wieloch *et al*. 1985), toxicity of exogenously applied glutamate *in vitro* (Choi *et al*. 1987;

Vornov *et al*. 1991) and the effect of glutamate receptor antagonists in different paradigms of cerebral ischemia (Dirnagl *et al*. 1999).

Glutamate receptors are divided into ionotropic, possessing an ion channel, and metabotropic receptors, the latter coupled to G-proteins. The ionotropic receptors are further subdivided into N-methyl-D-aspartate (NMDA), α–Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and delta subtypes (Petralia *et al*. 1998). Binding of glutamate to the AMPA-receptor increases the conductance of this ion channel to Na^+ and K^+ and rapidly depolarizes the cell membrane. Depolarization relieves the Mg^{++}-block from the NMDA-receptor, a prerequisite for its subsequent activation by glutamate. As glutamate binds to the NMDA-receptor this ion channel opens and allows influx of calcium and sodium down their respective gradients and further but slower membrane depolarisation. The metabotropic glutamate receptors modulates excitatory neurotransmission either by an increase in the level of cyclic AMP (class II and III) or by an activation of protein kinase C and a release of calcium from the endoplasmatic reticulum (class I) (Nicoletti *et al*. 1996; Pellegrini-Giampietro 2003).

AMPA-receptors are composed of four subunits, GluR 1-4, and have a mainly post-synaptic localisation. The presence of GluR2 renders the ion channel impermeable to Ca^{++} and a post-ischemic down-regulation of GluR2 subunits have been implicated in the pathogenesis of stroke (Opitz *et al*. 2000). The relative proportion of AMPA receptors containing GluR2 subunits increase in hippocampal neurons during the first 20 days in culture (Pickard *et al*. 2000).

NMDA-receptors have six subunits. NR1 is always present and in addition one or more of the regulatory subunits NR2A-D and NR3A (Cull-Candy *et al*. 2001). This agonist-operated ion-channel has a number of regulatory sites (Dingledine and McBain 1999). Thus, redox modulation of cysteine residues can decrease (through oxidation) or increase (reduction) NMDA-evoked currents (Choi and Lipton 2000). Nitric oxide inhibition of the NMDA-receptor occurs through a specific nitrosylation of the NR2A subunit (Choi *et al*. 2000) and acidosis (protons) has a strong inhibitory action through a modification of the NR1 subunit (Tang *et al*. 1990).

During physiological conditions, a glutamatergic synaptic signal is terminated by the reuptake of the transmittor by glutamate transporters into the nerve terminal or adjacent astrocytes (Danbolt 2001). In astrocytes, glutamate could either be converted to glutamine and metabolized in the citric acid cycle or transported to neurons and converted back to glutamate for reuse as transmittor.

During ischemia, glutamate can be released mainly through two

different mechanisms. Either through the calcium-dependent and vesicular mode illustrated in figure 3 or through the reversed operation of glutamate transporters (Jabaudon *et al*. 2000; Rossi *et al*. 2000). The reversed uptake mechanism and the neuronal glutamate-pool is believed to dominate during ischemia (Danbolt 2001; Ottersen *et al*. 1996).

Adenosine

Adenosine is an inhibitory neuromodulator in the central nervous system with diverse physiological functions such as the regulation of cerebral blood flow, the state of arousal and the coupling of cerebral blood flow to energy demand (Dunwiddie and Masino 2001). During ischemia the levels of extracellular adenosine increase (Hagberg *et al*. 1987) mainly as a result of ATP catabolism (Von Lubitz 1999). Four types of G-protein coupled receptors (A_1, A_{2a}, A_{2b} and A_3) mediate the effects of adenosine in the brain (Fredholm *et al*. 2001). The A_1-receptor has a widespread distribution in the brain and is generally believed to be responsible for the neuroprotective action of adenosine during cerebral ischemia (Von Lubitz 1999). An inhibitory effect on excitatory neurotransmission by the A_1-receptor is executed mainly through a presynaptic inhibition of glutamate release (Wu and Saggau 1997). However, another action of A_1-receptor agonists is systemic hypothermia, by itself a strong neuroprotectant. A transgenic mouse lacking the functional A_1-receptor has been developed. The phenotype includes increased anxiety, hyperalgesia and decreased recovery of EPSPs after *in vitro* hypoxia (Johansson *et al*. 2001).

Free radical species

A free radical is a molecule that contains an unpaired electron. The free radicals are highly reactive substances that oxidize double bonds in unsaturated fatty acids and SH-groups on proteins or deaminate bases in the DNA. Free radicals are continuously produced during normal physiological circumstances mainly as a by-product to the mitochondrial respiration and purine metabolism. Oxidation and reduction of many signalling molecules in the cell modifies their function which illustrates that free radicals have a physiological role (Wieloch 2002).

The free radicals of most importance in cerebral ischemia are the reactive oxygen species nitric oxide (NO), the superoxide anion ($O_2^{-}\cdot$) and the hydroxyl radical (OH^-).

The superoxide anion is produced as a result of adenine nucleotide metabolism, through the oxidation of hypoxanthine (1), as a result of phospholipid degradation (2) and as a by-product from the electron transport chain.

(1) Hypoxanthine + O_2 → Xantine + $O_2^{-}\cdot$

(2) Phospholipid → Arachidonic acid → prostanoids + $O_2^{-}\cdot$

Reaction of the superoxide radical with superoxide dismutase (SOD) form hydrogen peroxide (H_2O_2).

$$O_2^{-} + 2H_2O \xrightarrow{SOD} 2\ H_2O_2$$

Hydrogen peroxide has a low toxicity but can readily pass cell membranes and can be transformed to the more reactive hydroxyl radical through the Fenton reaction:

$$Fe^{++} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH\cdot$$

This reaction is favoured by the low pH and increase in free iron during ischemia.
Nitric oxide is a gas and a neuromodulator with diverse actions both within and outside the nervous system. It is a stable free radical but it can react with the superoxide anion to form the more reactive peroxynitrite.

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

The subsequent decomposition of peroxynitrite to yield hydroxyl radicals is, as with the Fenton reaction, accelerated at low pH (Siesjö et al. 1996).

The production of free radicals, including nitric oxide, is increased during ischemia and more so during the reperfusion phase following global ischemia and in the penumbra of a focal ischemic lesion where there is oxygen present (Siesjö 1992b). A vast number of experimental studies have clearly showed that free radical species have a role in the pathogenesis of both focal and global brain ischemia and that strategies to ameliorate oxidative stress are neuroprotective (Lipton 1999).

Objectives

The overall objective of this thesis was to establish and characterise a model of *in vitro* ischemia in the murine hippocampal organotypic tissue cultures. The specific aims were:

1. To develop an *in vitro* model of global cerebral ischemia in the murine organotypic tissue culture. Cell death should be reproducible and mimic the delayed and selective cell death pattern seen *in vivo* (Paper I).

2. To characterize and investigate the principal mechanisms of hyperglycaemic ischemic cell death *in vitro* (Paper II).

3. To assess the importance of calcium ions, free radical species and nitric oxide for the cell death processes in the models of *in vitro* ischemia and hyperglycaemic *in vitro* ischemia (Paper III).

4. To study the effect of acidosis on the regional selectivity of neuronal death following *in vitro* ischemia (Paper IV).

5. To use the model of *in vitro* ischemia for the study of genetically modified animals lacking the adenosine A_1- receptor (Paper V).

Methods

Animals

All handling of experimental animals was approved by the Malmö/Lund ethical committee on animal experiments. Pregnant, time-mated, Balb/c mice were purchased from Taconic M&B, Ry, Denmark (formerly Møllegaard). The offspring of these mice were used for the preparation of all tissue cultures except for the transgenic cultures used in the study of adenosine A_1-receptors (paper V).

The A_1R-transgenic cultures were prepared from the littermate pups of intercrossed heterozygous A_1R (129/OlaHsd/C57BL) mice. Tail biopsies were obtained one day before the preparation of cultures and the genotype determined by PCR.

Organotypic tissue cultures

Mice pups, 5-7 days old, were decapitated and the brains placed in ice-cold dissection medium. The hippocampi were dissected out and cut into 250 μm thick slices on a McIllwain tissue chopper. The transverse hippocampal slices were sorted under a dissection microscope and slices with perfect morphology, from the middle portion of the hippocampus, were chosen. These slices were then plated on Millicell culture inserts, one slice per insert, and placed in culture medium.

The dissection medium consisted of HBSS (Hank's balanced salt solution), buffered with 2mM HEPES to maintain pH 7.2 in air and supplemented with 100 units Penicillin/Streptomycin and 6 mg/ml D-glucose.

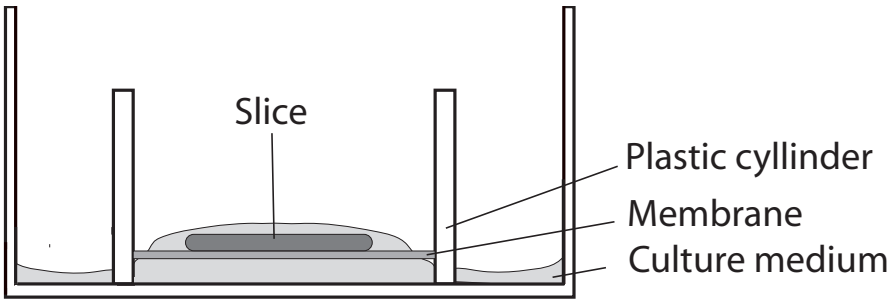

Figure 4. The interface method for culture of tissue slices. The slice lies on a porous membrane between the culture medium and air.

The initial culture medium (paper I) was composed of 50% MEM (Eagles with Earl's balanced salt solution), 25% heat inactivated horse serum, 18% HBSS, 2% B27 supplemented with 4 mM L-glutamine and 50 units Penicillin-Streptomycin/ml**.** The pH was adjusted to 7.4 by adding 0.5 ml sodium bicarbonate (7.5%) and the glucose-concentration adjusted to 40 mM by the addition of 6 mg/ml D-glucose. The osmolarity of the medium was approximately 330 mOsm.

Penicillin-Streptomycin was omitted from the culture medium one day before experiment. In paper II-V, B 27 was omitted after the first week in culture, a modification that improved the quality of our cultures (see Results section for details).

In paper II we varied the glucose-concentration in the culture medium and replaced D-glucose with sucrose (20, 30 or 35 mM) to preserve osmolarity. In subsequent experiments (paper III-V) a glucose-concentration of 20 mM and a sucrose concentration of 20 mM were used.

The culture medium was changed on the second day *in vitro* (div 2) and then three times a week. Cultures were housed in a humidified CO_2-incubator (Forma Scientific) at 35°C. All substances used for preparation and maintenance of cultures, except D-glucose (Sigma), were obtained from GibcoBRL, Life Technologies,

In vitro ischemia (IVI)

To accomplish aglycemia, glucose was omitted from the medium during the insult. To accomplish anoxia an anaerobic incubator (Electrotec, England) was custom built and equipped with a rapid entry for 24-well plates through which the cultures could be transferred rapidly in and out at the start or end of experiment. It had a slight overpressure and was filled with an anaerobic gas (85%N_2, 10%H_2, 5% CO_2). A palladium catalyst removed traces of oxygen in the atmosphere and a digital temperature control maintained the temperature at 35±0.3°C during experiments. A liquid oxygen indicator was continuously bubbled with the atmosphere of the anaerobic incubator during experiments to ensure that anoxia was maintained.

The medium used during the anoxic-aglycemic insult was varied as part of the experiments. It was bubbled with the anaerobic gas mixture inside the anaerobic incubator for 30 minutes, dispended into the wells of a 24-hole plate and allowed to equilibrate with the incubator atmosphere for another hour before experiments. The pH of the IVI medium was routinely measured in a standard micro-sampler blood-gas monitor (ABL 50 Radiometer Copenhagen, Copenhagen, Denmark). In Table 1, the four mediums used as standards are displayed. Concentrations are given in mM and osmolarity in mOsm.

	Paper I		Paper II-V	
	aCSF	iCSF	iCSF	iCSF+G
NaCl	125	70	70	70
KCl	2.5	70	70	70
NaH_2PO_4	1.25	1.25	1.25	1.25
$MgSO_4$	2	2	2	2
$CaCl_2$	2	0.3	0.3	0.3
$NaHCO_3$	25	5.25	5.25	5.25
sucrose	10	10	40	0
glucose	0	0	0	40
pH	7.4	6.8	6.8	6.8
Osmolarity	327.5	323	353	353

Table 1. The four standard compositions of ischemic medium used in this thesis. The osmolarity of iCSF was increased by 30 mOsm in paper II-V to maintain isoosmolarity with hyperglycaemic iCSF (iCSF+G).

In the first study (paper I) modifications of aCSF were made by raising potassium, decreasing calcium and decreasing pH. The combination of these modifications was termed ischemic CSF (iCSF). In the second study (paper II) a "hyperglycaemic" iCSF (iCSF+G) was accomplished by adding 40mM D-glucose to iCSF. In this and following studies the sucrose content of iCSF was increased to 40 mM to ensure isoosmolarity with iCSF+G.

The three constituents of the in vitro ischemia (IVI) thus were anoxia, aglycemia and the composition of the ischemic medium. In paper I, a hyperglycaemic IVI was accomplished by adding 40 mM glucose to the iCSF (iCSF+G). As the glucose concentration in the culture medium was decreased to 20 mM in the following studies it became necessary to pre-incubate the slices for one hour in culture medium with 40 mM glucose to induce the hyperglycaemic effect (Fig 1, paper III).

Measurement of cell death

The fluorescent cell death marker propidium iodide (PI) (1μg/ml) was added to the culture medium on the day before the insult and was included throughout the duration of the experiment (Fig. 1, paper I). Images were obtained using an inverted fluorescence microscope equipped with a 12-bit monochrome cooled fluorescence camera (Apogee Instruments, U.S.A.). For image processing the commercial software Image-Pro Plus 4.0 (Media Cybernetics, U.S.A.) was used. Fluorescence intensity was measured in standardized areas in the CA1- and CA3-regions and background staining was measured in a small hexagon placed in the cell-band in the CA2/CA3 region (paper I) or outside the cell band in the same region (paper II-V).

Two different approaches were used to estimate the amount of cell death

in this thesis. Values of absolute cell damage were obtained by subtracting mean fluorescence intensity (MFI) in the background area from the MFI levels in the standardized CA1 or CA3-area for each time-point.

The relative amount of cell death was expressed as percentage of the maximal amount in the experiment on A_1R-transgenic animals (paper V) and in the comparison between the CA1 and CA3-region (paper IV) according to the following formula:

Relative neuronal death = $(CA1^{24h/48h}-CA1^{0h})/(CA1^{MAX}-CA1^{0h})$.

The advantage of this formula is that it compensates for inherent differences between groups of slices or sub-regions, such as the total amount and density of neurons. The major disadvantage is that it does not take into consideration the clearance of PI-positive dead neurons between the first insult and the measurement after the second, maximal, insult at 72 hours of recovery. This will exaggerate the amount of damage which explains why values above 100% are sometimes seen.

Drugs

For studies of glutamate receptors (paper I, II and V), the non-competitive NMDA-receptor antagonist dizocilpine maleate (MK-801), the competitive NMDA receptor antagonist D-2-amino-5-phosphonopentanoic-acid (D-APV) and the AMPA-receptor blocker 2,3-dihydro-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX) were used. Intracellular calcium ions were chelated with the cell permeant 1,2-bis(2-amino-5-fluorophenoxy)ethane-N,N,N´,N´-tetraacetic acid tetrakis(acetoxymethyl) ester, BAPTA-AM (paper III).

To inhibit the production of free radical species (paper III) the nitric oxide synthase (NOS)-inhibitor N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), the iron chelator deferoxamine or the free radical scavengers N-tert-butyl-α-phenylnitrone (PBN) and N-acetyl-L-cysteine (NAC) were used.

The adenosine A_1R selective antagonist, 8-cyclopentyl-theophylline (8-CPT) was used as a pharmacological control to the transgenic animals (paper V).

All drugs were dissolved to stock concentrations in sterile water with the exception of BAPTA-AM, which was dissolved in DMSO.

Histological staining

For the histological evaluation of damage following IVI, cultures were fixed in paraformaldehyde (4%), embedded in Agar 100 (Link Nordiska, Sweden) and sectioned (2.5μm) on an ultratome (paper I). Sections were stained with 0.5% methylene blue / 0.5% azure blue in borax (1%).

A mouse monoclonal anti-NeuN antibody (Chemicon, U.S.A.) was used to visualize the neuronal population in unsectioned slice cultures in paper II.

Unsectioned cultures exposed to hyperglycaemic IVI were stained with the specific neuronal cell death marker Fluoro-Jade (0.001%, Histo.Chem inc, U.S.A.) in paper II to verify that the cell death, measured by PI-staining, was neuronal.

Statistics

From one mice pup we usually derived 12 cultures. On the experimental day these cultures were checked for the presence of PI-staining or bad morphology and10-20% of cultures were usually discarded on these grounds. The remaining cultures were divided into an equal amount of experimental groups. Thus, one mouse pup contributed one culture to each group in one experiment. These groups were exposed to insult together in the same 24-well plate and one such experiment was always repeated at least three times on different dates.

Two-way analysis of variance (ANOVA) with Scheffé´s post-hoc test was used to evaluate differences between groups, repeated measures ANOVA with Scheffé´s post hoc test was used in figures 2-4, paper I. Variability in experimental conditions between experimental dates was compensated for by including date as a factor. For statistical analyses the commercial software Statview 4.0 (Abacus Concepts Inc, Berkley, U.S.A.) was used.

Electrophysiology

For electrophysiological recordings the slice on its membrane was placed in a recording chamber and submerged in room-tempered aCSF consisting of (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, 1 NaH_2PO_4 and 11 glucose, which was gassed with 95% O_2 - 5% CO_2.

For the recording of extracellular field excitatory postsynaptic potentials (EPSPs) in the CA1-region, both the stimulating and recording electrode were placed in the stratum radiatum (paper I).

Whole-cell patch-clamp recordings were performed in voltage-clamp or current clamp mode. Two, slightly different pipette solutions were used in paper I, II, V and paper IV. In paper I, II and V, the Schaffer collaterals were stimulated in the stratum radiatum and excitatory postsynaptic currents (EPSCs) recorded from patch-clamped pyramidal neurons in the CA1-region. In addition, mossy fibers were stimulated in the hilus of the dentate gyrus when the CA3-pyramidal neurons were patch-clamped in the study on the regional effects of acidosis (paper IV). Signals were amplified and filtered at 2.9 kHz and sampled at 10 kHz with an EPC-9 patch-clamp amplifier. The GABA-antagonists picrotoxin (100 μM) or SR95531 (10μM) were used to block

inhibitory signalling and NBQX was used to block excitatory AMPA receptor activity when appropriate. Adenosine (20mM) was added to the perfusate and EPSCs recorded to measure the inhibitory effect of adenosine in transgenic A_1R animals (paper V).

The pH of the perfusate aCSF was altered (in paper IV) by exchanging NaHCO3 for NaCl to maintain osmolarity. Samples from the recording chamber were repeatedly checked in a blood-gas monitor to ensure that the pH could be reliably altered.

Results

A pathophysiological adaptation of the medium during *in vitro* ischemia led to a more selective and delayed cell death that was aggravated by glucose (Paper I)

Organotypic tissue cultures from the mouse hippocampus were established and a reproducible protocol for oxygen and glucose deprivation was developed.

Initial experiments were performed in glucose-free aCSF in accordance with previous reports from other groups using rat cultures. Oxygen and glucose deprivation (OGD) with this medium induced a cell death that developed faster and was less selective to the CA1-region then what is found following global ischemia *in vivo*. Based on aCSF and on extracellular recordings of the changes in ionic concentrations during ischemia in vivo we composed a new medium, with decreased $[Ca^{2+}]$, increased $[K^{+}]$ and acidotic pH. We termed this medium ischemic CSF (iCSF) to stress the resemblance to the extracellular fluid during brain ischemia *in vivo*. The combined effect of the ionic changes in iCSF was a lower level of cell death and a delay in the development of cell death compared with aCSF when slices were incubated concomitantly for 15 minutes in the anaerobic chamber. Histological evaluation with methylene blue/azure blue revealed that the iCSF-induced cell death was neuronal and that 15 minutes incubation killed approximately 25% of the neuronal population in CA1.

The individual contribution of $[K^{+}]$, $[Ca^{2+}]$ and acidosis (pH 6.8) was investigated in a separate experiment using aCSF as the standard. A high level (70 mM) of extracellular potassium could be expected to depolarize the plasma membrane and this was electrophysiologically confirmed in oxygenated cultures. This depolarization, by itself, did not induce cell death in oxygenated sham cultures and cell death was not affected by high $[K^{+}]$ during OGD. Both a decrease in $[Ca^{2+}]$ and acidosis, in the extracellular fluid during OGD, ameliorated damage, but in somewhat different fashion. While a lowering of $[Ca^{2+}]$ resulted in overall reduction in cell death, the low pH typically reduced the damaged area more and the density of dead cells less.

Combined oxygen and glucose deprivation in aCSF has repeatedly been shown to induce neuronal death in cultures in a glutamate-mediated way. To study the involvement of ionotropic glutamate-receptors in our system, we blocked NMDA-receptors with the use-dependent antagonist MK-801 (20μM) or the competitive antagonist APV (150μM) and AMPA-receptors with the competitive antagonist NBQX (100μM). A very potent inhibition of cell death was found with MK-801 or APV and a less potent but still significant effect was seen with NBQX. Thus, cell death was highly dependent on ionotropic glutamate receptors.

Addition of glucose during anoxia in aCSF has repeatedly been shown to inhibit damage. In contrast, we found that addition of 40mM glucose to the iCSF increased the amount of cell death in the CA1-region at 48 hours of recovery. Thus, iCSF induced cell death showed similarity with global ischemia *in vivo* in that it was delayed, selective to the CA1-region and increased by glucose.

Characteristics of hyperglycaemic *in vitro* ischemia (IVI) (Paper II)

Two major concerns regarding the culture protocol was whether the glucose concentration of 40 mM could be regarded as "normoglycaemia" and whether the survival of our cultures could be improved to allow for a prolonged culture period before experiments to ensure maturation.

B27 is a complement to serum-free medium containing vitamins, essential fatty acids, hormones and anti-oxidants. It had previously been found to improve the quality of murine slice-cultures in our laboratory. We questioned whether its prolonged use could contribute to the development of cell death in the central portion of the CA1-region most frequently encountered when the culture period was extended beyond 12-14 days. We thus cultured slices with or without B27 and found that the presence of B27 was necessary during the first week of culture to prevent a thinning of the cultures in the region of CA2/CA3 with a disruption of the neuronal band as a consequence. If, however, B27 was omitted after the first week in culture, the slices flattened out and the central CA1-damage was avoided. Apparently, slices could now be kept in culture for 3-4 weeks or longer.

We next cultured parallel groups of slices in 5, 10, 20 and 40 mM glucose and found that 5 and 10 mM resulted in a low yield of viable cultures while no differences was found between slices grown in 20 or 40mM glucose respectively. Electrophysiological measurements showed no differences in basic properties between cultures grown in 40 or 20 mM when performed at three weeks of age. We decided to regard 20 mM glucose as the physiological level in our system and 40 mM glucose as "*in vitro* hyperglycaemia" since it was twice the concentration needed for maintenance of healthy cultures.

In the first study on the effect of hyperglycaemic IVI, the effect of glucose-supplementation (40mM) during IVI was confirmed in three-week old cultures, grown in 40 mM glucose, and the temporal development of cell death studied. It was found that hyperglycaemia during IVI resulted not only in an aggravation of damage but also in a further delay.

When, instead, cultures were grown in medium containing 20 mM glucose, addition of this concentration of glucose during IVI was clearly protective. A more pronounced damage was seen with an increase to 40 mM glucose during IVI but an additional pre-incubation in 40mM glucose was

necessary to reproduce the pattern with delayed and aggravated cell death seen in the previous experiment.

In the following, a hyperglycaemic IVI represents culture in 20 mM glucose for three weeks, one hour pre-ischemic incubation in 40 mM glucose and iCSF containing 40 mM glucose during IVI.

Glucose is metabolized to lactate during anaerobic conditions. An increased lactate production and thereby an aggravated acidosis has been proposed as an important factor in the aggravated tissue damage seen with hyperglycaemic ischemia *in vivo*. To study the effect of the lactate molecule *per se* we exchanged glucose for lactate during ischemia with fixed pH 6.8 and found that lactate did not reproduce glucose-toxicity if pH was controlled. We also considered the possibility that aggravated acidosis could be the determinant factor in hyperglycaemic IVI but found no aggravation or delay of cell death when pH was further reduced to 6.3 during IVI.

On the other hand, when pH was increased during IVI, the delay and aggravation of cell death by hyperglycaemia was abolished.

To study whether hyperglycaemic IVI was still dependent on glutamate toxicity, APV and NBQX were used to block the ionotropic receptors of NMDA and AMPA sub-type respectively. In contrast to the previous experiments with glucose-free iCSF, no protective effect of either drug was seen with hyperglycaemic IVI.

Finally, Fluoro-Jade staining was used to confirm that the cell death seen after hyperglycaemic IVI was neuronal as had previously been shown with IVI (Paper I).

Comparisons between IVI and hyperglycaemic IVI concerning the relative importance of calcium ions and free radical species, including nitric oxide, for development of cell death (Paper III)

In this study we subjected parallel cultures to IVI or hyperglycaemic IVI and compared the responses to manipulation of intra-ischemic extracellular calcium ion concentration, intracellular calcium-chelation and pharmacological inhibition of free radical species.

In the glucose-free IVI paradigm, an omission of extracellular calcium during the insult prevented cell death almost completely. In hyperglycaemic IVI, on the other hand, calcium withdrawal had no effect.

When the intracellular calcium-chelator BAPTA-AM (200μM) was given both as a one-hour pre-treatment, during the insult and during the recovery phase, damage was significantly increased following IVI. A similar increase was seen in control-cultures treated with the vehicle, 1% DMSO, only. The same treatment-protocol was followed for hyperglycaemic IVI. In

this setting, no effect of the vehicle was seen. BAPTA-AM caused a more rapid development of cell death in hyperglycaemic IVI but to a lower final level at 48 hours of recovery. Inclusion of BAPTA-AM only during the recovery phase after hyperglycaemic IVI did not affect cell death.

Five different drugs with documented effect against free-radical species were tested for efficacy in the two IVI paradigms. The unselective NOS-inhibitor L-NAME, the iron chelator deferoxamine, and the free radical scavengers PBN and NAC were all ineffective in preventing cell death after IVI or hyperglycaemic IVI. Instead deferoxamine and PBN at a higher dose, significantly increased damage following IVI. The free radical scavenger MnTBAP had a dark stain when dissolved in medium that quenched PI-fluorescence and made impossible interpretation of the effect.

Cell death in the hippocampal CA3-region following IVI was selectively prevented by acidosis (Paper IV)

This study was initiated by the observations that acidosis seemed to reduce the area affected by cell death following OGD in aCSF and that cell death appeared in the CA3-area if pH was elevated above 6.8 during IVI.

Parallel groups of slices were subjected to IVI in iCSF at pH 6.2, 6.5, 6.8 and 7.4 for 12, 15 or 20 minutes and the relative amount of cell death was measured in the CA1 and CA3 region respectively. We found that cell death in the CA3-region was prevented with a decrease in pH from 7.4 to 6.8 independent of the insult duration or the time of recovery. A further protection by a decrease from 6.8 to 6.5 became evident only when the insult was prolonged to 20 minutes at the 48-hour recovery time-point. In the CA1-region no neuroprotective effect of acidosis was seen except for the 24-hour recovery time-point after the 20 minute IVI where the group exposed to IVI in pH 6.2 displayed less damage than groups exposed to pH 7.4 or 6.8 respectively. This effect was not preserved at the 48-hour recovery time-point. Thus, acidosis selectively protected CA3-neurons against IVI-induced death.

We questioned whether neuronal cell death in the CA1- and CA3-region was executed through similar mechanisms or not. We therefore performed IVI at pH 7.4 and chose the IVI-duration 20 minutes that previously had been shown to induce maximal damage in both regions at this pH. Omission of extracellular calcium and NMDA-receptor blockade by APV (150μM) effectively prevented damage in both regions while AMPA-receptor blockade by NBQX (100μM) was without effect. We concluded that IVI induced neuronal death in a highly NMDA and calcium-dependent way in both regions. Since acidosis has been shown to inhibit NMDA receptors we next studied if a different degree of inhibition could account for the regional differences in cell death.

For this purpose, patch-clamp recordings were obtained from pyramidal neurons in the CA1- and CA3-regions at pH 7.4 and 6.5. These recordings were performed in oxygenated aCSF. Evoked NMDA-receptor mediated EPSCs were depressed to approximately 30% of baseline, with no difference between the regions, when the low-pH perfusion medium was washed in. A significantly slower reversal of the inhibition was found in the CA3-region as the low-pH medium was washed out. Thus, a more prolonged inhibition of the NMDA-receptors by acidosis was found in the CA3-region.

The effect of adenosine A_1 receptors (A_1Rs) on the development of cell death following IVI and following global ischemia *in vivo* – a transgenic approach (Paper V)

To study the neuroprotective potency of A_1Rs, a strain of transgenic mice with a deletion in the gene encoding the A_1R was used for preparation of organotypic slice cultures and for the *in vivo* global ischemia experiments.

Excitatory postsynaptic currents were recorded from patch-clamped pyramidal neurons in the CA1-region of slice-cultures and verified a functional A_1R-knock out by the lack of inhibition by exogenously applied adenosine.

The relative amount of cell death was measured in the CA1-region of slices following a 12-minute-IVI. Slices from knock-out animals and their wild-type littermates were found to develop equal amounts of cell death. Similar results were found following 12 minutes of global ischemia *in vivo* where no difference in the amount of cell death was found between wild-type and knock-out animals in any of the six brain regions examined.

Pharmacological inhibition of A_1Rs by the competitive antagonist 8-CPT increased damage in the *in vivo* global ischemia paradigm. *In vitro*, no effect on cell death following a 12-minute-IVI was seen by 0.1, 1 or 10 mM 8-CPT.

Discussion

This thesis describes a new approach to the study of *in vitro* ischemia. When we applied pathophysiological concentrations of ions during the insult the injury became more similar to what is found *in vivo*. For the first time, an aggravating effect of glucose on the ischemic cell death *in vitro* was revealed and characterized. The two new models of *in vitro* ischemia (IVI) and hyperglycaemic IVI were compared on important aspects of calcium toxicity and free radical species. Finally an interesting finding regarding the selective vulnerability of hippocampal CA1 neurons was investigated.

Methodological considerations

B 27

Our original protocol for the culture of murine organotypic hippocampal slice cultures was a modification from the description of the interface method for corresponding cultures from the rat hippocampus (Stoppini *et al*. 1991). To improve the quality we made the cultures thinner (250 vs. 400μm) to enhance oxygenation and availability of nutrients. We also decreased the temperature during culture (35 vs. 36°C) to diminish microglia activation and added B27 to enhance development. This yielded a reasonable fraction (50-75%) of undamaged cultures at 10 days *in vitro*. Buchs had described that the clearing of debris after the dissection and the greatest modifications of synaptic contacts occurs during the first week of culture (Buchs *et al*. 1993). Furthermore, the microglial reaction to the dissection trauma had been shown to culminate on the second day *in vitro* and then gradually subside until day 10 (Coltman and Ide 1996). In concordance with Noraberg (Noraberg *et al*. 1999) we found that an increasing number of cultures developed damage in the central portion of the CA1-region after 12 days in culture and therefore settled for day 10 as the day of experiment.

After a systematic evaluation of the effect of B27 as a supplement to our cultures we changed the culture protocol in the second study and omitted B27 after the first week. The improved protocol yields a high fraction (70-95%) of slices with intact morphology and without signs of cell death. B27 contains both trophic factors and antioxidants (Brewer *et al*. 1993). Which component is beneficial during the first week of culture can only be speculated upon but possibilities are that the recovery of neurons and glia from the trauma of dissection is improved by trophic factors and that antioxidants prevent microglial attack on the tissues. With prolonged use of B27 the trophic effect seems to prevent the thinning of cultures and central necrosis develops.

Prolonged survival of cultures was gratifying since we now had stable cultures at three weeks of age, a time where synaptic morphology and transmission has matured in culture and *in vivo* (De Simoni *et al*. 2003).

The level of glucose

We used a high level of glucose (40mM) in the original protocol. This was the concentration in the original description of the interface method (Stoppini *et al*. 1991), recommended in the literature (Bergold and Casaccia-Bonnefil 1997; Gähwiler 1998) and used by most authors. We could find no explanation as to why this high concentration of glucose is used. Also, there were no systematic evaluation of the effect of different concentrations of glucose on the development of organotypic cultures and we therefore did a limited study in our own system. The conclusion of this study was that 10 mM glucose is too little but 20 mM glucose is enough to maintain healthy slices. Gross morphological appearance, survival or electrophysiological characteristics did not seem to differ between 20 or 40 mM cultured slices. We designated 20 mM glucose "normoglycaemia" in our system and 40 mM was accordingly designated "hyperglycaemia" since it was twice the concentration needed for survival.

Clearly, there is a potential for further improvement of our culture protocol but any systematic evaluation of culture medium components is obscured by the variations in culture quality induced by other factors such as dissection trauma, age of mice and handling during medium change. Evaluations are therefore both costly and time-consuming.

Propidium iodide as a marker of cell death

Our system for evaluation of damage is based on the fluorescent cell death marker propidium iodide (PI). PI had previously been shown to correlate with the loss of electrophysiological vital parameters in dissociated cell cultures (Macklis and Madison 1990), and with histological measures of cell death and lactate dehydrogenase release in organotypic slice cultures (Laake *et al*. 1999; Noraberg *et al*. 1999; Strasser and Fischer 1995). We found that PI could be safely included in the culture medium during experiments and that the subsequent degree of incorporation was correlated to the duration of the preceding hypoxic/aglycemic insult. PI is not a specific neuronal stain. We therefore performed a histological evaluation of damage following IVI with methylene blue/azure blue (paper I) and following hyperglycaemic IVI with the specific neuronal cell death marker Fluoro-Jade (paper II) and found both types of cell death to be of neuronal origin.

The method for induction of anoxia/aglycaemia

Anoxia can be accomplished in different ways in an *in vitro* setting. We tried an air-tight box filled with nitrogen and a system for submersion of slices but in both systems there was a variable degree of damage. The anaerobic incubator solved several problems. Anoxia was effectively induced both in the medium and the atmosphere during experiment and temperature was stable. Within the enclosed system, sterility could be maintained. The rapid entry system for 24-well plates allowed the simultaneous induction of anoxia in up to 24 slices divided in 4-6 experimental groups. The combined effect was a highly reproducible damage after a brief anoxia/aglycemia similar to what is found following 10-15 minutes of global ischemia *in vivo*. As discussed below, a possible drawback of this system is that the anoxia is actually too severe to allow some pathophysiological processes of importance during ischemia *in vivo* such as the production of free radicals.

The model of *In Vitro* Ischemia (IVI)

Extracellular ion concentrations

In our initial attempts to simulate brain ischemia we used aCSF, a salt-solution with an ionic composition similar to what is found during physiological conditions in the brain extracellular fluid. When the slices were subjected to anoxia/aglycemia in aCSF cell death developed rapidly and it was not selective to the CA1-region as *in vivo*.

We recognized that a major difference between ischemia in a cell culture system and the intact brain is the changes in ionic concentrations in the extracellular fluid that occurs during the first 1-2 minutes. A prerequisite for these changes is the volume relationship between the intra- and extracellular space. The extracellular space in the brain constitutes approximately 20% of the tissue volume and decrease to 6-10% during ischemia (Hansen and Olsen 1980; Lundbaek and Hansen 1992). Consequently, ionic fluxes over the plasma membrane will have a major impact on the extracellular concentrations. In the cell culture systems the culture medium can be regarded as an extension of the extracellular space, at least for small molecules such as ions. Since the volume of the medium usually is a 100-fold larger than the total volume of the cells, the medium will act as a buffer during ischemia and prevent changes in extracellular ionic concentrations. This will have consequences for the intracellular ionic concentrations as well as the levels of ions tend to equalize over the plasma membrane during energy failure. Thus, during OGD in aCSF, significantly higher intracellular calcium levels are recorded than *in vivo* and intracellular acidosis does not occur (Silver et al. 1997).

We reasoned that an alteration of the extracellular medium during the anoxic/aglycemic insult could be a way to approach *in vivo* conditions. When we used concentrations of ions that have been measured during ischemia *in vivo*, cell death became more selective to the CA1-region, less pronounced and delayed. The delayed pattern of cell death could be a result of the less severe insult produced by the new medium but could also reflect that other pathogenic mechanisms are involved. In support of the latter, hyperglycaemia induced further delay of cell death and aggravation through a different mechanism. A similar argument could be raised against the increased selectivity for the CA1-region with the new medium. We found, however, that a decrease in insult duration with aCSF only produced a more variable damage without markedly increased selectivity (data not shown). Furthermore, as is shown in paper IV, acidosis was a prerequisite for the selective cell death pattern.

Previous studies on oxygen and glucose deprivation in rat organotypic slice cultures have used considerably (30-60') longer insults than we (Abdel-Hamid and Tymianski 1997; Barth et al. 1996; Laake et al. 1999; Pringle et al. 1997; Strasser and Fischer 1995). One study with chemical anoxia/aglycemia in mouse slice cultures, however, reported selective CA1-cell death after a 7'-chemical anoxia/aglycaemia-insult (Bernaudin et al. 1998). The short duration of insult in our model of IVI is similar to *in vivo* models of global ischemia in rat (Pulsinelli et al. 1982a; Smith et al. 1984) and mouse (Olsson et al. 2003) and probably reflects the severity of energy depletion. Nevertheless, mice slice cultures differs from rat cultures in that they are smaller and thinner. This and perhaps also a higher metabolism in mice tissue (Singer 2001) could contribute to the decreased tolerance to IVI.

Importance of ionotropic glutamate receptors

Our studies with the ionotropic glutamate receptor antagonists NBQX, MK 801 and APV clearly showed that cell death following IVI was dependent on the activation of these receptors. The effect of NMDA receptor antagonists was profound and found both in 10 and 21 day old cultures (paper I and II). Furthermore, it was found not only at pH 6.8 but also at 7.4 (paper IV) and 6.2 (data not shown). The effect of the AMPA-receptor blocker NBQX, on the other hand, was less pronounced and was seen only at pH 6.8 in the 10-day old cultures. The function of AMPA-stimulation during synaptic transmission is thought to be a depolarisation of the postsynaptic membrane and thereby a facilitation of the NMDA-receptors. A profound depolarisation is achieved in our iCSF through the high potassium concentration (70 mM). The question is therefore why there was an effect of NBQX in the first study. An explanation might be a change in the composition of AMPA-receptor subunits. Since the

proportion of calcium impermeable AMPA receptors with a GluR2 subunit increase during the first 20 days in culture (Pickard et al. 2000), calcium entry through AMPA-receptors may have been important at 10 but not 21 days *in vitro* in our studies.

Calcium toxicity

Withdrawal of extracellular calcium had a strong protective effect, very similar to NMDA-receptor antagonists, suppressing cell death to background levels (paper III and IV). This could be due to both presynaptic and postsynaptic effects. The vesicular release of glutamate is mediated by a presynaptic increase in calcium ions entering through voltage-gated calcium channels (VGCC). Consequently, removal of extracellular calcium could inhibit this type of glutamate release. However, the major portion of glutamate release during dense ischemia has been shown to occur through the reversed operation of glutamate transporters (Jabaudon et al. 2000; Ottersen et al. 1996; Rossi et al. 2000). In our model, a dense metabolic inhibition is afforded by anoxia and aglycemia similar to the studies by Jaubadon and Rossi but different from many other *in vitro* studies. In one such study Abdel-Hamid and Tymianski exposed slice cultures to a 60-minutes OGD and found protection by the calcium chelator BAPTA-AM. They concluded that this protective effect was due to a presynaptic inhibition of glutamate release since BAPTA-AM had no effect on NMDA toxicity (NMDA acting on the postsynaptic neuron) in the same cultures (Abdel-Hamid and Tymianski 1997). We loaded our cultures with BAPTA-AM in the same way as Abdel-Hamid and Tymianski but found no protective effect. This probably reflects that vesicular release of glutamate contributes little to the excitotoxicity in our model.

Does calcium-entry through non-NMDA routes contribute to cell death in our model? The depolarisation by potassium can be expected to open postsynaptic VGCCs as well as presynaptic ones. The next to complete protection afforded by NMDA-antagonists could therefore mean that a less and thus non-toxic amount of calcium enters through VGCCs or that the calcium entering through the NMDA-channel is more toxic to the cell (Hardingham and Bading 2003). Several studies favour the latter hypothesis. Thus, calcium-loading via NMDA-receptors is toxic while identical amounts of calcium loads incurred through VGCCs is harmless (Sattler et al. 1998). NMDA-receptor activation leads to a specific calcium-accumulation in the mitochondria (Nicholls and Budd 2000; Peng and Greenamyre 1998). This, in turn, induces the mitochondrial permeability transition (MPT) with the opening of a large channel, osmotic influx of water, swelling and eventually disruption of the inner mitochondrial membrane (Haworth and Hunter 1979).

As a result, energy production will fail and calcium and pro-apoptotic factors will be released to the cytoplasm. The link between NMDA-receptor activation, calcium and mitochondria has recently been strengthened by the findings that iCSF induced cell death is prevented by cyclosporin A and NIM 811, blockers of the MPT (A. Rytter et al, unpublished results).

Acidosis

That acidosis had a protective effect against OGD (paper I) fits well with a model dependent on excitotoxicity and calcium entry through activated NMDA-receptors. Acidosis inhibits NMDA-receptors through a regulatory site on the NR1 subunit (Traynelis et al. 1995) and protects against glutamate- and OGD-induced cell death (Giffard et al. 1990b). In spite of the 70% reduction in NMDA-receptor mediated EPSCs found with acidosis (paper IV) cell death was still completely dependent on extracellular calcium (paper III). This is striking since the calcium concentration in iCSF is only 0.2 mM but highly relevant since these are the levels found during ischemia *in vivo* (Hansen 1985).

The excitotoxic concept is the basis of our present understanding of acute ischemic brain damage (Bramlett and Dietrich 2004; Brott and Bogousslavsky 2000; Dirnagl et al. 1999; Lee et al. 2000; Siesjö 1992a). The causal relationship between glutamate transmission and hypoxic injury was first demonstrated in neuronal cell cultures (Rothman 1984). Goldberg showed that injury after hypoxia (Goldberg et al. 1987) and OGD (Goldberg and Choi 1993) could be inhibited by NMDA-antagonists. A protective role for NMDA-antagonists in OGD-models has since been repeatedly confirmed in organotypic tissue cultures (Laake et al. 1999; Newell et al. 1995; Pringle et al. 1997; Strasser and Fischer 1995). Thus, our data were also confirmatory regarding the new ischemia medium, iCSF, and the murine slice culture.

Free radicals

The production of free radical species is generally regarded as an important downstream mechanism for excitotoxic cell death. Therefore the negative results on free radical scavengers must be explained and fitted into the model of iCSF-induced neuronal death.

The drugs used in this study were selected to target different mechanisms of free radical production and the doses were obtained from studies showing effect in studies similar to our own. N-acetyl-cysteine, deferoxamine, PBN and L-NAME are all water-soluble, and widely used drugs with low toxicity. These drugs were chosen to target the production of free radicals at different sites (see background section). N-acetyl-cysteine is a mucolytic that has been shown to

reduce free radicals, $O_2^{-}\cdot$ and H_2O_2 in particular (Moldeus et al. 1986), and to protect against OGD in brain slices (Monje et al. 2000). Deferoxamine is an iron chelator with protective effect against H_2O_2-induced damage (Ying et al. 1999) and dopamine induced toxicity (Alagarsamy et al. 1997) *in vitro*. PBN is a spin-trap that scavengers free radicals (Kotake et al. 1999). A protective effect of PBN has been documented against global ischemia (Carney and Floyd 1991; Li et al. 2001) and focal ischemia (Zhao et al. 1994) *in vivo* and against NMDA- and OGD-induced damage *in vitro* (Almli et al. 2001; Newell et al. 1995). Finally, L-NAME is an unselective NOS-inhibitor which forcefully hinders NO-production in hippocampal slices (Kojima et al. 2001).

No protective effect of any of the drugs was seen. Instead a dose-dependent toxicity was found with PBN, NAC and deferoxamine. L-NAME was not investigated at higher doses. Interestingly, DMSO, which had a toxic effect by its own in the BAPTA-AM experiment has been shown to have hydroxyl scavenging properties in higher concentrations (Panganamala et al. 1976). A possible explanation for the toxic effect of free radical scavengers in our IVI-model is that a shift in the redox state accentuate NMDA-channel opening. That reducing agents can induce spontaneous glutamate toxicity in neuronal cultures (Levy et al. 1990) and increase vulnerability to OGD in organotypic slices (Pringle et al. 2000) has previously been shown.

The explanation for the lack of protection by this battery of antioxidant drugs must be sought in the specific features of our new IVI-model. In this model, calcium withdrawal and NMDA-receptor blockade is protective, but only during the insult. Likewise, intra-ischemic but not post-ischemic hypothermia ameliorates damage (A. Rytter, unpublished results). Thus, the decisive events seem to occur during IVI and not after. Our model represents a very dense ischemia, reflected by the short time needed for reproducible damage and accomplished by the complete anoxia in medium and atmosphere (H_2 and palladium catalyst), depolarisation-stress by high potassium and aglycemia in combination. Indeed it is not likely that free radical species would be produced under such circumstances since these reactions are oxygen-dependent. Therefore, previous reports concerning the production and effect of free radical species during OGD needs to be interpreted with regard to the methods that was used to accomplish anoxia. Consequently, is not contradictory to our results that Perez Velazques et al found production of free radicals in a superfusion system where aglycemic aCSF was bubbled with 95% N_2/5%CO_2 since it is very difficult to produce complete anoxia in such a system. More conspicuous are the findings by Newell et al who showed neuroprotection of PBN (100μM) against a 35' anoxic/aglycemic insult using a similar system for anaerobic conditions as ours with a palladium catalyst and a Forma anaerobic

incubator. The fact that they used a much longer insult and do not seem to have made their medium anoxic by bubbling might indicate remnant oxygen, at least in the fluid phase during insult. In support of our findings are studies showing that NO is not produced until after a hypoxic/aglycemic insult (Kojima et al. 2001) and that PBN was without effect on OGD-induced derangement of glucose metabolism in acute slices (Murata et al. 2000). Particularly interesting is the finding by Monje that NAC prevented damage from anoxia/aglycaemia only if calcium was concomitantly removed from the OGD-medium (Monje et al. 2000). This study indicates the calcium challenge to mitochondria during OGD is the determinant step. Subsequent production of free radicals will only have a role if the calcium-load is decreased.

Data regarding the production of free radicals during cerebral ischemia *in vivo* are contradictory. While no production of free radicals is found in the core during focal ischemia (Gidö et al. 2000; Solenski et al. 1997), some authors report increased radical production during global ischemia (Zini et al. 1992) while others do not (Cao et al. 1988). Methodological differences concerning residual blood flow to the ischemic tissue and how the radicals were detected could be responsible.

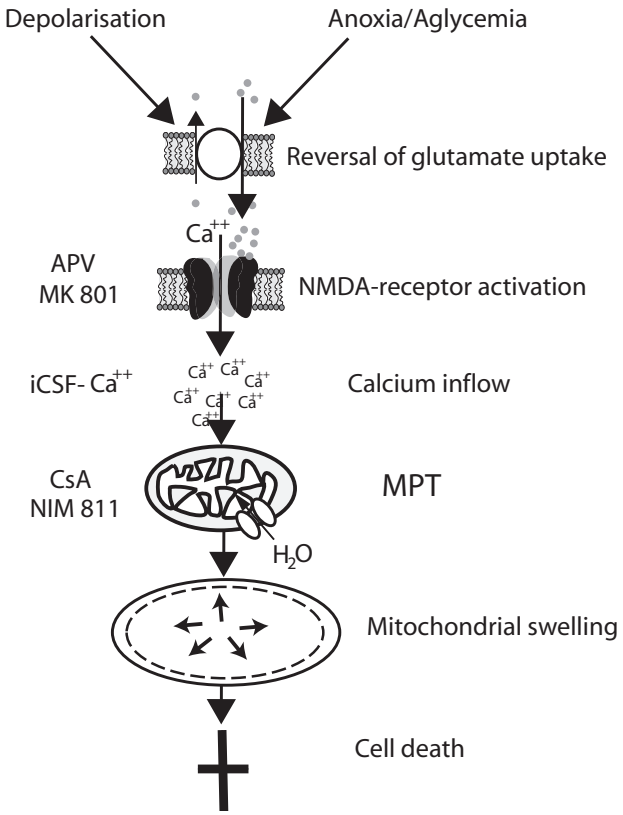

Figure 5. A model of IVI-induced neuronal death. The crucial events are the reversal of glutamate reuptake, the NMDA-receptor stimulation by glutamate, the calcium-link between the NMDA-receptor and the mitochondria and the activation of MPT. Following MPT, mitochondrial osmotic swelling leads to irreversible damage and cell death due to energy failure.

Selective vulnerability

The relevance of a pathophysiological adaptation of the medium during *in vitro* ischemia is illustrated by the findings on the effect of acidosis for selectivity of cell death (paper IV). This study shows that, in the context of iCSF, a degree of acidosis, very similar to what is found *in vivo*, selectively inhibits cell death in the CA3-region and is responsible for the selective cell death pattern in our IVI-model. The lack of inhibition by acidosis in the CA1-region might seem contradictory to previous results (paper I), showing an acidosis-induced protection also in this region. The former study, however, was performed in aCSF with a low potassium level (2.5 mM). As has been discussed earlier, the route of glutamate release might differ between our model, using iCSF and conventional models using aCSF. Thus, the high potassium level in iCSF might reverse the glutamate uptake and increase glutamate levels. In such a context, a 70 % inhibition of NMDA-receptors evidently is insufficient to reduce cell death in spite of the fact that the same cell death is completely blocked by NMDA-receptor antagonists or the withdrawal of calcium from the extracellular space. We explored the hypothesis that a higher degree of NMDA-receptor inhibition protected CA3-neurons while the CA1 neighbours succumbed. An equally strong inhibition was found during acidosis but a slower reversal of the evoked responses in the CA3 neurons on return to pH 7.4 might still indicate a subtle difference. Alternatively, there might be other regional differences downstream of the NMDA-receptor in the chain of events leading to cell death. Indeed, mitochondria from the CA3-region have a higher propensity to undergo permeability transition when challenged with calcium (Mattiasson et al. 2003) and intracellular increases in calcium concentration have a stronger depressant action on NMDA-evoked currents in the CA3 than in the CA1-region (Grishin et al. 2004).

Adenosine A_1-receptors

We used a transgenic and a pharmacological approach to study the possible involvement of adenosine A_1-receptors for the development of cell death following IVI and global ischemia *in vivo*. A strong inhibition of EPSPs by exogenously applied adenosine in slices from the wild-type but not from the knock-out animals confirmed that functional A_1Rs were present in cultures at three-weeks of age and also that the knock-out was functional.

However, no increase in the amount of cell death following IVI was found in cultures from the A_1R-knockout mice or slices treated with the A_1R–antagonist 8-CPT and the conclusion is therefore that A_1Rs are not involved in IVI-induced damage. A likely explanation is what is stated in the previous discussion on glutamate release during IVI. According to the hypothesis that

glutamate-release during IVI occurs mainly as a reversed uptake and not as a vesicular release, A_1R –manipulation will have little effect since the main action of A_1R –stimulation is to inhibit glutamate release.

How should we interpret the results from the *in vivo* model, where 8-CPT aggravated damage and knock-out was ineffective in modulating cell death? There are at least two main alternatives. Either important mechanisms, such as the mode of glutamate release, differs between the *in vitro* and *in vivo* model or that 8-CPT had a systemic effect in the *in vivo* model not seen *in vitro*. In the *in vitro* system we expose the cultures to altered ionic concentrations from the start of the insult and thereby may overlook an initial phase of synaptic glutamate release that actually occurs *in vivo*. If this is explanation is correct we still need to explain the discrepancy between the transgenic and pharmacological A_1R-antagonism *in vivo*. A possible explanation is the development of compensatory mechanisms in the transgenic animals. Such compensation has been indicated in the A_1R-knock out animals by a persisting inhibition of field EPSPs following an anoxic insult (Johansson et al. 2001). Systemic side effects of 8-CPT were not seen during our experiments but a subtle modulation of physiological parameters is still a possibility given the widespread distribution of A_1Rs (Fredholm et al. 2001).

The model of hyperglycaemic IVI

An interesting finding in the new model of IVI was that glucose no longer protected against hypoxia but instead increased damage. The dose-dependency of this effect was shown in the second study where 20mM glucose during IVI was protective and a pre-incubation in 40 mM glucose was required to reproduce the glucose-toxicity in slices cultured at a physiological glucose level.

This hyperglycaemic-ischemic cell death is clearly distinct from the IVI-induced type of cell death described above. It is further delayed and it is not mediated by the influx of calcium through activated NMDA-receptors (paper II and III). However intracellular calcium is involved since BAPTA-AM had a protective effect. In analogy with the IVI-induced cell death, production of free radical species does not seem to be involved (paper III).

Since this was the first time glucose-toxicity was studied in an IVI-paradigm we first performed some basic studies on the effect of lactate and acidosis. In hyperglycaemic ischemia *in vivo*, increased anaerobic glycolysis leads to an accumulation of lactate and thereby aggravated acidosis (Li et al. 1995; Siemkowicz and Hansen 1981). We investigated whether the lactate

molecule or an aggravated acidosis (pH 6.3) *per se* mediated the glucose-toxicity in our model of hyperglycaemic IVI but found that neither exchange of glucose for lactate nor an aggravated intra-ischemic acidosis reproduced the characteristic cell death pattern seen with high glucose (paper II). On the other hand, a moderate (pH 6.8) acidosis was a necessary prerequisite for glucose toxicity. Thus, the glucose molecule had the potency to cause cell death that was dependent on moderate acidosis but not enhanced by further aggravation of this acidosis.

How should the lack of effect by ionotropic glutamate-receptor antagonists or withdrawal of extracellular calcium in the hyperglycaemic paradigm best be understood? Clearly, they indicate that NMDA-receptor activation does not occur, at least not in a harmful way. In the presence of oxygen, anaerobic glycolysis could still provide sufficient energy to uphold a sodium-mediated glutamate uptake (Longuemare et al. 1994). Vesicular release of glutamate did not seem to be of importance in the IVI-paradigm, possibly due to an overwhelming effect of a reversal of glutamate uptake. In the hyperglycaemic paradigm a damaging effect of vesicular glutamate release may thus have been unmasked.

The effect of BAPTA-AM

Calcium-chelation by BAPTA-AM had a protective effect in the hyperglycaemic paradigm in contrast to what was found with IVI. Treatment during the recovery period only had no effect.

There are several possible explanations for the apparent discrepancy between the effect of BAPTA-AM and the lack of effect of calcium withdrawal in this paradigm. In the presynaptic terminal BAPTA-AM could prevent vesicular glutamate release (Abdel-Hamid and Tymianski 1997) and in the postsynaptic structure it could chelate calcium released from the endoplasmatic reticulum following activation of metabotropic glutamate receptors. This however assumes that NMDA-receptor activation is prevented. This could be accomplished through a modification of the NMDA-receptor or receptor-mediated pathways by, for example, glycation.

Alternatively, the protective effect of BAPTA-AM could have been to scavenge an influx of calcium during the early recovery phase. Interesting findings in astrocytic cultures supports the latter. Bondarenko and Chesler used a medium similar to ours and exposed their cultures to hypoxia. They found that damage was induced by a reversed operation of the Na^+/Ca^{++}-exchanger and could be inhibited by calcium-free medium in the reperfusion phase, by BAPTA-AM or by the specific inhibitor of Na^+/Ca^{++}-–exchange KB-R7943 (Bondarenko and Chesler 2001). This mechanism could also occur in the

post-synaptic neuron but there are other circumstantial evidence suggesting a role for glia in the development of hyperglycaemic IVI induced neuronal death.

A role for astrocytes in hyperglycaemic IVI?

That astrocytes are the prime target for hyperglycaemic ischemic brain injury has long been suggested (Plum 1983) and based mainly on findings *in vivo* and in cultures of isolated astrocytes. *In vivo*, a hallmark of hyperglycaemic cell death is pan-necrosis where, in addition to neurons, glial cells also die (Pulsinelli et al. 1982b). It has been proposed that the presence of glucose leads to an increased anaerobic glycolysis in the astrocytes, enhanced lactate production and aggravated intracellular acidosis (Lascola and Kraig 1997). Already in 1961, a dose-dependent toxicity of glucose during anoxia in acute cerebellar slices was shown (Friede and Van Houten 1961). These authors showed that astrocytes, subjected to anoxia in the presence of glucose developed morphological changes termed klasmatodendrosis that could be prevented by inhibitors of glycolysis. Astrocytes have a high capability for anaerobic glycolysis and generally are resistant to hypoxic injury (Swanson et al. 1997). However they are more sensitive than neurons to acidosis (Giffard et al. 1990a) and their ability to withstand hypoxia decrease with increasing acidification of the medium (Swanson et al. 1997).

Although we have a neuronal cell death following hyperglycaemic IVI, astrocytes may still be critically involved. Findings *in vitro* has suggested that astrocytes supply neurons with lactate following ischemia (Schurr et al. 1997). This mechanism could be important also following global ischemia *in vivo* since an inhibition of the monocarboxylate transporter aggravated the neuronal death (Schurr et al. 2001). It has lately been shown that the trophic factor erythropoietin is produced in astrocytes and protects neurons subjected to OGD (Ruscher et al. 2002).

In a speculative model (Figure 4) of astrocyte-mediated neuronal death following hyperglycaemic IVI the astrocytes are reversibly injured during the insult and fail to support the survival of neurons in the critical post-ischemic phase. While the astrocytes eventually regain their function, neurons are irreversibly injured and die in a delayed fashion.

Further morphological and pharmacological investigations are obviously needed to decipher the mechanism of hyperglycaemic IVI and for this the slice culture is a suitable system. A critical question for the astrocytic hypothesis is whether glucose toxicity during IVI could be reproduced in pure neuronal cultures. An argument for the involvement of astrocytes in our slices is namely that previous studies have shown that hypoxic cell death in isolated neuronal

cultures is glutamate mediated (Goldberg et al. 1987; Rothman 1984) while it is glutamate independent in slice cultures (Newell et al. 1995) and *in vivo* (Pearigen et al. 1996).

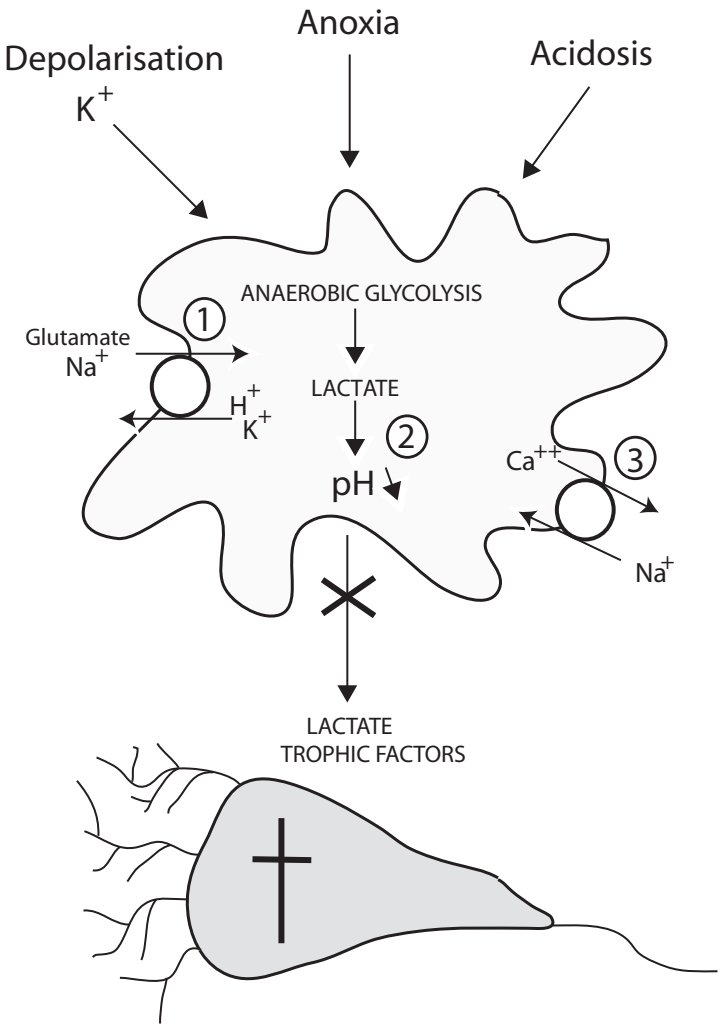

Figure 6. Possible mechanisms for astrocytic dysfunction and subsequent neuronal death following hyperglycaemic IVI. 1. Sequestration of glutamate. 2. Compartmentalized acidosis due to anaerobic glycolysis. 3. Reversed operation of the Na^{+}/Ca^{++}-–antiporter. A deranged astrocytic function in the recovery phase following hyperglycaemic IVI leaves the neuron without lactate and trophic factor support and leads to neuronal death while the astrocytes eventually recover function and survive.

Populärvetenskaplig sammanfattning

När blodförsörjningen till hjärnan upphävs uppstår ett tillstånd av blodbrist, ischemi. När hela hjärnan drabbas, som vid ett hjärtstillestånd, kallas detta global ischemi medan fokal ischemi betecknar att blodflödet är upphävt i en del av hjärnan, som vid stroke. Hjärt- och kärlsjukdomar, till vilka stroke också räknas, utgör idag ett dominerande hälsovårdsproblem i västvärlden och kostnaderna kan förväntas öka i takt med att befolkningen blir äldre.

Våra kunskaper om de mekanismer som leder fram till att nervceller skadas och dör till följd av ischemi har ökat avsevärt under de senaste årtiondena till följd av en omfattande grundforskning inom området. Kunskaper om de faktorer som påverkar skadeutvecklingen, som tex. kroppstemperaturen och blodsockernivån har bekräftats i kliniska studier och blivit en del av det rutinmässiga omhändertagandet vid stroke och hjärtstillestånd. Ett stort antal substanser har visat sig skydda mot ischemisk hjärnskada i djurmodeller men hittills har ingen av dessa, trots ett antal kliniska studier, visat sig minska hjärnskadan hos människor. Detta dystra faktum understryker behovet av en fortsatt kartläggning av de grundläggande mekanismerna vid ischemi i hjärnan och av att skapa försöks-modeller som mer efterliknar de kliniska tillstånden. Experimentella modeller av hjärnischemi kan grovt delas in i *in vivo* modeller där hela djur används och *in vitro* modeller där istället cellkulturer eller färsk vävnad används.

Vi har etablerat en metod för att odla vävnadssnitt från mushippocampus i vårt laboratorium. I dessa snitt bibehålls kontakterna mellan nervceller och samspelet mellan nervceller och stödjeceller, astrocyter. Hippocampus är en hjärnregion där nervcellerna är särskilt känsliga för ischemi, ffa. i den sk. CA1-regionen . För att efterlikna ett tillstånd av ischemi utsatte vi kulturerna för syre- och glukos brist och studerade vilka effekter detta hade på cellöverlevnad. Vi fann att om vi samtidigt med syre-och glukosbrist ändrade halterna av viktiga salter i det medium som omger kulturerna till de nivåer man uppmätt i hjärnan vid ischemi (med högt kalium, lågt calcium och lågt pH-värde) så förändrades utvecklingen av skada. Skadan begränsades nu till CA1-regionen, den blev mildare och den utvecklades långsammare. Var för sig hade såväl en sänkning av calcium som ett sänkt pH-värde en skyddande effekt. Sammantaget fick vi en skadebild som mer liknar den man ser hos försöksdjur och människor efter en motsvarande lång global ischemi. Till skillnad från tidigare *in vitro* modeller fann vi också att glukostillförsel ökade skadan i vårt anpassade medium och gav en ytterligare fördröjd skadeutveckling.

Höga glukoshalter i blodet (hyperglykemi) leder till en ökad produktion av mjölksyra, laktat, en ytterligare sänkning av pH-värdet och en svårare

hjärnskada vid stroke. Eftersom glukos skyddar effektivt mot syrgasbrist i en balanserad saltlösning har detta fenomen inte tidigare kunnat studeras i *in vitro* sammanhang. I separata försök fann vi att laktat eller en förstärkt pH-sänkning (pH 6.3) var för sig inte kunde förklara den skadliga effekten av glukos i vårt system. En måttlig sänkning av pH var däremot nödvändig för att den skadliga effekten av glukos skulle uppstå.

Vi hade nu tillgång till två nya modeller för in vitro ischemi (IVI) och hyperglykem IVI och vi jämförde dessa på en rad områden. Vid IVI kunde celldöden helt förhindras genom att blockera den calciumgenomsläppliga NMDA-receptorn eller genom att använda ett calciumfritt medium vid skadetillfället. Ingen av dessa åtgärder hade någon effekt vid hyperglykem IVI. Där hade istället BAPTA-AM, som binder calcium inne i celler, en skyddande effekt.

Att reaktiva och potentiellt skadliga syreföreningar, sk. fria radikaler bildas efter ischemi i hjärnan har tidigare visats liksom en skyddande effekt av substanser som hämmar dessa. Vi fann emellertid inget skydd av sådana substanser i någon av de två modellerna.

I en separat studie fann vi att den skyddande effekten av acidos vid IVI var betydligt mer uttalad i hippocampus CA3-region jämfört med den närliggande CA1-regionen. Eftersom NMDA-receptorn blockeras vid lågt pH misstänkte vi att skillnader i blockering tex. pga. olika regional sammansättning av receptorn, kunde förklara våra fynd. Vi studerade NMDA-förmedlade signaler i CA1 och CA3 vid olika pH och fann en likvärdig och kraftig signalblockering i de båda regionerna men en mera kvarstående effekt i CA3-regionen, vilket kan representera en ökad regional inhibition och därigenom ett ökat skydd.

Fördelen med att använda musvävnad är den rika tillgången på sk. transgena djur i vilka man förändrat uttrycket av enskilda gener för att studera dessas betydelse i sjukdomsprocesser.

Adenosin är ett ämne som ingår i ämnesomsättningen och som förekommer rikligt i kroppen. Adenosin frisätts vid ischemi och kan, genom att binda till den sk. A_1 –receptorn, minska frisättningen av glutamat och därigenom skydda mot skada. Vi odlade kulturer från djur som saknar A_1 –receptorer och utsatte dessa för IVI men fann inte att skadeutvecklingen skiljde sig jämfört med kontrollkulturerna. Inte heller påverkades skadeutvecklingen av 8-CPT, en substans som blockerar A_1 –receptorer. Inte heller vid global ischemi i en *in vivo* modell fann vi någon ökad skada hos djuren som saknade A_1 –receptorer. Däremot gav 8-CPT en ökad skada *in vivo*. Dessa resultat kan bero på en skadlig system-effekt av 8-CPT. En alternativ förklaring är att adenosinberoende glutamatfrisättning är av mindre betydelse i vår *in vitro* modell jämfört med *in vivo* och att effekterna av A_1 –receptor-avsaknad kompenserats genom en

uppreglering av andra mekanismer i de djur som använts i *in vivo* försöken.

Våra modeller för IVI och hyperglykem IVI representerar två skilda celldödsprocesser. IVI-inducerad celldöd följer ett klassiskt, NMDA- och calciumberoende mönster medan hyperglykem IVI leder till en annorlunda och hittills okänd celldödsmekanism. Vårt system lämpar sig väl för studier av mekanismer vid ischemisk hjärnskada då farmakologiska, genetiska, elektrofysiologiska och flourescens-mikroskopiska tekniker är lätta att applicera. Ett noggrant kartläggande av mekanismerna bakom IVI- och ffa. hyperglykem IVI-inducerad celldöd kan leda fram till nya behandlingsstrategier vid ischemisk hjärnskada.

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