ASTROCYTIC COMMUNICATION AND CELL DEATH DURING METABOLIC DEPRESSION AND OXIDATIVE STRESS

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Abstract
Stroke is a major cause of death and adult disability in the western world. Most often, stroke is caused by the occlusion of a brain artery. Within the perfusion territory of the occluded vessel, various degrees of necrotic and delayed programmed cell death will occur if the occlusion persists, leading to expanding tissue damage. Astrocytes are the most numerous cells in the brain, but the astrocytic response to ischemic conditions and the extent to which these cells can recover after an ischemic insult is not well understood. An increasing amount of evidence indicates that astrocytes are more sensitive to ischemic injury than previously thought. Astrocytic functions are vitally important for neuronal activity during physiological conditions and probably during various pathological situations, including stroke. Astrocytes are highly coupled by intercellular gap junction channels that enable the formation of large cellular networks. These networks provide the basis for several important astrocytic functions including intracellular signalling and transport of molecules and metabolites.

In order to investigate astrocytic reactions during metabolic depression we used the glycolytic blocker iodoacetate (IA) in primary astrocyte cultures. This treatment induced a reproducible and concentration-dependent ATP decrease which was associated with a profound increase in the activity of reactive oxygen species (ROS). This suggests that metabolic depression induced oxidative stress. Moreover, programmed cell death was initiated in individual astrocytes or small cell clusters and spread to include large clusters of astrocytes. However, when gap junction communication was inhibited during metabolic depression, programmed cell death was initiated in individual cells but no expansion into large cell clusters was observed. This suggests that gap junction permeable substances contribute to the spreading of cell death in astrocytes. The observed programmed cell death involved translocation of apoptosis inducing factor from the mitochondria to the nucleus. Similar results were observed in a model of oxidative stress using 3-morpholinosyndomine (SIN-1), a compound known to produce equimolar amounts of superoxide and nitric oxide which react to form peroxynitrite. Caspase-activation was not observed in astrocytes exposed to metabolic depression or oxidative stress.

Astrocytes and several other cell types express endogenous antioxidant systems. The expression of many of the enzymes involved in this cellular defense is regulated by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2). The potential protective effect of the Nrf2 system in astrocytes was investigated by using the Nrf2-activating phytochemicals sulforaphane (naturally occurring in broccoli) and curcumin (from turmeric) or the commonly used food additive tert-butylhydroquinone. Exposing the astrocytes to these substances before adding IA or SIN-1, prevented oxidative stress, enabled the astrocytes to maintain their ATP levels and efficiently prevented cell death. Similar results were observed when the exogenous ROS scavengers trolox (a vitamin E analogue), tempol (a superoxide dismutase analogue) or the free radical scavenger cocktail B27 were used.

Finally, we investigated the possibility for the astrocytes to recover following a simulated reperfusion injury where metabolic depression was reversed by washing out IA. Although metabolic depression was interrupted early during the ATP decrease, the astrocytes were not able to recover their ATP levels and widespread cell death occurred. However, pre-treatment with Nrf2 activators or addition of exogenous ROS scavengers enabled recovery of ATP levels and prevented cell death.

In summary, these results show that astrocytic cell death mediated by metabolic depression and oxidative stress involves the translocation of apoptosis inducing factor. In addition, gap junction communication was important for the spreading of cell death during metabolic depression. Finally, astrocytes were efficiently protected by activation of Nrf2-regulated endogenous antioxidant systems, which may represent an interesting target for the limitation of ischemic injury.

Key words: astrocyte, iodoacetate, SIN-1, gap junction, ATP, ROS, oxidative stress, Annexin V, AIF, programmed cell death, Nrf2, scavenger

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Stroke är den vanligaste orsaken till bestående handikapp i vuxen ålder samt den tredje vanligaste dödsorsaken i västvärlden. Stroke orsakas vanligtvis av att en blodpropp fastnar i en av hjärnans artärer och stoppar blodflödet. Därmed minskar blodtillförseln till den del av hjärnan som försörjs av den aktuella artären. Detta leder i många fall till omfattande celldöd och betydande funktionsnedsättning hos den drabbade.

Astrocyter är den vanligaste celltypen i hjärnan och de tillhör familjen gliaceller i centrala nervsystemet. Astrocyter har flera funktioner som är av vital betydelse för nervcellernas förmåga att fungera dels under normala förutsättningar och även vid flera skade- och sjukdomstillstånd i hjärnan, inklusive stroke. Astrocyterna är sammankopplade via intercellulära kanaler, så kallade gap junctions och bildar på så sätt nätverk vilka möjliggör flera av deras viktiga funktioner. Kunskapen om hur astrocyter reagerar, hur deras funktioner påverkas samt i vilken utsträckning de kan återhämta sig vid en ischemisk skada är idag begränsad.

Arbetet i denna avhandling har fokuserats på centrala cellulära reaktioner hos astrocyterna efter metabol och oxidativ stress. För att kunna studera dessa reaktioner i detalj har vi använt astrocyter som odlats i cellkulturer. I dessa kulturer användes ett ämne (jodacetat) som blockerar ämnesomsättningen i cellen i syfte att simulera den metabola stress som uppstår efter en stroke. Genom att först karaktärisera och sedan använda denna modell fann vi på ett reproducerbart sätt att energinivåerna sänktes i astrocyterna, att nivåerna av fria radikaler ökade och att cellerna så småningom gick under via en välreglerad form av celldöd, så kallad programmerad celldöd. Celldödsprocessen initierades i tillsynes känsligare astrocyter och spred sig sedan och omfattade till slut stora cellgrupperingar. Uppkomsten av grupperingar av döende astrocyter kunde förhindras om kommunikationen via gap-junctions hämmades, vilket tolkades som att spridning av celldödsinitierande signaler sker via dessa intercellulära kanaler. För att ytterligare belysa dessa mekanismer användes en modell av oxidativ stress baserat på inverkan av fria radikaler.

Astrocyter och andra celltyper i kroppen har egna inre försvarssystem mot fria radikaler och andra cellskadande ämnen. Vissa av dessa viktiga försvarssystem är reglerade på gennivå av en faktor som kallas nuclear factor E2-related factor 2 (Nrf2). Förutom att oxidativ stress kan aktivera Nrf2-styrda system har det visat sig att flera naturligt förekommande ämnen i vår föda kan aktivera detta system. Vi undersökte den potentiellt skyddande effekten av de Nrf2- aktiverande ämnena sulforafan (från broccoli), curcumin (från gurkmeja)och ett vanligt förekommande tillsatsämne i föda (tert-butylhydrokinon; E319). Efter behandling med dessa ämnen fann vi att astrocyter i våra modeller för cellulär stress kunde upprätthålla sina energinivåer längre och celldöden hämmades.

Vi studerade även vissa av de mekanismer som är involverade i återhämtningsprocessen efter en period av metabol hämning. Trots att energinivåerna bara var delvis sänkta när den metabola hämningen avbröts kunde astrocyterna inte återhämta sig, utan utbredd celldöd observerades istället. Däremot, om astrocyterna hade förbehandlats med de Nrf2-aktiverande ämnena eller andra hämmare av fria radikaler, kunde astrocyterna återhämta sig, energinivåerna återställas och celldöden förhindras.

På grund av den mekanistiskt komplexa skadesituation som uppstår efter en stroke och andra hjärnskador är det sannolikt fördelaktigt att använda flera olika behandlingsstrategier parallellt. En intressant möjlighet är att skydda astrocyter från den skada som uppstår vid till exempel stroke och därmed skapa förutsättningar för nervcellers överlevnad eftersom dessa är till stor del beroende av funktionella astrocyter. En stimulerande tolkning av resultaten från denna avhandling är att man med hjälp av astrocyternas egna försvarssystem skulle kunna uppnå ett bredspektrumskydd mot oxidativ stress i centrala nervsystemet.

PAPERS INCLUDED IN THE THESIS

This thesis is based on the following papers:

I. Nodin, C., Nilsson, M. and Blomstrand, F.
Gap junction blockage limits intercellular spreading of astrocytic apoptosis induced by metabolic depression.
Journal of Neurochemistry 2005, Aug;94(4):1111-23.

II. Nodin, C., Nilsson, M. and Blomstrand, F.
Exogenous free radical scavengers or activation of Nrf2-regulated defense systems prevent astrocytic cell death induced by metabolic depression.
Manuscript

III. Nodin, C., Zhu, C., Blomgren, K., Nilsson, M. and Blomstrand F.
Metabolic depression and oxidative stress induce astrocytic cell death involving translocation of apoptosis inducing factor.
Manuscript

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ABBREVIATIONS

AIF	apoptosis inducing factor
ARE	antioxidant response element
ATP	adenosine triphosphate
CNS	central nervous system
Cbx	carbenoxolone
DMSO	dimetylsulfoxide
G3PDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
HBSS	Hank's buffered salt solution
IA	iodoacetate
LDH	lactate dehydrogenase
MCA	middle cerebral artery
MEM	minimum essential medium
MPT	mitochondrial permeability transition
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced)
Nrf2	nuclear factor erythroid 2-related factor 2
PAR	poly(ADP-ribose)
PARP-1	poly(ADP-ribose) polymerase-1
PBS	phosphate buffered saline
PI	propidium iodide
PS	phosphatidylserine
ROS	reactive oxygen species
SIN-1	3-morpholinosyndomine
SEM	standare error of the mean
tBHQ	tert-butylhydroquinine
tPA	tissue plasminogen activator

BACKGROUND

Cerebral ischemia

Stroke or cerebral ischemia is a major cause of death and the primary cause of adult chronic disability in the western world. In Sweden, approximately 30 000 new cases are diagnosed each year.

Based on pathological classifications, three types of stroke has been described; 1) ischemic stroke, caused by the occlusion of an artery in the brain, resulting in a focal ischemia (approximately 80%), 2) primary intracerebral hemorrhage (approximately 15%), and subarachnoid hemorrhage (approximately 5%) [1].

In focal ischemic stroke, the reduction of blood flow of a main brain artery is caused by either an embolus or local thrombosis [2]. There will be a gradient of hypoperfusion, being maximal at the ischemic core where it causes depletion of oxygen and energy metabolites. The consequence will be loss of ionic homeostasis and membrane depolarization [3,4]. In turn, excessive amounts glutamate will be released, leading to excitotoxicity. All together, these factors contribute to cell dysfunction and extensive cell death, leading to development of an infarct. In the tissue surrounding the infarct core, the penumbra zone, partial blood flow is remained by collateral blood vessels and the hypoperfusion gradient will decrease towards the periphery. In the penumbra zone, the blood flow is too low to maintain the electrical activity but sufficient to remain the function of ion channels [4,5]. However, deleterious ions such as potassium ions and glutamate can spread from the core area and increased production of reactive oxygen species (ROS) and several other factors will lead to delayed tissue damage and cell death [2,6].

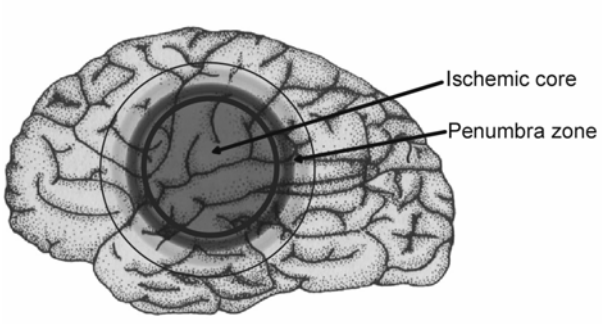

Fig. 1. The occlusion of a brain artery results in a region of low perfusion forming the ischemic core. In the surrounding penumbra zone there will be a gradient of hypoperfusion. From the onset of the occlusion, the core and penumbra are dynamic in space and time. Adapted from Dirnagl et al. 1999 [2]

The ischemic brain injury is very complex and multiple factors are involved in the pathophysiology. For example, the degree and timeframe of the hypoperfusion and whether

the occlusion is permanent or reversed, are important determinants of the extension of the infarct[7-9]. Reversal of the occlusion can occur spontaneously or as a result of thrombolytic treatment. However, reperfusion must be initiated within a defined time window after onset of ischemia since the risk of reperfusion damage otherwise can exceed the benefits of treatment[10,11]. Reperfusion itself can initiate deleterious responses, including generation of a variety of reactive oxygen species, and it is possible that different factors contribute to the tissue damage during ischemia and reperfusion[12,13]. Some treatments initiated during reperfusion has been shown to reduce the cell loss after cerebral ischemia in animal models[14,15]. This suggests that a fraction of cells are viable at onset of reperfusion and can be protected but also that reperfusion events are critical for the development of damage.

Today the only clinically approved treatment of acute focal cerebral ischemia is thrombolysis using tissue plasminogen activator (t-PA)[11,16]. Several other substances have been evaluated in clinical trials but although promising results have been shown in animal models, none have been successful [17-19]. Due to the complexity of the pathology, it is possible that several therapeutic approaches complementing each other, could improve the outcome for patients suffering from cerebral focal ischemia.

Astrocytes

Astrocytes are one of the members of the glial cell family and are the most abundant cell type the brain. Astrocytes represent up to 30% of the cell volume in cerebral cortex, with regional variability and species differences [20,21]. Astrocytes form a heterogeneous cell group and are classically subdivided into three types according to their morphology and their spatial organization in the brain. The protoplasmic astrocytes are mainly found in the gray matter, fibrous astrocytes are predominantly located in white matter and radial glia extend their processes from the ventricular zone [22-24]. However, astrocytes located at the border of white and gray matter regions can display morphology intermediate between protoplasmic and fibrous astrocytes [25]. It was recently described that astrocytes in humans and higher primates display a larger complexity than for example rodents and it is likely that astrocytes are much more important in contributing to brain function than previously thought [26,27].

Astrocytes have traditionally been identified by staining for the astrocytic intermediate filament protein glial fibrillary acidic protein (GFAP). GFAP is strongly expressed in cultured astrocytes, reactive astrocytes and fibrous astrocytes. However, protoplasmic astrocytes have

been described with no detectable GFAP staining, albeit having astrocyte morphology, astrocyte electrophysiological properties and immunoreactivity for glutamine synthetase [28,29]. GFAP staining reveals a star-like conformation of astrocytes. However, by using dye filling it has been shown that protoplasmic astrocytes have a bush-like shape with specific domains arranged with minimal overlapping [30-32]. These astrocytic domains consists of fine processes that cover several thousands of synapses in rodents and in humans this number could be over a million [26,27]. This close contact with neurons enables astrocytes to regulate neuronal signaling [33].

The glial cells were for a long period of time considered as electrically silent, not participating in the information processing in the central nervous system (CNS). However, it has become evident that astrocytes play an essential role in the integration of information in the brain and may shape neuronal responses [34,35]. Astrocytes express receptors for many neurotransmittors, various ion channels and second messenger systems, previously thought to be exclusive for neurons [36-38]. Astrocytes are strategically positioned around neurons and blood vessels. They enclose the synapse and by responding to neuronal release of neurotransmitters and by releasing 'gliotransmittors' astrocytes are enabled to feed back regulate the neuronal activity [37,39]. This have given rise to the expression "the tripartite synapse" [40].

Recactive gliosis

Astrocytes become reactive as a response to various brain pathologies, including stroke. The process is known as reactive gliosis and is characterized by hypertrophy of astrocytes and proliferation of for example microglia and astrocytes [41]. Well known hallmarks of reactive gliosis is up-regulation of the intermediate filament proteins GFAP and vimentin, re-expression of nestin and hypertrophy of astrocyte processes [41,42]. In addition, a number of enzymes, growth factors, cytokines and recognition molecules are up-regulated in reactive astrocytes [41,43].

The reactive astrocytes form a glial scar by a meshwork of tightly interwoven astrocytic processes [43]. The glial scar has been shown to be protective and enhance healing after CNS injury by separating the uninjured regions from the lesion which may provide beneficial and stabilizing functions for the CNS tissue [32,44-46]. Nevertheless, the glial scar and the dense meshwork of reactive astrocytic processes may be an obstacle for regeneration [47-49].

Gap junctions

Intracellular communication via so called gap junction channels is essential for regulating and synchronizing functions in various organs, such as heart, lung, liver and brain [50-52]. The existence of gap junction channels between astrocytes was first shown in the 1960s [53,54]. The gap junction channels are built up by so called connexons, which are hexamers of the membrane bound protein connexin (Cx) [51]. A gap junction channel is formed when a connexon from one cell conjugates with one on a neighbouring cell [51,52]. It is not fully understood how the opening of the first channel occurs but occasional gap junctions are not sufficient to permit intercellular communication. Rather several hundred gap junction channels, forming a gap junction plaque, are needed to enable cell-cell communication [55,56].

Connexins

Connexins are expressed in several cell types in the brain including astrocytes, neurons, oliogodendrocytes, microglia and ependymal cells. Some connexins are generally expressed whereas others are cell specific [57,58]. In astrocytes the most frequent gap junction protein is connexin43 (Cx43) and in Cx43 knockout mice the gap junction coupling was decreased to 5%, compared to wild type [59]. However, astrocytes express several other connexins [60] and different connexins can be simultaneously expressed in the same astrocyte [61].

Gap junction communication

Gap junction channels enables transfer of ions and small metabolites via diffusion. Gap junctions are approximately 1.0-1.5 nm in diameter and permeable to substances with molecular weight up to 1.2 kDa [51,58]. The permeability for charged molecules is depending on the connexins forming the channel. Gap junctions formed by Cx43 is permeable to both positively and negatively charged substances, whereas others are more charge specific [50,62].

Astrocytic gap junction communication is dynamic and can be regulated in a long time perspective (hours/days) by regulation of transcriptional, translational and degrading connexins or in the short time perspective (seconds-minutes) by open probability, open time and phosphorylation and internalization of channels already present in the gap junction plaque [58,63]. These factors enable a large plasticity in the state of coupling.

Regional heterogeneity in gap junction permeability have been shown in astrocytes cultured from different brain regions [64]. Moreover, two phenotypes of astrocytes have been shown to be represented in the hippocampus, where one type is gap junction coupled but the other is

not [65]. Similar results showing that not all cortical astrocytes are involved in the gap junction communication were recently presented [63].

Besides forming gap junctions, single connexons can function as channels to the extracellular space and are then referred to as hemichannels [62]. Hemichannels have been reported to open during certain conditions, both physiological and pathological, which may be functional or detrimental depending on the situation [62,66,67].

Several endogenous factors modulate gap junction communication. For example, increased extracellular levels of glutamate and potassium can increase communication [68-70]. Addition of H_2O_2 has been shown to increase gap junction communication [71], whereas increased intraracellular levels of Ca^{2+}, acidocis and NO may inhibit gap junction communication [58,72]. Neuroactive peptides of the endothelin-group are potent inhibitors of astrocytic gap junction communication as shown in culture [73,74] and in acute slice preparation [61].

Exogenous gap junction blockers

Besides the endogenous factors regulating gap junction coupling, several substances have been used to block gap junction communication [75]. For example, carbenoxolone and the related substance glycyrrhetinic acid have been commonly used [75-78]. Carbenoxolone is considered as one of the more specific blockers, although other effects besides gap junction blockage has recently been reported [79,80]. The alcohols octanol and heptanol also potently block gap junction communication, albeit less specific [75]. Flufenamic acid is yet another structurally different gap junction blocking substance [67,81]. In an effort to achieve more specific gap junction inhibitors, connexin mimetic peptides have been developed and used to block gap junctions and hemichannels [82,83], although the specificity was recently questioned[84].

Gap junction mediated astrocytic functions

The gap junction channels enable astrocytes to form vast syncytiums of interconnected cells. These networks provide the morphological basis for main astrocytic functions which are essential for normal brain function.

Spatial buffering of potassium

Astrocytes have an important role in the regulation the extracellular homeostasis in the brain. Accumulation of extracellular potassium occurs at regions of intense neuronal activity.

Potassium is taken up by astrocytes via potassium channels and is distributed along its concentration gradient within the astrocytic syncytium, thus leading to so called spatial buffering of potassium [85,86]. In relation to the potassium uptake, the extracellular osmolarity is regulated by water flux through aquaporins [87,88]. Moreover, astrocytes are involved in the regulation of extracellular levels of sodium, chloride and hydrogen ions [86].

Glutamate uptake

Rapid removal of glutamate from the extracellular space is of major importance for normal function and survival of neurons. Astrocytes are primarily responsible for the glutamate uptake and can thereby participate in the regulation of the glutaminergic synapses [89]. Glutamate is efficiently taken up by Na^+-dependent glutamate transporters and can be distributed via gap junctions [89-91]. A large proportion of the glutamate is converted to glutamine by the astrocyte-specific enzyme glutamine synthetase, but glutamate can also enter the tricarboxylic acid (TCA) cycle [92,93]. Glutamine, which does not act as a neurotransmitter, is transported to the extracellular space to be taken up by neurons [94,95] and is thereafter converted back to glutamate to restore the neurotransmitter pool [93,96,97].

Calcium signaling

Astrocytes respond to a variety of external stimuli such as neurotransmitters, hormones or mechanical stress by generating changes in the intracellular levels of Ca^{2+} [68,98-100]. Astrocytes respond by oscillating increases of intracellular Ca^{2+}, and importantly, the Ca^{2+} signaling can be propagated as a wave to neighboring cells [101]. Astrocytes are non-excitable cells and the Ca^{2+}-signaling is believed to be an astrocytic form of 'excitability', enabling intercellular communication [102,103]. Two pathways have been suggested for mediating the communication. One involves gap junction communication [64,104,105] and the other release of astrocytic ATP or glutamate that activates membrane receptors on neighboring astrocytes [106,107]. Most likely, these two pathways work in conjugation to coordinate the communication [102]. Increase of intracellular levels of Ca^{2+} can lead to astrocytic release of neuroactive substances, including glutamate [105,108]. This enables astrocytes to sense, integrate and respond to external stimuli released by e.g. neurons and to spread the signal via the network [37,39,40]. Astrocytic Ca^{2+} signaling has mainly been studied *in vitro* and *ex vivo*, but recently astrocytic Ca^{2+} signaling was shown to occur in vivo [109].

Astrocytic metabolism

Glucose is the major energy source for the brain and enters the brain via glucose transporters in the epithelial cells in the capillary walls. Astrocytic expansions, so called endfeet surround the blood vessels in the brain. These endfeet are enriched in glucose transporters, enabling glucose to be further transported to the astrocytes [97,110]. Glucose is gap junction permeable and can be spatially distributed to provide astrocytes and neurons with energy [73,111]. Astrocyte metabolism has been described to be more dependent on glycolysis than on oxidative phosphorylation [96,112,113]. However, astrocytes have about equivalent oxidative capabilities as neurons and that the main energy production in astrocytes has been suggested to occur via oxidative phosphorylation [114,115]. However, the thin outer extensions of astrocytes are too narrow to accommodate mitochondria. Therefore, in these parts the energy demand during for example uptake or release of neurotransmitters, is depending on glycolysis, glycogenolysis and probably diffusion of ATP production [114]. Astrocytes are the main storage sites of glycogen in the brain and the levels substantially decrease during brain activation [116-118]. The increased metabolism of glycogen constitutes a rapid source for astrocytes in order to meet important energy dependent demands [119]. Moreover, astrocytes lack the enzyme necessary to form glucose from glycogen and the glycogen-mediated metabolites cannot be released from the astrocytes to the extracellular space [111].

For a long time it was believed that glucose was the exclusive substrate for the energy support in the brain. However, lactate has been suggested to be of even greater importance than glucose [120]. An astrocyte-neuron lactate shuttle has been postulated and according to this hypothesis, neurons are supplied with lactate generated by glycolytic activity in astrocytes, especially during periods of high neuronal activity [121-123]. Increased neuronal activity and glutamate release stimulates glutamate uptake by astrocytes, which leads decreased levels of ATP, triggered glycolytic activity and glycogenolysis and increased lactate production and release from astrocytes, despite sufficient levels of oxygen [118,124-126]. The lactate is taken up by neurons and converted back to pyruvate and metabolized in the TCA cycle to generate ATP needed during neurotransmission. However, the importance of this pathway has been questioned. It has been demonstrated that neuronal activity induced a slow, delayed increase of lactate levels which extended well beyond the activation [127]. In addition, there is a lack of direct evidences that neurons oxidize lactate to keep up synaptic activity *in vivo* [115,128]. Although much research has been done with the purpose of understanding brain metabolism,

it is not fully understood to what extent metabolic substrates are transferred between cell types in the brain and more research in this field is needed [129].

Programmed cell death

The term apoptosis was first proposed in 1972 as one possible way of cell death, the other being necrosis [130]. Apoptosis was described as an active process with the purpose to remove unwanted cells without leaving any tracks [131]. In contrast, necrosis was described as an uncontrolled way of death, involving cell lysis and leakage of cellular constituents to the environment which provokes a substantial inflammatory response. Today several highly regulated pathways for programmed cell death have been described, where apoptosis is described as one. Necrosis is now also believed to be a well controlled programmed form of cell death, in contrast to the earlier view [132,133]. Programmed cell death is thought to be a dynamic process where a cell can use different mechanisms with underlying apoptotic or necrotic features [134-136].

Programmed cell death is an essential progress during development, not the least in the brain. It serves to remove excess astrocytes and neurons with improper connections, without causing inflammation [137-140]. However, programmed cell death is also associated with several pathological situations in the brain such as stroke, Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS).

Several different pathways for programmed cell death have been suggested and the most general are briefly described here.

Caspase dependent apoptosis

Classic apoptosis is characteristically associated with caspase dependent programmed cell death. Caspases are cystein-dependent aspartate-specific proteases and expressed in at least 14 different forms of which 11 are known in humans [141]. They are normally expressed in their inactive form as pro-caspases which are activated when the inactive subunit is cleaved off. Activation of caspases leads to cleavage of downstream pro-caspases or other key target proteins [142,143].

The caspase dependent cell death is generally accomplished by the so called extrinsic or intrinsic pathways. The extrinsic pathway, or death-receptor pathway, is initiated by the activation of cell membrane bound death receptors which activates caspsase-8 and in turn caspase-3 [144]. Activation of the intrinsic pathway leads to release of mitochondrial pro-

apoptotic factors including cytochrome c [145,146]. Once in the cytosol, cytochrome c binds to the scaffolding protein apoptotic protease activating factor 1 (Apaf-1) and caspase-9 to form the so called apoptosome, which in turn activates downstream effector caspase-3. Both the extrinsic and the extrinsic pathways lead to activation of several pathways which results in chromatin condensation, cleavage of DNA and the dismantling and removal of the cell [147].

Caspase independent programmed cell death

Programmed cell death independent of caspase activation can be conducted by the translocation of the mitochondrial protein apoptosis-inducing factor (AIF) to the nucleus, where it is involved in chromatin condensation and large scale DNA fragmentation [148-150]. Cell death mediated by AIF has received increasing interest and has been described to induce programmed cell death in animal and human models due to various stimuli [136].

AIF is a mitochondrial flavoprotein with vital physiological functions. It and has been shown to be required for the maintenance or maturation of complex I in the respiratory chain and to have free radical scavenger functions [151,152]. AIF is essential during embryogenesis and AIF knockout mice die early during embryonic development because of deficient organogenesis[153]. Conditional knockout of AIF has been shown to induce severe organ deficiency [151,154,155]. Moreover, in Harlequin mice the AIF expression is reduced to 10-20% of the normal value due to a hypomorphic mutation. These animals show cerebellar neurodegeneration and blindness due to retinal degeneration [152].

AIF is synthesized as a 67 kDa precursor protein and transported to the mitochondria where it is cleaved and anchored to the mitochondrial intermembrane space [148,156,157]. When programmed cell death has been initiated, AIF is further truncated in the mitochondrial intermembrane space and released to the cytosol [158,159]. It is not fully understood how the release occurs but it was recently shown that the cleavage of AIF is dependent on calpains [160-162]. AIF is further translocated to the nucleus but the mechanism remains elusive. However, both translocation and chromatinolysis by AIF has recently been shown to be facilitated by interaction with cyclophilin A [163,164].

Translocation of AIF has been strongly associated as a downstream factor of poly(ADP-ribose) polymerase-1 (PARP-1)-activation [160,165,166]. PARP-1 is a nuclear protein involved in the DNA repair system. PARP-1 uses nicotinamide adenine dinucleotide NAD^+ to form poly(ADP-ribose) (PAR) and at excessive DNA damage, PARP-1 can be over-activated which leads to NAD^+ depletion [168]. The depletion of NAD^+ in turn, has been suggested to be

involved in the signaling leading to AIF release [167,168]. Moreover, it was recently suggested that polymers of PAR promotes the release of AIF [166]. However, the exact mechanism of AIF translocation remains to be explored [160,166].

Mitochondrial release of cell death mediating factors

The release of proteins including AIF, cytochrome c, SMAC /DIABLO, Omi/HtrA2 and Endonuclease G from the intermembrane space in mitochondria is one of the essential events in programmed cell death [136,169,170].

Several lines of evidence suggest that the Bcl-2 protein family is of major importance for the maintenance of mitochondrial integrity. Bcl-2 and Bcl-X_L are factors preventing programmed cell death, whereas the so called BH3-only proteins e.g. Bax, Bak and Bid are pro-apoptotic[171-173]. Bax and Bid are activated by cleavage, which can be mediated by several proteases, including caspases and Ca^{2+}-dependent calpains [136,161,173]. The truncated forms of Bid and Bax together with Bak have been suggested to interact in the formation of pores in the mitochondrial outer membrane allowing release of pro-apoptotic intermembrane space proteins [136,174,175].

The first mechanism proposed to mediate the release of mitochondrial intermembrane constituents was the mitochondrial permeability transition (MPT) [172]. It is described as a Ca^{2+}-dependent increase of mitochondrial membrane permeability, leading to loss of the mitochondrial membrane potential, mitochondrial swelling and rupture of the outer mitochondrial membrane. MPT is thought to occur after the opening of a channel known as the mitochondrial permeability transition pore (MPTP) [172,176]. The pore allows unspecific transport of molecules less than 1.5 kDa which leads to ion-transport and subsequent swelling of the mitochondria and depolarization of the mitochondrial membrane potential [172,177]. Cyclosporine A and bongcrecic acid are common blockers of the MPTP and prevention of cell death by the use of these have supported the existence of the pore [165,178]. It has been described that Bcl-2 and Bcl-X_L can block the opening of MPTP, however, it is not fully elucidated if the BH3-only and the MPTP represent separated pathways or if they interact [170,177,179].

Translocation of phosphatidylserine

The removal of cells undergoing programmed cell death by phagocytosis prevents the eventual release of pro-inflammatory factors from the dying cells [180,181]. In a healthy cell the

phospholipid phosphatidylserine (PS) is expressed on the inside of the cell membrane. However, during the early steps of programmed cell death, it is translocated to the outside of the cell membrane and plays an important role for the recognition and removal of dying cells by phagocyting cells, including microglia [182-184]. Translocation of PS has been reported to occur both during caspase dependent [185,186] and AIF-dependent programmed cell death [148,187]. The extracellular translocation of PS have been used as a experimental tool to identify cells undergoing programmed cell death [188-191].

ATP levels and programmed cell death

Programmed cell death is an active process and several steps require ATP. For example, it is conceivable that ATP depletion interferes with the activity of the apoptosome, since ATP is required for the formation of the protein complex [192,193]. Moreover, ATP is also needed for the translocation of phosphatidylserine and caspase activity [194]. The relative importance of ATP levels for the molecular decision between programmed cell death or necrosis have been described in several cell types [195-198]. By manipulating the intracellular levels of ATP, it was shown that when ATP levels were depressed below a critical value (~ 15-20% of normal ATP levels), stimuli which normally induced programmed cell death instead caused necrotic cell death [195,198]. However, it has been shown that AIF translocation can occur although the ATP levels are depleted by simultaneous blockage of both glycolysis and oxidative phosphorylation [199,200]. Nevertheless, the translocation of AIF was associated with necrotic features [200]. Processes occurring during programmed cell death may also contribute to decreasing ATP levels. For example, opening of the mitochondrial permeability transition pore can lead to out-flux of H^+, which are required for the function of ATP synthase [177]. If mitochondrial dysfunction due to pore opening persists, or if the majority of the mitochondria in a cell are affected due to a severe insult, cell death may turn to necrotic pathways [177].

Oxidative stress

The human brain consumes approximately 20% of the oxygen utilized in the body but only represent 2% of the body weight. As a consequence, reactive oxygen species (ROS) will be continuously generated at high rate during oxidative phosphorylation [201]. The detoxification of ROS is therefore an essential task in the brain and is normally accomplished by enzymatic and small molecule antioxidant defenses. The enzymes include superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, and catalase as well as the small molecules

glutathione, ascorbic acid, vitamin E and a number of dietary flavinoids [202]. Glutathione is a central component in the cellular defense against oxidative stress, acting both as a direct scavenger of ROS in non-enzymatic reactions and as is the electron doner in the reduction of peroxides catalysed by glutathione peroxidase [201,203]. Astrocytes are believed to play a crucial role in the antioxidant defense in the brain [203,204]. They contain high concentrations of antioxidants and provide neurons with substrates for e.g. glutathione [10,201,203]. In particular, the mitochondrial pool of glutathione has been shown to be of major importance for the astrocytic defense against oxidative stress [178].

Oxidative stress has been described as an important factor involved in the patophysiology of several neurodegenerative disorders, including cerebral ischemia. The increased levels of ROS due to increased production, decreased cellular defense ability or both, will lead to oxidative stress and cellular damage. Like other cells, astrocytes are vulnerable to excessive production of ROS generated during ischemia and reperfusion [25]. The sources, mechanisms and time course of ROS generation during ischemia and reperfusion are not fully understood and it is possible that the origin of ROS varies during ischemia and reperfusion [205]. The mitochondria likely play a role, both as initiators and targets of oxidative stress [8]. Moreover, nitric oxide synthase (NOS), xantine oxidase, NADPH oxidase and other factors may contribute to the production of ROS in the brain [202,205,206].

The ROS particularly involved in oxidative stress include superoxide (O_2^-), hydroxyl radical ($^{\bullet}OH$) hydrogen peroxide (H_2O_2), nitric oxide (NO) and peroxynitrite ($ONOO^-$). The latter two are also described as reactive nitrogen species (RNS). Superoxide can be generated during mitochondrial impairment due to dysfunction of the enzymes in the respiratory chain. It can also be generated by xantine oxidase, NADPH oxidase and during certain conditions, by nitric oxide synthase (NOS) [207]. Superoxide can be enzymatically degraded by SOD, a reaction which in turn yields H_2O_2. H_2O_2 must be rapidly degraded by catalase since superoxide and H_2O_2 can react and form extremely reactive hydroxyl radicals by the iron-catalyzed Haber-Weiss reaction [207-209].

Nitric oxide is a water and lipid-soluble free radical with diverse biological activities, including neurotransmitter functions. It is generated by the activity of NOS and the production of NO can increase, due induction of expression and activation of NOS during pathological situations, such as cerebral ischemia [207,208,210]. NO is highly reactive and the chemistry of NO involves redox forms, including $NO^{\bullet}$ NO^+ and NO^- [194] Underlying many of the deleterious

effects of nitric oxide is the formation of peroxynitrite by the reaction of $NO^{\bullet}$ and superoxide.

ROS may bring about oxidative damage of lipids, proteins, RNA and DNA [210,211]. For example, peroxynitrite has been reported to modulate cell function via inhibition of mitochondrial respiration [8,212,213]. Inactivation of mitochondrial electron transport enzymes can lead to further increased production of radicals such as superoxide, thus further contributing to additional formation of hydroxyl radicals and peroxynitrite and aggravating the oxidative stress [214]. Peroxynitrite is a highly reactive molecule causing oxidation or nitrosylation of various proteins and excessive formation has been strongly implicated as a contributor to tissue damage following cerebral ischemia [215-217] and other brain injuries [214,218].

Oxidative stress can lead to the development of programmed cell death, although the exact mechanisms underlying ROS mediated programmed cell death are not fully understood. However, mitochondrial impairment can lead to release of e.g. AIF, cytochrome c, Smac/DIABLO, all factors involved in programmed cell death [8,210,211]. Moreover ROS mediated DNA damage can lead to extensive PARP-1 activation and cell death involving translocation of AIF [134].

The Nrf2 antioxidant system

Astrocytes, neurons and several other cell types in detoxifying organs such as liver and kidney and organs continuously exposed to different components of the environment such as skin, lung and the digestive tract express inducible endogenous defense systems against ROS [219,220]. Many of the genes involved in this so called phase II protection system contain a cis-acting enhancer region commonly referred to as antioxidant response element (ARE) [221,222]. The transcription of these genes is regulated by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), which is located in the cytoplasm and associated with Kelch-like ECH-associated protein1 (Keap1) [222-224]. Following cellular stress such as oxidative injury, Keap1 looses its ability to bind Nrf2. Nrf2 dissociates and translocates to the nucleus where it dimerize with small Maf proteins and binds to ARE [222,225]. In this way Nrf2 is capable of regulation the expression of all genes containing the ARE sequence. The repression of the Nrf2-mediated antioxidant response was recently suggested to be mediated by Keap1. Upon recovery of the redox homeostasis, Keap1 may translocate to the nucleus and escort Nrf2 out to the cytosol where the inactive Keap1-Nrf2 complex is re-established [226].

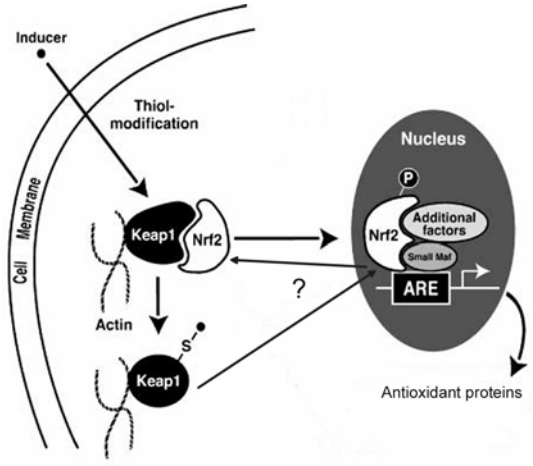

Fig. 2. As a response to cellular stress, Nrf2 dissociates from Keap1 and translocates to the nucleus where it binds to ARE. This leads to transcription of genes encoding for antioxidant proteins. Keap1 has been proposed to escort Nrf2 out of the nucleus back to the cytoplasm. Adapted from Zhang and Gordon, 2004 [222].

The most persuasive evidences that Nrf2 mediate regulation of ARE driven genes come from experiments using Nrf2 knockout mice [225]. Micro-array analysis of mixed neuron and astrocyte cultures from both Nrf2 knockout- and wild type-mice have revealed that over 200 genes are directly or indirectly regulated by Nrf2 [227,228]. These genes include detoxification- and antioxidant-genes, such as NAD(P)H quinine oxidoreductase (NQO1), heme oxygenase-1 (HO-1), Cu/Zn superoxide dismutase (SOD1), thioredoxin reductase, peroxiredoxin, ferritin, metallothionein, glutathione s-transferases, glutathione reductase and catalase as well as genes involved in inflammation, signal transduction and the maintenance of cellular reducing potential [227,228].

Activation of the Nrf2 system occurs as a response to oxidative stress but several small molecules have been reported to initiate the translocation of Nrf2 and activation of ARE driven genes. One of the most commonly used is tert-butylhydroquinone (tBHQ) [204,219,229]. Interestingly, several naturally occurring phytochemicals including sulphoraphane from broccoli, curcumin from the roots of turmeric, reservatrol from red grapes and ECGC from green tea can stimulate the dissociation of Nrf2 from Keap1 and activation of the phase II detoxifying and antioxidant defense enzymes [225,230].

In contrast, the ability of Nrf2 to activate ARE driven genes has been shown to be prevented by retinoic acid (from vitamin A) [231] and by the ochratoxin-A, a toxin which have been found in food products such as cereals, green coffee, cocoa, dried fruit and meat [232].

Astrocytes during ischemic conditions

Focal cerebral ischemia leads to various degrees of necrotic and programmed cell death of both neurons and astrocytes [6,134,210,233].

Increasing evidence suggests a substantial role for caspase-independent pathways involving mitochondrial release of AIF following ischemic brain injury [234]. In the brain, this has mostly been studied in neurons [156,175,235-238]. However, AIF translocation was also described in astrocytes in the penumbra zone following transient ischemia *in vivo* [6,239]

Astrocytes – more or less sensitive to ischemia?

Astrocytes have in general been described as more resistant than neurons to most stress conditions *in vitro*. However, regional differences showing that hippocampal astrocytes are more susceptible for ischemic conditions in comparison to cortical astrocytes *in vitro* have been described [240,241]. Moreover, protoplasmic astrocytes in acutely isolated hippocampus slices, and especially fibrous astrocytes in the optic nerve, have been shown to be vulnerable to oxygen and glucose deprivation [242]. Importantly, increasing evidence indicate that astrocytes may be more sensitive to ischemic injury *in vivo*, than previously thought [134,243-245]. Protoplasmic astrocytes that dominate the gray matter structures, have been shown to rapidly loose the GFAP immunoreactivity after onset of MCA occlusion, which is followed by signs of cell death [244,245]. Interestingly, fibrous astrocytes were less severely injured and formed the glial scar [244]. In contrast to these results, no decrease of immunoreactivity for GFAP or glutamnine synthetase (another astrocytic marker) was observed during reperfusion after MCA occlusion in rats [246]. Thus, the relative susceptibility of astrocytic markers may be sensitive to the investigated ischemic conditions. However these results may also reflect that different subtypes of astrocytes react differently to ischemic injury. Further enlightening this, at least a fraction of astrocytes has been shown to remain viable the infarct core after ischemic injury [247].

GFAP staining is a common way to identify astrocytes. However, since protoplasmic astrocytes only weakly express GFAP, are GFAP negative [28,29] or even loose the GFAP expression as a result of focal ischemia [244,245], it may be difficult to identify dying astrocytes. Moreover, rapid astrogliosis by surviving, less sensitive astrocytes, can mask an early loss of astrocytes and disguise the importance of acute astrocyte injury when post mortem samples are analyzed [242]. Nevertheless, the reason why some astrocytes appear to be very sensitive to

ischemic injury whereas other astrocytes survive among the dying cells in both core and penumbra is not known.

Astrocytic functions during ischemic conditions

Astrocytic functions are of major importance for neuronal function during physiological situations, but most likely also during pathological situations.

For example a major contributor to tissue damage is glutamate excitotoxicity. Astrocytic Na^+-dependent glutamate uptake is functionally coupled to the Na^+/K^+ ATPase [89]. ATP depletion can therefore lead to decreased glutamate uptake or even reversed function of the Na^+/K^+ ATPase, thus leading to efflux of glutamate [23]. Moreover, astrocytes respond to intracellular levels of Ca^{2+} by release of glutamate [105,108] and this may contribute to excitotoxicity. In addition, hemichannels can open during ischemic conditions [66]. It was recently shown that glutamate was released via hemichannels in astrocytes when extracellular Ca^{2+} was omitted [248]. Moreover, excitotoxicity occurred if astrocytic glutamate uptake was simultaneously inhibited. Energy depletion may also cause neuronal glutamate release due to membrane depolarization. However, it is not elucidated whether astrocytes or neurons are the primarily source of the excitotoxic glutamate [23].

Dysfunction of the Na^+/K^+ ATPase may also lead to increased levels of extracellular K^+ levels. Elevations of extracellular K^+ lead to neuronal depolarizations and may potentiate the effect of excitotoxicity [23]. Astrocytes are able to buffer the initial insult by up take and spatial distribution of potassium (as described above). However, if the ischemic period is prolonged, these functions may have consequences for other cellular functions [249]. For example, it has been suggested that increased spatial buffering of K^+ may contribute to the phenomenon of gap junction dependent spreading depression [23,250,251].

Astrocytic metabolism during ischemic conditions

Experiments *in vitro*, have shown that during inhibition of mitochondrial respiration, the ATP levels are restored due to a shift in metabolism from oxidative phosphorylation to anaerobic glycolysis using both glucose and glycogen as substrates [125,252-254]. Astrocytes constitute the main storage pool of glycogen and could therefore, in contrast to neurons, be less sensitive to oxygen depletion [210]. It is likely that the glycogenolysis and glycolytic production of ATP, leading to lactate formation is of importance also *in vivo* since several reports indicate that lactate levels increase following focal ischemia [255]. The lactate could be an energy source for

neurons during hypoglycemia or during reperfusion after transient ischemia [115,256], although the importance of the lactate shuttle from astrocytes to neurons have been questioned during neuronal activity [257].

Gap junction communication during ischemia – good or bad?

Astrocytes are extensively coupled via gap junctions. The gap junction communication has been reported to persist, although at decreased efficiency, during ischemic conditions [66,258,259]. According to experimental findings, it still remains obscure whether gap junction communication is beneficial or detrimental during ischemic conditions. For example, blocking gap junction communication reduced tissue injury after global [78,260] and focal ischemia [261-263]. In contrast, reduced astrocytic gap junction communication was demonstrated to aggravate neuronal damage *in vitro* after oxidative injury [264], glutamate toxicity [265] or NMDA induced injury [266]. Moreover, Cx43 heterozygote knock-out mice or mice with astrocytic conditional Cx43 knockout are more susceptible to infarct expansion after focal ischemia [267-269].

Several observations suggest complex alterations in astrocyte connexins following various brain injuries [58]. Following focal ischemia, the levels of Cx30 increased in Cx43 heterozygote knockout mice [268] and in rats, the Cx43 expression increased in the glial scar formation [270]. Similar results were found in human brain tissue where the Cx43 reactivity was increased following ischemic injury [271]. It is not fully understood what these changes in connexin expression represent but could be an adaptive response to increase the gap junction communication or be involved in the glial scar formation.

Bystander killing

Spreading of death signals or so called bystander killing mediated by gap junctions have been described in several cell types [272-274]. In astrocytes, gap junctions have been shown to remain open during programmed cell death and bystander killing in was observed [275]. Similar results have also been obtained in retina and modified C6 cells [276,277]. Moreover, during ischemic conditions, open hemichannels may be involved in the cell death signaling [272]. However, the key question is which signals pass through gap junctions and mediate the bystander killing. This is yet not answered but suggested molecules include Ca^{2+}, IP_3, cAMP Na^+ and ROS [272,274,277,278].

The significance of the Nrf2 antioxidant system

There is an increasing interest among neuroscientists for the endogenous Nrf2-regulated antioxidant system. The goal is to achieve protection from various brain diseases, including stroke. The neuroprotective potential of the Nrf2 system in the brain has recently been investigated. Results show that astrocytes and neurons derived from Nrf2 knockout mice are more susceptible to oxidative stress and that Nrf2 knockout mice have larger infarct volume following MCA occlusion [219,227,229]. Interestingly, the infarct volume following permanent focal ischemia was similar in Nrf2 knockout and wild type mice after 24h but significantly smaller in wild type mice after 7 days, suggesting that Nrf2 may play a role in shaping the penumbra [229].

The Nrf2 antioxidant system is most likely of major importance for the astrocytic defense of oxidative stress [229]. Astrocytes have a higher basal expression of Nrf2 than neurons *in vitro* [228] and when treating mixed cultures of astrocytes and neurons with Nrf2 activators, the ARE mediated gene expression was predominately up-regulated in astrocytes [219,228,279]. Interestingly, this was sufficient to protect both astrocytes and neurons against oxidative stress [219]. Administration of tBHQ has also been shown to reduce infarct volume after cerebral ischemia and protect against mitochondrial stress induced by 3-nitropropionic acid *in vivo* [229,280]. Moreover, sulforaphane has been shown to reduce the infarct volume when administrated during focal ischemia [281] and curcumin enabled neuronal survival and reduced infarct volume when administered after onset of reperfusion following transient focal ischemia [282,283].

It is not in detail investigated to what extent these substances pass the blood brain barrier. However oral or intraperitoneal administration of tBHQ [229] and sulforaphane [281] has been shown to induce Nrf2 activity in the brain. In addition, the presence of curcumin in the brain after systemic administration was recently shown [284]

AIMS OF THE STUDIES

- To set up *in vitro* models of metabolic depression and oxidative stress in primary astrocyte cultures

- To investigate the significance of gap junction-mediated communication during the progress of cell death following metabolic depression in cultured astrocytes

- To identify pathways that contribute to the progress of cell death during metabolic depression and oxidative stress in cultured astrocytes

- To identify factors that are important for the initiation and progress of astrocytic cell death and to study the extent to which these factors contribute to cellular injury, during metabolic depression and oxidative stress in cultured astrocytes

- To explore possible protective strategies for the prevention of astrocytic injury and cell induced by metabolic depression and oxidative stress in cultured astrocytes

MATERIAL AND METHODS

Primary astrocyte cultures (I, II, III)

Primary astrocyte cultures were prepared from newborn (P1-P2) Sprague-Dawley rats as previously described [285]. The rats were decapitated and the hippocampi were carefully dissected and mechanically passed through an 80 μm nylon mesh into minimum essential medium (MEM; Invitrogen, Belgium) supplied to the following composition: 20% (v/v) fetal bovine serum gold (PAA laboratories GmbH, Austria), 1% penicillin-streptomycin, 1.6 times the concentration of amino acids, and 3.2 times the concentration of vitamins, 1.6 mM L-glutamine (all from Invitrogen, Belgium), 7.15 mM glucose and 48.5 mM $NaHCO_3$. The cells were cultured at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed after three days in culture and thereafter three times a week. Cells were used after 14-17 days in culture when a confluent monolayer had been formed. The experimental protocol was approved by the Ethical Committee of Göteborg University (Dnr. 240-2001, 6-2004 and 65-2005).

Comments: Primary cultures of astrocytes have been widely used as a model system to study astroglial properties for more than 30 years [286,287]. Cell cultures provide less complex systems enabling studies of specific astrocytic physiological properties and responses to various stimuli responses such as cell death, gap junction communication studies or cell imaging experiments. Moreover, metabolic disturbances resulting from ischemia can be independently investigated to identify discrete mechanisms involved in the mediated cellular injury. However, simplified systems also have inherent disadvantages. For instance, the cultures are grown in an artificial milieu and the influence of other cell types is absent which can affect their properties. Moreover, gap junction coupling can only occur in two dimensions. The primary cultures in these studies originate from tissue obtained from immature animals and it is possible that receptors, membrane channels and other proteins are not expressed as in mature astrocytes in the intact brain. Thus, cell cultures should be considered as a model system and be used to study specific questions that are impossible or difficult to answer *in vivo*. Results from *in vitro* studies may be used to generate adequate hypothesis and design experimental paradigms in more complex model systems *in vivo*. Direct extrapolations or comparisons between the *in vitro* and *in vivo* situation should be made with caution.

Metabolic depression (I, II, III)

Metabolic depression was chemically induced by adding iodoacetate (IA; Sigma-Aldrich; Germany) alone (10 – 100 μM) or in combination with azide (5 mM; Sigma-Aldrich, Germany). Both substances were diluted to the final concentration in a HEPES buffered salt solution (HBSS; containing in mM; 137 NaCl, 5.4 KCl, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 1.26 $CaCl_2$ 0.64 KH_2PO_4, 3 $NaHCO_3$, 5.5 glucose and 20 HEPES, pH 7.4). Prior to addition of IA, alone or in combination with azide, the cell cultures were adapted to HBSS for 1h. All incubations occurred at 37ºC. In the washout model two set of cultures were used. After 2 h, the experiment was terminated in one set of cultures. The other set of cultures was carefully washed with HBSS. The cultures were thereafter kept in HBSS for 14 h.

Comments: IA potently inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (G3PDH) by binding to the SH-groups at the cystein residues in the active site of the enzyme. IA has been commonly used in astrocytic cultures to induce metabolic depression [66,277,288-290] and can easily be combined with other treatments (see below). IA has been described as a specific blocker of G3PDH at low concentrations (< 100 μM) [291]. At these concentrations, IA was shown to inhibit G3PDH but not glucose-6-phosphate dehydrogenase (a key enzyme of the pentose phosphate pathway) and did not affect the glutathione (GSH) levels in endothelial cells [292]. Moreover concentrations of IA < 25μM had similar effects as another glycolytic blocker, sodium fluoride in C6 glioma cells [293]. In higher concentrations IA may affect other systems. For instance, at concentrations above 400 μM, IA had a partial inhibitory effect on calpains in skeletal muscle [294]. In even higher concentrations (> 1mM), IA has been used to inhibit various enzymes. Using IA is an artificial way of achieving metabolic depression. An alternative to IA would be to use glucose deprivation in an oxygen-free chamber. However, this model was not compatible with several of the other treatments and assays used in this study without causing re-entrance of oxygen.

Azide blocks the cytochrome oxidase (complex IV) in the respiratory chain and provides an example of chemical hypoxia [254,295,296]. However, azide has been also been reported to scavenge singlet oxygen and hydroxyl radicals by an interaction with the radicals, likely via a charge transfer [297-299].

Combination of metabolic depression with gap junction blockage, Ca^{2+}-chelation and caspase inhibition (I)

Metabolic depression was combined with several treatments. All incubations occurred at 37ºC and all substances were diluted to the final concentration in HBSS.

Carbenoxolone (Cbx, 20-100 μM; Sigma-Aldrich, Germany) was used to establish gap junction blockage. As reference substances to Cbx we used the common, albeit unspecific, gap junction blockers flufenamic acid (200 μM), octanol (1mM), and heptanol (2mM), all from Sigma-Aldrich, Germany. Glycyrrhizic acid (100 μM), a structural inactive analogue to Cbx [75] was used as negative control. The cultures were incubated for 8 min with either a gap junction blocker or glycyrrhizic acid prior to addition of IA (or HBSS).

BAPTA-AM (Sigma-Aldrich, Germany) was used to chelate intracellular Ca^{2+}. BAPTA-AM was dissolved in dimethyl sulfoxide (DMSO) and prior to use diluted to a final concentration of 10 μM and 0.5% DMSO. Before addition of IA the cultures were pre-incubated with BAPTA-AM for 45 min at 37ºC.

The caspase inhibitor Z-VAD-FMK (Alexis, CA, USA) was used to evaluate the possible involvement of caspases in the cell death during the metabolic depression. Z-VAD-FMK was dissolved in DMSO and diluted to a final concentration of 50 μM and 0.5% DMSO. Preincubation with Z-VAD-FMK occurred for 30 min before addition of IA.

Comments: At present there is no specific gap junction blocker available and the exact mechanisms of action of the presently used gap junction blockers are elusive. However, Cbx is considered as one of the more specific blockers and has been widely used. Nevertheless, other effects but gap junction blockage has been reported [79,80]. Therefore, other structurally unrelated reference gap junction blockers, as well as the inactive analogue glycyrrhizic acid were used in this study to exclude other properties besides gap junction blockage to be responsible for the shown effects of Cbx.

Intracellular Ca^{2+} was chelated by using BAPTA in order to assess the possible involvement of increased intracellular Ca^{2+} levels in the astrocytic cell death processes. BAPTA is a development of the well known Ca^{2+}-chelator EGTA, and have higher and more selective affinity for Ca^{2+} than EGTA [300]. BAPTA is commonly used to clamp intracellular Ca^{2+} concentrations in various cell systems. The conjugation of acetoxymethyl (AM) ester groups to BAPTA improves the cell membrane permeability. In the cytosol, unspecific esterases will cleave the ester bonding, trapping BAPTA in the cell. Efficient chelation of intracellular Ca^{2+}

from this protocol was verified in Ca^{2+} imaging experiments (for details see section, Quantification of intracellular Ca^{2+} levels below).

The broad-spectrum caspase inhibitor Z-VAD-FMK was used in order to investigate if the cell death process during the metabolic depression involved caspase activation. It is constructed by a fluoromethylketone (FMK) coupled to a peptide sequence (VAD; valine-alanin-aspartate) which is a specific binding site for caspases. By synthesizing the sequence to a benzyloxycarbonyl group (Z), the cell membrane permeability is enhanced.

Oxidative stress generated by SIN-1 (III)

Similar to IA treatment, the experiments were initiated by replacing the culture medium with HBSS and the cultures were let to equilibrate 1 h before the addition of SIN-1.

SIN-1 was freshly dissolved for each experiment to 1 mM concentration in HBSS. The cultures were incubated with SIN-1 for 1 h and were thereafter carefully washed and further incubated in HBSS.

Comments: SIN-1 is a compound known to produce equimolar amounts of superoxide and nitric oxide forming peroxynitrite [218,301]. SIN-1 continuously generates superoxide and peroxynitrite. That is advantageous since peroxynitrite has a short half-life in aqueous solutions [302].

Superoxide, NO and peroxynitrite and the decomposing products of peroxynitrite all contribute to oxidative stress. Peroxynitrite formation has been shown to be an important factor contributing to the cellular damage after cerebral ischemia.

Scavenging of reactive oxygen species (II, III)

Scavenging of ROS was done by using B27 (Invitrogen, Belgium) the vitamin E analogue trolox or the superoxide dismutase mimic tempol (both from Sigma-Aldrich, Germany). B27 was purchased as a 50x concentration solution and diluted to 1x concentration. Trolox (0.5 mM) and tempol (5 mM) were dissolved as fresh solutions for each experiment. Trolox was dissolved in EtOH as a 0.5 M solution and rapidly further diluted to the final concentration in HBSS. Tempol was dissolved directly in HBSS to the final concentration. The scavengers were applied together with IA. In the washout model trolox and tempol (but not B27) were present at the same concentrations also during the washout period. When trolox (0.5 mM) or tempol (5 mM) were used in association with SIN-1, the scavengers were added to the

cultures 45 min before SIN-1 treatment and during the subsequent HBSS treatment, but not together with SIN-1.

Comments: B27 is a commonly used medium supplement which contains glutathione, superoxide dismutase, catalase, vitaminE, vitamin A, selenium, L-carnitine and transferring, all substanses able to degrade or scavenge ROS, but also albumin, insulin, biotin, corticosterone, putrescine, linolenic acid, etanolamine, galactose, triiodothyronine and progesterone [303,304]. Trolox is a water-soluble and cell permeable derivate of vitamin E, described to protect against peroxides and hydroxyl radicals [305]. For trolox the EtOH concentration never exceeded 0.1%, which did not have any protective effect per se. Tempol is a stable, cell-permeable nitroxide that acts as spin trap and as a metal independent super oxide dismutase mimic [306,307]. To avoid the potential risk of extracellular reactions, trolox and tempol were never added together with SIN-1.

Activation of Nrf2-regulated antioxidant systems (II, III)

The astrocytic Nrf2-regulated systems were stimulated by incubation with tBHQ (50 μM, 24 h), curcumin (30 μM, 24 h) or sulforaphane (15 μM, 48 h) (all from Sigma-Aldrich, Germany). The substances were dissolved in DMSO and stock solutions were stored at -20°C. The substances were diluted to the final concentration in culture media before addition to the cultures. Prior to addition of IA, the cultures were rinsed with warm HBSS and incubated with HBSS at 37°C for 1 h.

Comments: tBHQ, curcumin and sulforaphane are all electrophils and known to activate the Nrf2 system [220]. tBHQ is a common food additive and the phytochemicals curcumin and sulphoraphane are substances naturally occurring in roots of turmeric and broccoli sprouts, respectively. All three substances have been frequently used for their ability to activate Nrf2, including in astrocytes [219,308]. A biphasic dose-response relationship has been described for many phytochemicals where low concentrations give beneficial effects whereas higher concentrations may be toxic [230]. However, at the concentrations used in these experiments, the cultures remained viable after exposure. In our system, a 24 h pre-incubation with either tBHQ or curcumin was sufficient to protect against IA- or SIN-1-mediated stress. However, it was necessary to pre-activate the astrocytes for 48 h with sulforaphane to achieve protection. One explanation to this may be that tBHQ, curcumin and sulforaphane do not stimulate transcription of the exact same genes and that the efficiency and maximum

expression may vary. During the incubation the DMSO concentration never exceeded 0.25% which did not have any protective effect per se.

Staurosporine treatment (III)

Staurosporine (Sigma-Aldrich, Germany) was dissolved in DMSO and prior to use, diluted in HBSS to a final concentration of 1 μM and 0.1% DMSO. The astrocyte cultures were adapted to HBSS for 1 h prior to addition of staurosporine.

Comments: Staurosporine is an alkaloid originally isolated from *Streptomyces* bacteria. It is described as a broad protein kinase inhibitor and has been widely used to induce apoptosis in several different cell types. Staurosporine has been reported to induce caspase dependent cell death in astrocytes [309,310] but also translocation of AIF [165].

Quantification of intracellular Ca^{2+} levels (I)

Cell cultures were incubated at 37ºC with 8 μM of the Ca^{2+} sensitive probe Fura-2-AM and 0.03% Pluronic® F-127 (both from Molecular Probes- Invitrogen, Netherlands) in HBSS for 45 min. Thereafter the cultures were left for 15 min in HBSS to allow cleavage of the acetoxymetyl (AM) esters by intracellular esterases. After addition of IA alone or in combination with BAPTA, time lapse images were captured for at least 2 h 30 min at 0.2 Hz in a Photon Technology International (PTI) imaging system. Excitation was alternated at 340 and 380 nm and emitted light was captured at 510 nm. Ratios were then quantified from the emitted light intensity. Estimations of intracellular levels of Ca^{2+} were performed by comparing these data with calibration ratios achieved in solutions from Ca^{2+} calibration kit to which Fura-2 was added.

Comments: Fura-2 and several other fluorescent Ca^{2+} indicators were developed during the 1980s and have been widely used to monitor changes in intracellular Ca^{2+} concentrations. The excitation maximum of Fura-2 shifts from 380 nm in its unbound state to 340 nm when Ca^{2+} is bound [311]. The emitted light intensity is measured at each excitation wavelength and a ratio is calculated. The ratiometric determination of intracellular Ca^{2+} changes reduces potential problems with dye leakage, photobleaching, cell thickness or uneven dye loading.

Isolation and determination of intracellular levels of ATP (I, II, III)

To isolate ATP the cultures were rapidly rinsed in ice cold PBS, thereafter ice cold trichloroacetic acid (0.5%) (Sigma-Aldrich, Germany) was added to extract ATP. The samples

were stored frozen at -80ºC until analysis. A 1.25 M KOH / 1.0 M KH_2PO_4 buffer was added to a standardized volume of extract to get pH 7.6-8.0. Thereafter, an ATP Bioluminescence Assay CLS II kit (Roche Applied Science) was used following the manufacture's protocol. Samples were loaded into white, flat-bottomed 96-wellplates and luminescence was determined using a Victor II plate reader (Wallac, Finland). The ATP levels were calculated as percent of untreated control (four wells pooled per 24-well plate) for each independent experiment.

Comments: The ATP assay is based on the chemical reaction naturally occurring in the American firefly Photinus pyralis. In this reaction, the enzyme luciferase catalyzes a reaction involving ATP and luciferin which results in emission of light [312]. This light output is proportional to the consumption of ATP and can therefore be used as a determination of ATP content in the sample. The method is simple, stable, reliable and widely used for determination of ATP levels.

Determination of gap junction permeability – scrape loading/dye transfer (I)

Gap junction permeability was studied at room temperature (20-22ºC) using a scrape loading/dye transfer method. The cultures were treated with the substances of interest or HBSS and the conditioned incubation buffer was removed and saved. The cultures were rinsed in Ca^{2+}-free HBSS and two parallel scalpel cuts were performed in Ca^{2+}-free HBSS containing 0.1% (w/v) neurobiotin (Vector, CA, USA) or 0.1% (w/v) Lucifer Yellow (Sigma-Aldrich, Germany). The cultures were rinsed after 1 minute. Thereafter the conditioned incubation buffer was reintroduced and the cultures were left for 7 minutes. When using Lucifer Yellow, 6-8 photos of each culture were directly taken. When neurobiotin was used, the cultures were fixed in 2% formaldehyde (4ºC, 10 min) before permeabilization with PBS supplemented with 1% bovine serum albumine and 0.05% saponin. The cultures were incubated for 1 hr with fluorescein-conjugated avidin to visualize neurobiotin (1:200; Vector, CA, USA) in the supplemented PBS and thereafter rinsed and mounted before 6-8 photos of each culture were taken. Quantification of the dye spread was done by measuring the area of fluorescent cells of each side of the scalpel cut in a defined region of interest using the analysis program Easy Image Analysis 2000 (Tekno optik AB, Sweden). The area occupied by cut cells, defined by using a complete gap junction blocking protocol (100 μM, Cbx 8 min), was subtracted from the total fluorescent area to obtain the operational dye transfer area. The

results were compared as percent of the average effective dye spread area in controls treated with HBSS for 2 hrs for each dye.

Comments: The scrape loading/dye transfer method has been widely used to quantify gap junction communication in cell cultures, including astrocytes [64,313,314]. It is based on the spreading of a gap junctional permeable, but cell membrane impermeable substances (here Lucifer Yellow or neurobiotin). The dye is introduced to a row of cells by the cut and can then spread via gap junctions to neighboring cells. To avoid that the external, high Ca^{2+}-levels would block gap junctions in the cut cells, the dye is introduced in a Ca^{2+} free environment. It is essential to keep this period short (here 1-2 min) since a Ca^{2+} free extracellular milieu can be toxic, Moreover, it has been reported that omission of Ca^{2+} can lead to opening of astrocytic hemichannels, through which the dye can directly be taken up [67].

Visualization of processes of programmed cell death by Annexin V binding (I, II)

The progress of programmed cell death was investigated by using Vybrant® Apoptosis Assay Kit #2 (Molecular Probes-Invitrogen, Netherlands) which contains Alexa Fluor® 488-conjugated Annexin V and propidium iodide. The kit was used following the manufacture's instructions. After staining with Annexin V and propidium iodide, the cells were fixed with cold 2% formaldehyde (4ºC, 10 min). The cells were rinsed in PBS with 0.1% bovine serum albumin and 0.005% Saponine and stained with Hoechst 33258 (100 ng/ml, Sigma-Aldrich, Germany) for 7 minutes in order to stain all cell nuclei and also to reveal condensed chromatin. Thereafter the cells were carefully rinsed and mounted with fluorescent mounting medium. The results were quantified by counting the number of cells being Annexin V-positive and propidium iodide-negative, the number of cells being both Annexin V- and propidium iodide-positive (necrotic cells) and the total cell number (Hoechst nuclei stain) in randomly picked microscopic fields (8 per cover slip) using the analysis program Easy Image Analysis 2000 (Tekno optik AB, Sweden). In order to study formation of Annexin V-positive cell groups, specific cluster sizes were defined (single cells, 3-10, 11-50 or >50 cells) and the distribution of all Annexin V-positive cells into these was investigated. Moreover the term 'apoptotic unit' was introduced and defined as either a single Annexin V-positive cell or a cluster of positive cells. In order to investigate the distribution of varying unit sizes, the relative number of units involving either single cells, 3-10, 11-50 or >50 cells was calculated.

Comments: Annexin V has high affinity for phosphatidylserine (PS) and labeled Annexin V has been used to visualize the PS translocation in different cell types [188,189], including

astrocytes [190,191]. If the cell membrane is disrupted Annexin V can bind to intracellular phosphatidylserine. Therefore, to distinguish cells with disrupted cell mambrane from cells undergoing programmed cell death, propidium iodide which is taken up by cells with disrupted cell membrane and binds to DNA, was used. Appearance of Annexin V-positive cells indicates that apoptotic processes are initiated. However it is not possible to conclude what pathway of programmed cell death the cells will take. Moreover, cells being both Annexin V- and PI- positive may be a result of necrosis or incomplete apoptosis. In this study, not only the total number of Annexin V-positive cells was counted. The subdivision of the groups of Annexin V-positive cells in defined cluster sizes along with the definition of the term 'apoptotic unit' was made to make it possible to visualize how apoptotic processes were initiated and progressed over time.

Determination of extracellular levels of lactate dehydrogenase (I, II, III)

The release of lactate dehydrogenase (LDH) to the extracellular space was used to determine the amount of necrotic cell death or incomplete programmed cell death in the cultures. After the experimental treatment, the extracellular solution was collected and saved for analysis. The intracellular part was collected by lysing the cells with 0.25% Triton–X-100 (Sigma-Aldrich, Germany). Samples were frozen at –80ºC and analyzed within a week. The samples were analyzed by either a conventional assay or a commercial kit. The conventional assay was done by the procedure as follows: equal volumes of sample and 0.35 mg/ml pyruvate in 0.1 M Tris (pH 7.4 at 37ºC) was mixed. Just before starting the analysis, NADH 4 mg/ml in 5 mM HEPES was added. The decreasing NADH fluorescence was determined over time using Victor II plate reader (ex 355nm; em 460nm Wallac, Finland). Analysis performed with the kit (LDH cytotoxicity kit; Roche Applied Science, Germany) followed the manufactures protocol and was spectrophotometrically analyzed at 490 nm using the Victor II plate reader. The amount of LDH release was calculated and correlated to the total amount of LDH (intra- and extracellular) in non-treated sister cultures.

Comments: Lactate dehydrogenase is a stable cytosolic enzyme present in all cells and it is rapidly released to the extracellular solution upon cell membrane disruption. Both assays used here are based on the reaction where LDH catalyses the reduction of pyruvat to lactate in the presence of NADH which is oxidized to NAD^+. In the conventional assay, the level of LDH is determined by monitoring the kinetics of the reduction of NADH fluorescence. The

reaction in the kit has been slightly modified and the product of the reaction is colored and an end-point value is determined spectrophotometrically.

Mitochondrial staining (III)

All staining procedures were performed on astrocytes grown on cover slips. Mitochondria were visualized with the mitochondrion selective probe MitoTracker® Orange CM-H_2TMRos (Molecular Probes-Inviotrogen, Netherlands). The astrocytes were incubates with the substances of interest and MitoTracker® CM-H_2TMRos (75 nM) was added to the cell cultures 30 min before termination of the experiment. The cultures were carefully washed with HBSS (37°C) prior to fixation with ice cold 0.5% TCA and further immunostained as described below.

Comments: MitoTracker® Orange CM-H_2TMRos is a cell permanent dye that is concentrated by active mitochondria. MitoTracker® Orange CM-H_2TMRos contains a chlorometyl group which can react with thiols of peptides and proteins and retains the dye in the mitochondria after loading. The fluorescent signal of MitoTracker® Orange CM-H_2TMRos is brighter in active mitochondria than in mitochondria with depolarised membranes and can therefore be used to detect changes in the mitochondrial membrane potential.

Immunocytochemistry (III)

After fixation the cultures were washed in PBS. Non-specific binding was blocked for 45 minutes in PBS supplemented with 0.6% Triton-X-100 and 3% donkey serum. All antibodies were applied in PBS supplemented with 0.6% Triton-X-100 and 3% donkey serum. Antibodies against AIF (D-20, 0.8 μg/ml; Santa Cruz Biotecnology, CA, USA) and active caspase-3 (0.75 μg/ml; cat. no. 557035, BD Biosciences, Belgium) were incubated for 1 h at room temperature. These antibodies were visualized by fluorescence-tagged secondary antibodies Alexa Fluor® 488 donkey-anti-goat and Alexa Fluor® 488 donkey-anti-rabbit, both at 1:2500 dilution (both from Molecular Probes- Invitrogen, Netherlands). Hoechst 33258 (62.5 ng/ml) was added together with the secondary antibodies and used in order to stain all cell nuclei and to visualize chromatin condensation. For quantification of AIF, at least 7 pictures of randomly selected fields were taken for each treatment from 3 separate experiments. AIF positive astrocytes were counted into two different groups. They were either classified as cells with intense AIF staining in the nuclear area and displaying

depolarized mitochondria (lack of MitoTracker® orange staining) or as cells with intense AIF staining co-localized with mitochondria with punctate and dotted morphology and preserved membrane potential. Positive cells were counted using the analysis program Easy Image Analysis 2000.

Comments: Immunocytochemistry is a widely used method to demonstrate the presence and cellular distributions of antigens, although there is a possible risk of unspecific binding of the antibodies. The antibodies were therefore used at low concentrations within the range recommended by the manufacturer. In addition, negative controls where the primary antibodies were omitted were performed for the secondary antibodies. The AIF antibody is raised against the C-terminus of AIF and therefore binds to the truncated forms of AIF. The caspase-3 antibody binds to a conformational epitope which is exposed when caspase-3 has been activated by cleavage.

Caspase-3 activity measurements (III)

For caspase-3 activity measurements, astrocytes were treated as indicated. At the termination of the experiment the cells were detached by using trypsin (0.25% + EDTA; 5 min at 37°C) (Invitrogen, Belgium). The trypsin was neutralized by adding an equal amount of culture medium (see above). The cells were centrifuged, resuspended and sonicated in 150 µl sample buffer (1.5 mM Tris-HCl, pH 7.3, 0.1 mM DTT, 32 mM sucrose, 0.1 mM $MgCl_2$, 0.3 mM EDTA). Proteases were inhibited by the addition of complete tablets and 100 µM orthovanadate. Prior to analysis the cell lysates were stored frozen at -20°C. For the caspase-3 analysis, defined amounts of cell lysate was mixed with extraction buffer (50 mM Tris-HCl, pH 7.3, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1% protease inhibitor cocktail and 0.2% CHAPS) in a microtiter plate (Microfluor Dynatech, VA, USA). After incubation for 15 min at room temperature, the peptide substrate Ac-DEVD-AMC (50 µM; Peptide Institute, Japan) in extraction buffer without protease inhibitors or CHAPS but with 4 mM DTT, was added. Cleavage of Ac-DEVD-AMC was measured (excitation at 380 nm / emission at 460 nm) and the degradation was followed at 2 min interval for 2 h, and Vmax was calculated from the entire linear part of the curve. The activity was calculated as pmol AMC released per mg protein and minute and expressed as percent of control treated cultures.

Comments: Ac-DEVD-AMC is a synthetic non-fluorescent complex of the tetrapeptide DEVD (aspartate-glutamate-valin-aspartate) bound to the fluorescent AMC. Activated

caspase-3 specifically cleaves Ac-DEVD-AMC between D and AMC, thus releasing the fluorescent AMC.

Determination of intracellular levels of ROS (III)

To estimate the levels of ROS in astrocytes during the different treatments, we used CM-H_2DCFDA (Molecular Probes-Invitrogen, Netherlands). CM-H_2DCFDA was dissolved in DMSO as a 10 mM solution and further diluted to the final concentration (7 μM). Fresh solutions were made for each experiment. CM-H_2DCFDA was added to the cultures 30 min prior to termination of the experiment. At the termination the cells were carefully washed with HBSS (37°C) before they were detached by adding trypsin (0.25% + 1 mM EDTA) for (5 min, 37°C). The trypsin was neutralized by adding an equal amount of culture medium. The cell suspensions were rapidly transferred to a black walled 96-well plate and the fluorescence was measured (excitation at 485 nm / emission 535 nm). The cell suspensions were thereafter centrifuged and resuspended in PBS and stored at -20°C prior to protein analysis. The florescence value was related to the protein content and thereafter related to the control values for each individual experiment

Comments: CM-H_2DCFDA is a non-fluorescent substance which passively diffuses in to the cells. The acetate groups are then cleaved of by intracellular esterases. The chloromethyl group (CM) allows for covalent binding to intracellular components, which prolongs the retention in the cells. Subsequent oxidation by ROS yields the fluorescent adduct CM-DCF, which is trapped inside the cell. CM-DCF has been described to be sensitive to peroxides, hydroxyl radical and NO and less sensitive to superoxide [305,315,316].

RESULTS

Glycolytic blockage by iodoacetate induces ATP depression, altered cellular morphology, intracellular Ca^{2+} fluctuations and increased levels of ROS (I, II)

Inhibiting the glycolysis with iodoacetate (IA) depressed the astrocyte ATP levels in a time and concentration-dependent manner. When the lowest concentration of IA (10 μM) was used, the astrocytes were able to maintain the ATP levels longer and the ATP decrease occurred with a lower rate, in comparison to the higher concentrations (Fig. 3). The time from initial ATP depression to complete ATP depletion was about twice as long when IA 10 μM was used compared to the higher concentrations.

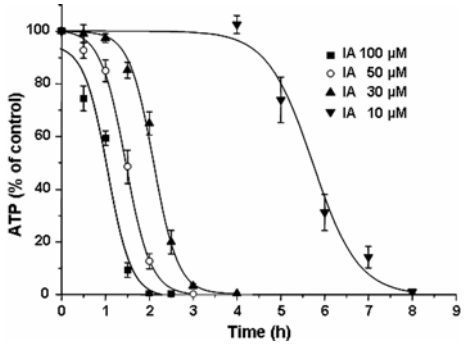

Fig 3. The glycolytic inhibitor IA depressed the ATP levels in a concentration and a time dependent manner. Data points represent mean ± SEM, (n = 3-23).

The first cellular responses that were observed after the IA treatment were rapid transient increases in the intracellular Ca^{2+}concentrations that occurred after approximately 15 min of IA treatment (30 μM). These followed a heterogeneous pattern - the Ca^{2+} increases were seen once in some cells, repeatedly in others and some cells did not respond at all. In a second phase, prolonged and more homogenous Ca^{2+} increments were observed. These were initiated at a timepoint which coincided with detectable ATP decreases in the cell cultures. In contrast to the earlier transient Ca^{2+} fluctuations, the prolonged Ca^{2+} increments did not reverse to basal levels. Moreover, they were first observed in a fraction of the astrocytes and then spread over time to involve all cells in a microscopic field.

The activity of reactive oxygen species (ROS) was also investigated during the IA-treatment. In astrocytes treated with 30 μM IA, the levels of ROS increased about 4 times above levels in non-treated cultures although the ATP levels were still at approximately 70% of control. The levels of ROS continued to increase and were about 5.5 times higher than in non treated cultures when the ATP levels were decreased to about 30% of control.

In astrocytes treated with 30 μM IA, distinct morphological changes were observed during the late phase of the metabolic depression. Dispersed cells or small groups of cells with visible, dark and round to oval shaped nuclei were first observed when ATP levels dropped to approximately 20% of control. The groups of astrocytes displaying morphological changes enlarged as the ATP levels continued to decrease. Parallel to the morphological changes, we detected extracellular release of LDH.

Gap junction blockage affects the iodoacetate induced ATP depression (I)

In order to investigate the involvement of gap junction-mediated communication in astrocytic resistance to metabolic stress, IA (30 μM) was combined with several concentrations of the gap junction blocker carbenoxolone (Cbx; 20, 50 or 100 μM). Interestingly, we observed that addition of Cbx resulted in an earlier onset of the ATP decrease, in comparison to IA-treatment alone. This effect was dependent on the Cbx concentration. When IA was combined with the highest concentration of Cbx (100 μM), the 50% reduction in ATP was advanced by approximately 1 hour. Cbx alone (100 μM) had no effects on the ATP levels. The structural analogue of Cbx, glycyrrhizic acid (100 μM), which does not block gap junctions did not affect the IA-induced ATP decrease.

We next investigated the gap junction communication when IA treatment was combined with Cbx in different concentrations. The scrape loading dye transfer experiments revealed that the gap junction blocking effect of Cbx was attenuated by the presence of IA. In presence of IA, 100 μM Cbx was needed for efficient blockage of the gap junction communication. The lower concentrations of Cbx (20 and 50 μM) failed to block dye transfer already after 1.5 h. Nevertheless, even in presence of 100 μM Cbx, the blockage was somewhat attenuated after 2h (ATP depressed to approximately 15 % of control). Cbx alone, in all concentrations used, potently blocked dye transfer in absence of IA in the studied time window (2.5 h).

Expanding areas of Annexin V-positive astrocytes emerge during metabolic depression – the area expansion, but not the initiation of programmed cell death, is inhibited by gap junction blockage (I, II)

The binding of Annexin V to extracellular phosphatidylserine was followed to evaluate the progress of programmed cell death in relation to the ATP decrease during the IA-induced metabolic depression. Cell death was evaluated in both gap junction communicating and non-communicating conditions.

When the ATP levels were only moderately decreased (about 70% of control), single cells or occasional small clusters of Annexin V-positive astrocytes were observed. The number of Annexin V-positive astrocytes increased and the clusters of positive cells enlarged during the subsequent ATP decrease. In the late phase of the ATP decrease (ATP levels < 20% of control), clusters of Annexin V-positive cells became united and large areas of positiove cells were observed. Typically, astrocytes in the center of such big clusters showed bright Hoechst staining, indicating condensed chromatin. Eventually, as the ATP levels decreased further, astrocytes that were both Annexin V- and propidium-positive were identified and extracellular LDH release was detected, indicating that cell membrane rupture had occurred. In control cultures, the number of Annexin V-positive cells did not increase in the studied time window.

Enlarging areas of Annexin V-positive cell groups were not seen in cultures where the gap junction communication was inhibited by Cbx (100 μM) during the IA-induced metabolic depression. Initiation of cell death was not prevented but interestingly, Annexin V-positive cells were only found as isolated individual cells. However, in the very late phase of the ATP depression (ATP levels < 15% of control) and when the gap junction blocking effect of Cbx was attenuated, clusters of Annexin V-positive astrocytes were discovered also in these cultures. To ensure that the Cbx effect was due to gap junction blockage, several control experiments were performed. The structurally similar, but inactive, Cbx-analogue glycyrrhizic acid (100 μM) did not mimic the effect of Cbx and the cell death pattern was indistinguishable from IA treatment alone. To exclude that the lack of early Annexin V-positive cell clusters was due to the earlier onset of ATP depression rather than on gap junction blockage, a higher concentration of IA (50 μM) was used. The ATP decrease induced by 50 μM IA related well in time with that of 30 μM IA combined with 100 μM Cbx. Annexin V-positive cells appeared when ATP was approximately 70% of control in both conditions, but enlarging groups of Annexin V-positive cells were only observed after treatment with 50 μM IA.

In summary, in this set of experiments Annexin V-positive cells first appeared at ATP levels around 70% of control, but at different time points in cultures treated with 30 or 50 μM IA or 30 μM IA + 100 μM Cbx. Expansion of Annexin V-positive cell clusters following IA treatment could only be efficiently prevented by a high concentration of Cbx.

Iodoacetate and SIN-1 induce cell death which is accompanied by AIF translocation, but not caspases (III)

To evaluate possible mechanisms involved in the astrocytoic cell death we investigated the nuclear translocation of AIF from the mitochondria. In addition to the model of metabolic depression (IA 10 μM), we also used a model of oxidative stress induced by SIN-1 (1 mM), a compound known to produce equimolar amounts of superoxide and nitric oxide which react to form peroxynitrite. During the SIN-1 treatment (1 h) a rapid decrease of the astrocytic ATP levels to approximately 60% of control was observed. After the subsequent washout of SIN-1, the ATP levels stabilized approximately at this level within the 8 h. The translocation of AIF was investigated by immunocytochemistry using the mitochondrial marker MitoTracker® orange and antibodies against AIF. Control cells displayed AIF staining that co-localized with the MitoTracker® orange staining observed in elongated tubular thread-like mitochondria.

Following IA- and SIN-1 treatment, different stages of AIF translocation and changes in the mitochondrial morphology were observed. In one fraction of cells, intense AIF staining was observed in the nuclear area indicating nuclear translocation of AIF. The non-nuclear AIF staining in these cells occurred typically in large dots and was more intense in comparison to untreated control cultures. The mitochondrial membrane potential was disrupted as indicated by the lack of MitoTracker® orange staining. Moreover, many of the nuclei had become pycnotic and were brightly stained with Hoechst, indicating condensed chromatin. In another fraction of cells, the mitochondrial staining showed round and punctate mitochondria with the mitochondrial membrane potential maintained. The AIF staining in these cells was more intense and commonly co-localized with the mitochondria. Some astrocytes in the IA- or SIN-1-treated cultures did not appear injured and were similar to control cells. The fraction of cells displaying AIF translocation to the nucleus, and cells with more intense AIF staining in the mitochondria with maintained membrane potential were markedly increased after both IA and SIN-1 treatment.

The possible involvement of caspase-dependent pathways for programmed cell death during IA or SIN-1 treatment was also investigated. In astrocyte cultures, IA (30 μM) was combined with the broad-spectrum caspase inhibitor Z-VAD-FMK (50 μM). However, the presence of Z-VAD-FMK did not alter the kinetics of the IA-induced ATP depression and the

enlargement of Annexin V-positive cell clusters was indistinguishable from cultures treated with IA alone.

The involvement of caspase-3 in cell death following IA and SIN-1 treatment (10 μM and 1 mM, respectively), was investigated by immunocytochemistry. We used an antibody specifically binding to activated caspase-3 and combined that with the mitochondrial marker MitoTracker® orange. Immunostaining for active caspase-3 could not be detected in IA- or in SIN-1-treated cultures. Still, astrocytes with altered mitochondrial morphology and depolarized mitochondrial membranes were identified. The activity of caspase-3 was also investigated by measuring the increase in fluorescent AMC over time, yielded by the caspase-3 specific cleavage of the the non-fluorescent Ac-DEVD-AMC. However, neither IA nor SIN-1 treatment induced any increase of the caspase-3 activity. Staurosporine, which has been reported as a caspase-3 activator in astrocytes [309,310] was used as a positive control. Staurosporine treatment (1 μM) significantly increased the caspase-3 immunoreactivity and the caspase-3 mediated DEVD cleavage in the astrocyte cultures.

The iodoacetate-mediated ATP decrease, cell death, ROS activity and morphological changes are prevented by intracellular Ca^{2+} chelation, pre-activation of the Nrf2-regulated antioxidant system or exogenous free radical scavengers (I, II, III)

We next investigated various possible cytoprotective strategies to gain further insight into the mechanisms involved in the IA-mediated cell death. As transient fluctuations followed by prolonged increases in intracellular levels of Ca^{2+} were observed during IA treatment, the Ca^{2+}-chelator BAPTA was used to investigate the extent to which increased intracellular Ca^{2+} levels contribute to programmed cell death. The presence of BAPTA (10 μM) during the IA treatment (30 μM) efficiently prevented the IA-induced Ca^{2+} increments. In addition, chelating intracellular Ca^{2+} decelerated the IA-induced ATP decrease. After an initial ATP drop to ~ 80% of control, the ATP levels continued to decrease with a slower rate, in comparison to IA treatment alone. The presence of BAPTA did not prevent the formation of expanding areas of Annexin V- positive cells. However, the increase in numbers of Annexin V-positive cells slowed down and was related to the slower kinetics of the ATP decrease.

As IA-treatment elevated the levels of ROS, the "astroprotective" potential of exogenous ROS scavengers was investigated. IA (30 μM) was first combined with the mitochondrial respiratory chain complex IV-blocker azide (5mM), which has ROS scavenging properties.

When IA and azide were combined, ATP levels decreased and the onset was more rapid than with IA alone. This is most likely due to the simultaneous blockage of both glycolysis and the respiratory chain. Interestingly, the ROS levels remained at control levels and the expansion of Annexin V-positive cell groups was prevented. Moreover, no morphological changes were observed.

The protective potential of several well known ROS scavengers was also investigated. IA (10 or 30 μM) was combined with the medium supplement B27 (1x), the vitamin E analogue trolox (0.5 mM) or the SOD analogue tempol (5 mM). The presence of these scavengers during the IA treatment enabled the astrocytes to maintain ATP levels longer and the ATP decrease was decelerated. The scavengers also inhibited the IA-induced increase of ROS and programmed cell death. The appearance of Annexin V-positive cells and the AIF translocation to the nucleus induced by IA (30 and 10 μM, respectively) was prevented. Moreover, in presence of the scavengers the cellular morphology was indistinguishable from control treated cultures, the release of extracellular LDH and the IA-induced ROS activity was decreased and the mitochondrial membrane depolarization was prevented.

We also investigated the protective potential of the endogenous Nrf2-regulated antioxidant system. Activation of the Nrf2-system was stimulated by treating astrocyte cultures with tBHQ (50 μM; 24 h) curcumin (30μM; 24 h) or sulforaphane (15μM; 48 h) prior to adding IA (10 or 30 μM). Pre-treatment with these substances enabled the astrocyte cultures to maintain the ATP levels longer in the presence of IA. tBHQ was the most efficient, but curcumin and sulforaphane pre-treatment also efficiently decelerated the IA-induced ATP decrease. Moreover, IA-mediated increase of ROS activity and cell death initiation was also efficiently prevented by pre-treatment with the Nrf2 activators. The number of Annexin V-positive cells was efficiently decreased and IA-induced AIF translocation was prevented. In addition, the IA-induced release of LDH was reduced, the IA-mediated cellular and mitochondrial morphology changes were not observed and depolarization of the mitochondrial membrane potential was not detected.

SIN-1 mediated ATP depression and AIF translocation is prevented by pre-activation of the Nrf2 system or addition of exogenous scavengers (III)

Pre-treatment with any of the three Nrf2-activators tBHQ (50 μM; 24 h), curcumin (30μM; 24 h) or sulforaphane (15μM; 48 h), or addition of the exogenous scavengers trolox (0.5 mM) or tempol (5 mM) 45 min before and after SIN-1 treatment (1 mM), significantly prevented the SIN-1-induced ATP depression. Moreover, the SIN-1-induced translocation of AIF was prevented. The astrocytes appeared as control treated and drastically different to astrocytes treated with SIN-1 alone.

Addition of exogenous scavengers or pre-activation of the Nrf2-system facilitates recovery of the ATP levels and prevents cell death after metabolic depression (II)

With the aim to simulate ischemic reperfusion in culture, we investigated the ability of astrocytes to recover following a transient period of IA-induced metabolic depression. After 2h of IA treatment (30 μM), ATP levels were decreased to about 70% of control. At that time point, IA was washed out and the cultures were left to recover for 14 h in HBSS. Cultures treated in this manner were not able to recover from the IA-induced stress. The ATP levels were obliterated and massive cell death occurred after washout of IA. The majority of the cells were Annexin V- and propidium iodide-positive and high levels of extracellular LDH were detected. When IA was combined with azide (5 mM), ATP levels were further depressed than with IA alone at the onset of washout. Interestingly however, these cultures recovered their ATP levels and cell death was prevented. Moreover, if the exogenous scavengers B27 (1x), trolox (0.5 mM) or tempol (5 mM) were present during the IA-treatment (trolox and tempol also during the washout perid) or if the Nrf2 system had been pre-activated by tBHQ (50 μM; 24 h), curcumin (30 μM; 24 h) or sulforaphane (15 μM; 48 h), the astrocytes were able to recover their ATP levels after washout. Cell death was efficiently prevented as visualized by lack of Annexin V staining and low levels of extracellular LDH. Moreover, in this experimental paradigm, astrocytic recovery and survival was observed even when the IA-induced ATP depression was not fully prevented at the onset of washout.

DISCUSSION

With the aim to investigate various astrocytic reactions during a gradual ATP decrease, we set up and characterized a model of metabolic depression. We used primary hippocampal astrocyte cultures and the glycolytic inhibitor IA. This model was used in all three papers and induced a reproducible and concentration dependent ATP decrease.
In the same primary astrocyte culture system, a model of oxidative stress was set up and characterized. Astrocytes were exposed to SIN-1, which promotes NO and superoxide production, which in turn react to form peroxynitrite. SIN-1 was chosen since peroxynitrite has been strongly implicated as a contributor to tissue damage following cerebral ischemia [215-217] and other brain injuries [214,218].

In paper I and II, the formation of enlarging clusters of Annexin V-positive astrocytes was observed during the ATP decrease. In the later phase of metabolic depression, astrocytes with condensed nuclei (brightly stained with Hoechst) were seen in the center of large Annexin V-positive clusters. Translocation of phosphatidylserine (to which Annexin V binds) to the outer leaflet of the cell membrane has been associated with programmed cell death [182-184]. Moreover, nuclear condensation and increased Hoechst fluorescence discriminate cells undergoing programmed cell death from cells dying via necrotic pathways [196]. This suggests that programmed cell death was initiated during metabolic depression. In the later phase of ATP depression, we also observed morphological changes in astrocytes. Similar to the Annexin V stainings, these were initiated in a few cells and subsequently involved larger cell clusters. Annexin V-positive astrocytes and morphological changes have also been shown following hypoxia *in vitro* [190], showing that these features are not exclusive for metabolic depression induced by IA.

Our findings indicate that the sensitivity and response of cultures astrocytes to IA varied and that cell death appeared to spread from initially more susceptible astrocytes to neighboring cells. Astrocytes are extensively coupled via gap junction channels, which has been shown in cultures [313] and in acutely isolated brain slices [61]. Moreover, gap junction communication has been reported to persist, although at decreased efficiency, during ischemic conditions [66,259]. These findings were also confirmed in our model. Thus, it is likely that small molecules (less than 1.2 kDa) diffuse between cells with different sensitivities to ischemic injury. We

hypothesized that gap junction communication could be involved in the observed spreading of cell death. In paper I, we investigated the effect of gap junction inhibition during metabolic depression on the subsequent ATP decrease and programmed cell death. Our data showed that several structurally unrelated gap junction blockers induced an earlier onset of the IA-induced ATP decrease. Thus, diffusion of endogenous low molecular weight substances appears to be important for maintaining astrocytic ATP levels following IA-induced metabolic depression.

Astrocytic gap junction communication is favorable for neuronal survival in response to oxidative stress [264] or glutamate toxicity [265], in co-cultures of neurons and astrocytes. However, it remains obscure whether gap junction communication is beneficial or detrimental during ischemic conditions [317,318]. A functional network may be beneficial since deleterious substances can be spatially distributed within the astrocytic network. On the other hand, in a dying and gap junction-coupled astrocyte, deleterious metabolites or ions can accumulate and spread to neighboring cells and induce injury there. Another possibility that has been suggested is that important metabolites such as ATP are drained from healthy cells, making these more susceptible to further stress [56]. The two latter statements could be a possible explanation for our findings that inhibition of gap junction communication during metabolic depression prevented the expansion of Annexin V-positive astrocytic clusters. Importantly, although the expansion was prevented, the initiation of cell death was not. Clusters of dying cells have also been demonstrated in gap junction-coupled carcinoma cells [274]. Moreover, gap junction-mediated bystander killing, which could be inhibited by gap junction blockers, has been described in HIV-infected astrocytes [275], in gap junction communicating C6 cells during metabolic depression [277] and in intact retina preparations by intracellular administration of cytochrome c [276]. Factors suggested to mediate bystander killing include Ca^{2+}, IP_3, cAMP Na^+ and ROS [272,274,277,278]. However, further studies are needed in order to reveal the mechanism behind bystander killing in astrocytes exposed to various stressors.

Caspase independent programmed cell death is characterized by translocation of the mitochondrial protein apoptosis-inducing factor (AIF) to the nucleus, which results in large scale DNA degradation [148-150]. In paper III, we showed that IA-mediated metabolic depression or SIN-1-mediated oxidative stress both induce nuclear translocation of AIF in

cultured astrocytes. Thus, AIF translocation probably contributes to the astrocytic cell death triggered by metabolic depression and oxidative stress. The importance of AIF in triggering astrocytic cell death has also been reported in several other stress models such as following PARP-1 activation induced by the alkylating agent MNNG [165], cobalt toxicity [315], complex I blockage by MPP^+ [319] and exposure to the NO donor SNP [320]. Increasing evidence also suggests a substantial role for caspase-independent pathways involving mitochondrial release of AIF following ischemic brain injury in both astrocytes [6,239] and neurons [175,235-238],.

Intensified AIF staining which co-localized with MitoTracker® Orange staining was observed in rounded or punctuate mitochondria during the IA- and SIN-1 treatment. The altered mitochondrial morphology preceded the IA- and SIN-1-mediated AIF translocation. Rounded punctate mitochondria have previously been described in astrocytes during PARP-1-mediated AIF translocation [165] and subsequent to the release of the pro-apoptotic factor cytochrome c due to glucose deprivation [321]. Moreover, in neurons, punctate mitochondrial staining has been observed after cerebral hypoxia-ischemia [322] and neuronal mitochondrial morphology described as "abruptly shortened" has been reported after treatment with IA or the NO donor SNOC *in vitro* [323]. It is possible that these morphological changes reflect mitochondrial impairment due to decreased ATP production or depolarization of the mitochondrial membrane [323].

In paper I, we investigated if caspases were involved in the IA-induced cell death by using the broad-spectrum caspase inhibitor Z-VAD-FMK. However, Z-VAD-FMK was unable to inhibit the ATP decrease and the increased number of Annexin V-positive cells induced by IA, indicating that caspases were of activated. In paper III, we again showed that caspases are not the main actors in IA-mediated cell death using immunocytochemistry and enzymatic activity measurements. Similar results were observed in the SIN-1-mediated model of oxidative stress. In contrast to IA and SIN-1, staurosporine induced caspase-3 activity, indicating that caspases are expressed and can be activated in our system. Caspase-3 is one of the downstream effector caspases and lack of caspase-3 activity indicates that upstream caspases are probably not activated either. In line with this data, the NO donor sodium nitroprusside (SNP) induced AIF translocation and mitochondrial depolarization in astrocytes without activating caspases [320]. Lack of caspase-3 activity has also recently been reported after treatment with the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) and

SIN-1 in astrocytes [310]. Moreover, caspase activation was not observed in astrocytes subjected to oxygen and glucose deprivation although mitochondrial membrane depolarization and cytochrome c release occurred [324]. A possible explanation for these findings is that NO inhibits caspases (and other proteins) by nitrosylation [194]. Similarly, peroxynitrite formed following neurotrauma in a fluid percussion model, inhibited the activation of caspases [218].

In all three papers we detected elevated extracellular levels of LDH. When using Annexin V to detect dying cells, we observed cells double positive for Annexin V and propidium iodide. Both of these features indicate that rupture of the cell membrane had occurred. However, this was only observed when the ATP levels were severely decreased by the IA-treatment, but occurred also during SIN-1 treatment. Programmed cell death is an active process, which requires ATP. A switch from programmed cell death to necrosis due to ATP depletion has previously been described [195-197]. However, in contrast to our results, exposure of extracellular phosphatidylserine and chromatin condensation was absent in cells undergoing necrotic death due to depleted ATP exposure in these studies [195,196]. In addition, loss of plasma membrane integrity has been described as a late feature in programmed cell death and predominately seen in cultures [196]. Cells undergoing programmed cell death *in vivo* are generally phagocytosed before the plasma membrane breaks down [196]. However, it is likely that programmed cell death was initiated in our model of metabolic depression but that at severely decreased ATP levels such processes cannot be completed and cell death continues via necrotic pathways. AIF translocation has previously been observed *in vitro* although the ATP levels were depleted by simultaneous blockage of both glycolysis and oxidative phosphorylation [199,200]. The translocation of AIF was then associated with necrotic features[200].

Programmed cell death is probably a dynamic process where apoptotic and necrotic mechanisms can occur simultaneously [134-136]. Features of both programmed cell death and necrotic pathways was recently shown to occur in the same cell in brains of neonatal rats subjected to hypoxia-ischemia [325] and in neuronal cultures exposed to hypoxia [326]. This has been described as "programmed necrosis" and it has been suggested to contribute to infarct development after stroke [234,326]. Moreover, it is now strongly suggested that programmed cell death cell death occurring during pathological conditions such as cerebral ischemia is essentially different from physiological programmed cell death [134,140,144].

In paper II, we observed that IA-treatment strongly increased the intracellular levels of ROS. Immediate and rapid increases in ROS production have also been shown in astrocytes following metabolic depression induced by glucose deprivation [327,328]. Therefore, in paper II and III, we investigated the potential protective effects of exogenous ROS scavengers. When IA was combined with the medium supplement B27, the vitamin E analogue trolox or the SOD analogue tempol, ROS levels decreased. This enabled the astrocytes to maintain their ATP levels longer and prevented extracellular phosphatidylserine exposure and morphological changes during IA treatment. Trolox and tempol also potently prevented the translocation of AIF induced by IA or SIN-1. Trolox has previously been shown to protect astrocytes against oxidative stress induced by H_2O_2 and $FeSO_4$, a generator of peroxides and hydroxyl radicals [305], and tempol has been used as a scavenger of superoxide radicals in astrocytes [329].

An exciting alternative to the use of exogenous scavengers for astrocytic protection is activation of endogenous protection systems against oxidative stress. In paper II and III we found that stimulation of Nrf2-regulated antioxidant systems prior to IA or SIN-1 treatment was highly protective and potently inhibited the IA-induced ROS increase, ATP decrease, and AIF translocation. The protective potency of the Nrf2 system against injury mediated by glycolytic inhibition has, to the best of our knowledge, not previously been shown in cultured astrocytes. However, the importance of the Nrf2 system in astrocytes has previously been highlighted in models of oxidative stress. For instance, in mixed cultures of neurons and astrocytes, tBHQ and sulforaphane were shown to activate the Nrf2 system primarily in astrocytes [219]. Interestingly, this activation protected not only astrocytes but also neurons against H_2O_2-induced oxidative stress. Transplanted astrocytes over-expressing Nrf2 were shown to decrease brain injury induced by oxidative stress several weeks after transplantation, thus further showing the potency of the Nrf2 system [330,331]. Moreover, stimulation of the Nrf2 system using tBHQ, sulforaphane or curcumin have been shown to reduce infarct volume after focal cerebral ischemia [229,281-283].

In paper I, we concluded that the pattern of astrocytic cell death *in vitro* was dependent on the level of ATP depression. Moreover, the programmed cell death, involving expanding groups of coupled astrocytes, occurred at significantly but not severely depressed ATP levels. In paper II and III we confirmed that increased activity of ROS is an important factor for astrocytic cell death during metabolic depression. Interestingly, when ROS levels remained

low, the astrocytes were able to survive even severely depressed ATP levels, as shown when IA was combined with azide. In contrast, IA treatment alone led to significant cell death at similar ATP levels. Moreover, our data indicates that increasing ROS levels may contribute to the aggravation of the ATP depression. In cultures where the IA-mediated ROS elevation was efficiently prevented by the presence of scavengers or by pre-activation of the Nrf2-antioxidant system, the astrocytes were able to maintain their ATP levels at control levels for an extended period of time. In contrast, the levels of ROS were markedly elevated during the ATP decrease in cultures treated with IA alone. In addition, in paper III we observed that the ATP levels were rapidly decreased when astrocytes were exposed to oxidative stress mediated by SIN-1.

In paper I, we observed early and transient increases in the intracellular Ca^{2+}- concentration in a subset of astrocytes during the IA-treatment. These transient Ca^{2+} fluctuations were followed by prolonged Ca^{2+} increases, which did not reverse to basal levels and appeared to spread from cell to cell. Accumulation of cytotoxic levels of Ca^{2+} has been described as a crucial factor, influencing several pathways of programmed cell death [332]. This prompted us to investigate the effect of Ca^{2+}-chelation during metabolic depression. Ca^{2+}-chelation led to a slower progression of the ATP decline. However, cell death was not prevented but the expansion of Annexin V-positive astrocyte clusters was slower. These results indicate that Ca^{2+} mobilization may contribute to the aggravation of the ATP depression but does not seem to have a ubiquitous role for the initiation the astrocytic cell death. Moreover, the bystander killing effect is probably not directly mediated by Ca^{2+}. However, it is possible that the increased intracellular levels of Ca^{2+} and increased activity of ROS may be linked. Other studies have shown that hypoxic and hypoglycemic conditions or oxidative stress in astrocytes induce non-transient prolonged increases of cytoplasmic of Ca^{2+} [333,334,335] suggesting that increased ROS activity may cause intracellular release of Ca^{2+}. In addition, increased intracellular Ca^{2+} was shown to increase ROS activity in astrocytes[336]. Furthermore, increased intracellular levels of Ca^{2+} levels in astrocytes led to depolarization of the mitochondria [334]. This in turn can accelerate the oxidative stress and lead to release mitochondrial death factors [177,337].

Oxidative stress has been strongly implicated as a factor contributing to cell death during both ischemia and reperfusion [10,12,338,339]. In paper II, we investigated whether cultured

astrocytes were able to recover from metabolic depression in a simulated "reperfusion" situation. Metabolic depression was reversed by washing out IA. At the onset of washout, ATP levels were only moderately decreased but ROS levels were markedly elevated in IA-treated astrocytes. These astrocytes were not able to recover their ATP levels and widespread cell death occurred after washout. However, pre-treatment with Nrf2 activators or addition of exogenous ROS scavengers enabled recovery of ATP levels and prevented cell death. In these cultures, the levels of ROS were significantly decreased at onset of washout, in comparison to IA alone. Interestingly, although the ATP levels were decreased at the onset of washout, recovery of the ATP levels occurred and cell death was prevented. These results suggest that decreased ROS activity during the initial metabolic depression is important for astrocytic recovery *in vitro*. However, oxidative stress probably continues during washout. Indeed we observed that the scavengers trolox and tempol had to be present also during washout to be protective, indicating that ROS activity is important also later in this injury paradigm. An additional increase of already elevated ROS levels were also recently described when oxygen and glucose was reintroduced in oxygen-and-glucose deprived neurons [205]. Interestingly, we observed that the pre-activation of the Nrf2-system or addition of B27 only during the IA treatment was sufficient to be protective also during washout, suggesting an advantage of a broad-spectrum protection against oxidative stress.

In summary, our results show that astrocytes in culture are sensitive to metabolic depression and oxidative stress. Astrocytic cell death mediated by metabolic depression and oxidative stress involves the translocation of AIF from the mitochondria to the nucleus, but not caspase activation. In addition, gap junction communication is important for the spreading of cell death during metabolic depression. Cell death induced by metabolic depression and oxidative stress in cultured astrocytes can be prevented by exogenous scavengers or pre-activation of the Nrf2 antioxidant system. Broad-spectrum protection against ROS-induced damage in astrocytes might be a promising way to enhance the effect of neuroprotective strategies. On a speculative basis, Nrf2-activation may be useful as a prophylactic treatment in high-risk patients and in extending the therapeutic window for acute treatment of stroke patients with thrombolytics. The potential of the Nrf2 system will most likely be extensively studied in the near future. Cell cultures and stress models like the two investigated here, can be a helpful base to investigate several important parameters of known Nrf2 activators. This can be for instance activation mechanisms, cell type specific responses, the kinetics of up-

and down-regulation and the relative protective potency. Moreover, these models can be used to screen for new potent neuroprotective candidate substances. Such *in vitro* studies would provide the basis for the design of relevant *in vivo* experiments.

CONCLUSIONS AND RESPONSES TO GIVEN AIMS

- *In vitro* models of IA-induced metabolic depression and SIN-1-induced oxidative stress were set up in primary astrocyte cultures. Both models induced reproducible responses, enabling them to be used for the studies in this thesis

- Gap junction communication contributed to the spreading of cell death from single cells to large clusters of cells during metabolic depression in cultured astrocytes

- Astrocytic cell death was accompanied by AIF translocation and not by caspase activation, suggesting that the AIF-mediated pathway induces cell death in cultured astrocytes following metabolic depression or oxidative stress

- ROS activity and ATP levels were identified as important factors for the initiation and progress of cell death after metabolic depression and oxidative stress in cultured astrocytes. ROS activity and increased intracellular Ca^{2+} are likely to contribute to the aggravation of the astrocytic injury following metabolic depression

- Pre-activation of the endogenous Nrf2-regulated antioxidant systems, or addition of exogenous scavengers, efficiently protected cultured astrocytes from cellular injury mediated by metabolic depression or oxidative stress

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