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PATHOPHYSIOLOGY OF SUBARACHNOID HEMORRHAGE IN THE RAT

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ABSTRACT

Subarachnoid hemorrhage (SAH) causes brain damage, but the underlying mechanisms are poorly understood. An obstacle in SAH research is the lack of an adequate animal model. In this thesis, we developed a new approach to simulate SAH that involves the injection of blood into the prechiasmatic cistern of the rat. This model had several advantages when it was compared to the two commonest SAH rat models in the literature: unlike the endovascular perforation of the internal carotid artery model, it was reproducible, easy to perform and the mortality rate was acceptable, while when compared to the injection of blood into the cisterna magna model, the blood distribution and histological, hemodynamic and metabolic changes more closely resembled that found in patients with SAH.

We studied various mechanisms that can cause brain damage after SAH. Among the many factors occurring during the first minutes-hours after SAH, the global reduction in cerebral blood flow (CBF), but not the changes in intracranial and perfusion pressure, seemed to be a main determinant of brain injury: the degree of its reduction was correlated with the amount of subarachnoid blood, the acute changes in the expression of the N-methyl-D-aspartate (NMDA) receptor subunits, the severity of subsequent inflammation, and most importantly, with delayed cell death and the mortality rate. Signs of acute metabolic derangements after severe SAH were detected by a reduction in cerebral oxygen extraction and altered levels of extracellular glucose, lactate and pyruvate. These changes in metabolism could not always be explained by ischemic episodes.

Dying cells, mainly neurons, were seen in various areas of the brain in a large percentage of the surviving animals at 2 and 7 days after SAH. The involvement of apoptotic pathways in the brain damage after SAH was evidenced by the

morphological (chromatin condensation and/or apoptotic bodies) and molecular features (upregulation of Bax and active caspase 3) of the damaged cells. Activation of an inflammatory cascade, comprising both parenchymal and vascular tissue, was also detected in the brain by the induction of intercellular adhesion molecule 1, OX6, ED1, tumor necrosis factor α, inducible nitric oxide synthase and nestin. In accordance with the view that inflammation caused brain damage, we found a marked overlapping between areas with dying cells and those with inflammation.

Using *in situ* hybridization, a CBF-dependent early downregulation of the hippocampal NR2A, NR2B and NR3B subunit mRNA levels after SAH was observed. Although these changes may have played a pathogenic role following SAH, their causal relationship to subsequent cell death could not be established.

In conclusion, this thesis describes a new and suitable SAH model in the rat. We showed that acute ischemic episodes, early metabolic derangements, a secondary inflammatory reaction and apoptosis are probable mechanisms which contribute to brain damage after SAH. The fact that cells were still dying at least 7 days later indicates that there is a temporal window during which adequate treatment may improve the final outcome after SAH.

Key words: apoptosis, inflammation, cell death, cerebral blood flow, cerebral metabolism, NMDA receptor subunits, subarachnoid hemorrhage.

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CONTENTS

LIST OF ABBREVIATIONS

aCBF	Autoradiographic cerebral blood flow
AMPA	amino-hydroxy-methyl-isoxalone propionic acid
$AVDO_2$	Arterio-venous oxygen difference
BBB	Blood brain barrier
CBF	Cerebral blood flow
$CMRO_2$	Cerebral metabolic rate of oxygen
CNS	Central nervous system
CPP	Cerebral perfusion pressure
CSF	Cerebrospinal fluid
Cy3	Indocarbocyanine
DNA	Deoxyrybonucleic acid
DPX	Distrene 80, dibutyl phthalate, xylene
FITC	Fluorescein isothiocyanate
GFAP	Glial fibrillary acidic protein
ICAM	Intercellular adhesion molecule
ICP	Intracranial pressure
IL	Interleukin
INOS	Inducible nitric oxide synthase
LDF	Laser-Doppler flowmetry
MABP	Mean arterial blood pressure
MHC	Major histocompatibility complex
mmHg	Millimeters of mercury
mRNA	Messenger ribonucleic acid
NeuN	Neuronal nuclei protein
NMDA	N-methy-D-aspartate
NMDAr	N-methy-D-aspartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
OxyHb	oxyhemoglobin
PBS	Phosphate-buffered saline
s.e.m.	Standard error of the mean
SAH	Subarachnoid hemorrhage
TdT	Terminal deoxynucleotidyl transferase
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling

INTRODUCTION

Clinical aspects of SAH

Subarachnoid hemorrhage (SAH) is defined as the deposition of blood in the subarachnoid space. Spontaneous SAH is caused by the rupture of an aneurysm in 85% of the cases, but it can also be due to perimesencephalic hemorrhages, dural arterio-venous malformation, hypertension, brain tumors and cocaine abuse (van Gijn and Rinkel 2001). The incidence of spontaneous SAH is about 10 in 100.000 persons (Linn *et al.* 1996). It accounts for 5% of deaths from stroke and 27% of all stroke-related years of potential life lost before the age of 65 years (Johnston *et al.* 1998). About 50% of the patients suffering from SAH die within 1 year and many of the survivors suffer permanent significant neurological and cognitive disturbances (Hop *et al.* 1997). Only a very small percentage of all those affected recover completely; the negative consequences of the disease on the patients, their families and society in general are thus considerable (Buchanan *et al.* 2000; Hop *et al.* 1998).

The effects of SAH increase substantially if the traumatic type is also included. Several studies have reported an incidence of 32-61% of SAH in traumatic brain injured patients and have suggested that the presence of blood in the subarachnoid space worsens the outcome (Eisenberg *et al.* 1990; Gaetani *et al.* 1995).

The pathophysiology of SAH can be divided into two phases: the acute phase that comprises the first hours after SAH, and the late phase that ensues during the days after the bleeding. The events taking place during the acute phase are largely unknown. Studies in patients are hampered by the fact that during this period they are usually not receiving medical attention. Reports of rebleeding episodes have given some clues about the initial events, but they may not mimic a first SAH. Moreover, experimental studies have paid little attention to the acute effects of SAH, mainly because few patients are hospitalized immediately afterwards. However, the events taking place during the first minutes/hours are considered to play a decisive role in the subsequent outcome, and their understanding could substantially help to find specific and adequate treatment for SAH (Edner *et al.*

1992; Jakobsen *et al.* 1991; Kassell *et al.* 1990; Ljunggren *et al.* 1984; Saveland and Brandt 1994).

More is known about the late phase of SAH, during which patients doing well may deteriorate clinically. In many cases, this is ascribed to delayed cerebral arterial vasospasm (Hijdra *et al.* 1986; Kassell *et al.* 1985). Although extensive research has provided us with more knowledge, the underlying mechanisms of vasospasm are not fully understood. During the days following SAH, the patients may also suffer from other harmful conditions, like alterations in cerebral blood flow (CBF) unrelated to vasospasm, cerebral metabolic depression, hypothalamic dysfunction, hydrocephalus, loss of autoregulation, signs of cerebral inflammation, systemic electrolyte disturbances, edema and seizures (Carpenter *et al.* 1991; Claassen *et al.* 2002; Grubb *et al.* 1977; Hasan *et al.* 1990; Kassell *et al.* 1990; Kolluri and Sengupta 1984; Lin *et al.* 1999; Martin *et al.* 1984; Ryba *et al.* 1992; Voldby *et al.* 1985a). Experimental studies have confirmed the clinical observations, and have thrown a little light on the particular pathways causing these changes.

The current management of SAH includes the prevention of rebleeding by early surgical clipping or coiling of the aneurysm, prevention of delayed ischemic neurological deficits with the Ca^{++}-channel blocker nimodipine and the treatment of delayed symptomatic cerebral vasospasm with 3-H therapy (hypertensive, hypervolemic, hemodilution therapy) or transluminal balloon angioplasty to improve cerebral perfusion (Kassell *et al.* 1982; Pickard *et al.* 1989; Romner and Reinstrup 2001; van Gijn and Rinkel 2001; Zubkov *et al.* 1984). The poor prognosis for these patients suggests that more effective treatment is needed.

Animal models of SAH

To extend our knowledge of the mechanisms causing brain damage after SAH, an adequate animal model is a prerequisite. An ideal experimental model of SAH should simulate the mechanism of hemorrhage and blood deposition after the rupture of an aneurysm, it should result in substantial pathological and pathophysiological findings, but with an acceptable mortality rate, and it must be reproducible and simple to perform. The first *in vivo* model of SAH was reported in 1928, when Bagley described

the injection of blood into the subarachnoid space of dogs (Bagley 1928). Since then, various species have been used to study the disease, but during the last two decades, the rat has become increasingly popular due to its easy availability and the extensive knowledge of its biology.

The commonest experimental models of SAH in the rat include the injection of blood into the cisterna magna (cisterna magna SAH) and the intraluminal perforation of the internal carotid artery intracranially (perforation SAH) (Bederson *et al.* 1995; Delgado *et al.* 1985; Veelken *et al.* 1995). Each model has drawbacks as regards to the ideal criteria. The cisterna magna SAH model is easy to perform, has very low mortality rate and is adequate for studing the occurrence of vasospasm. However, the blood is dispersed in the posterior cranial fossa and spinal canal, unlike the clinical picture in which about 90% of aneurysms rupture involve the anterior circulation (Kassell *et al.* 1990; Velthuis *et al.* 1998). The perforation SAH model closely resembles the mechanism of an aneurysmal rupture and the blood distribution, but the amount of the hemorrhage can not be controlled and it has a high mortality rate. Therefore, many animals are needed to obtain reliable data. Although other SAH models have been used in the rat that deliver blood into the basal cisterns, they have the drawbacks of requiring complicated surgery or the need of specialized equipment (Klinge *et al.* 1999; Piepgras *et al.* 1995; Zhao *et al.* 1999).

Possible mechanisms of brain damage after SAH

SAH causes brain damage, as shown by the development of cognitive and neurological deficits after rupture of an aneurysm, and confirmed by the presence of infarcted areas on CT scans, MRIs and autopsies of patients dying of SAH (Crompton 1964; Hadeishi *et al.* 2002; Hijdra *et al.* 1986). Little is known about the fundamental causes of the brain damage, but many pathological mechanisms are probably involved.

Ischemia

Much evidence suggests that SAH induces changes in CBF. It is well established that SAH leads to a sudden reduction in CBF in various laboratory species (Bederson *et al.* 1995; Jackowski *et al.* 1990; Kamiya *et al.* 1983; Petruk *et al.* 1972; Trojanowski 1984;

Umansky *et al.* 1983; Veelken *et al.* 1995; Zhao *et al.* 1999). Cerebral hypoperfusion in SAH patients on admission have been reported, which suggest that clinical SAH probably also cause an immediate reduction in CBF (Hayashi *et al.* 2000). Moreover, pathological findings consistent with an ischemic injury have been seen in the brains of patients dying shortly after SAH (Crompton 1964). Although CBF reductions may result in cellular injury (Siesjo 1992), the contribution of the acute hypoperfusion to the damage after SAH is poorly understood. Measurements of CBF during the days following a ruptured aneurysm showed both global and focal reductions (Ferguson *et al.* 1972; Grubb *et al.* 1977; Heilbrun *et al.* 1972; Jakobsen *et al.* 1991; Kawamura *et al.* 1992; Matsuda *et al.* 1990; Mickey *et al.* 1984; Voldby *et al.* 1985b); similar findings have been noted in experimental studies (Delgado *et al.* 1986a; Delgado-Zygmunt *et al.* 1993).

Low CBF levels are caused by various conditions, and many of them probably take place after SAH. An increase in intracranial pressure (ICP), especially within the first minutes of an aneurysm rupture, has been proposed as a main factor leading to ischemia due to a reduction in cerebral perfusion pressure (CPP) (Grote and Hassler 1988; Nornes 1973; 1978; Weaver and Fisher 1994). However, this view is based on observations made in patients during recurrent bleeding, a situation that may not reflect the events during a first hemorrhage. Experimental studies have shown that CPP changes can hardly explain the changes in CBF after SAH (Bederson *et al.* 1995; Dorsch *et al.* 1989; Jackowski *et al.* 1990; Umansky *et al.* 1983). Hydrocephalus due to obstruction of CSF outflow by blood is one of the 3 major neurological complications during the first days after SAH and may also increase ICP, which contributs to hypoperfusion in some patients (Chang *et al.* 1999).

Narrowing of the cerebral arteries, a well-documented phenomenon both immediately and shortly after SAH (Bederson *et al.* 1998; Delgado *et al.* 1985; Weir *et al.* 1978), may also cause ischemic deficits. Clinically, delayed cerebral vasospasm plays a significant and harmful role: it probably accounts for poor outcomes in about 6% and death in about 7% of the SAH patients surviving the initial insult (Kassell *et al.* 1990). However, the reductions in CBF are frequently not due to arterial narrowing (Ferguson *et al.* 1972; Grubb *et al.* 1977; Heilbrun *et al.* 1972; Martin *et al.* 1984; Mickey *et al.* 1984).

Under normal conditions, CBF is coupled to the metabolic demands. Thus, a decrease in metabolism causes a reduction in CBF (Magistretti 1997). Low metabolic activity is one of the major effects of aneurysms rupture (Carpenter *et al.* 1991; Grubb *et al.* 1977; Hayashi *et al.* 2000; Jakobsen *et al.* 1991; Martin *et al.* 1984; Voldby *et al.* 1985b), but it is uncertain whether a low CBF reduces metabolism after SAH or whether a low metabolism reduces CBF.

Metabolic alterations

Since the pioneer studies of Fein (Fein 1975; 1976), several lines of evidence suggest that SAH leads to metabolic alterations. Clinically, a decrease in the cerebral oxygen consumption and changes in the levels of metabolites related to the cellular oxidative energy are common after SAH (Carpenter *et al.* 1991; Grubb *et al.* 1977; Hayashi *et al.* 2000; Jakobsen *et al.* 1991; Kawamura *et al.* 1992; Martin *et al.* 1984; Voldby *et al.* 1985b). Experimental studies have also found changes in metabolism after SAH, including a reduction in $CMRO_2$, both an increase and a decrease in glucose utilization, increases in lactate, free fatty acids and extracellular glutamate levels, and reductions in phosphocreatine and hexokinase levels (Bederson *et al.* 1998; d'Avella *et al.* 1996; Delgado *et al.* 1986a; Fein 1975; 1976; Gewirtz *et al.* 1999; Glenn *et al.* 2002; Sahlin *et al.* 1987; Solomon *et al.* 1987; Yin *et al.* 2001).

The etiology of the reduction in metabolic activity is not known. In many SAH patients, ischemia probably plays an important role in causing changes in metabolism (Jakobsen *et al.* 1990). However, several clinical and experimental studies have shown metabolic derangements after SAH with no changes in CBF (Carpenter *et al.* 1991; Fein 1975; 1976; Voldby *et al.* 1985b), which shows that causes other than hypoperfusion are primarily responsible in many cases. Spreading depression, which has been suggested to take place during the first minutes after SAH (Beaulieu *et al.* 2000; Hubschmann and Kornhauser 1980), may also lower cerebral metabolism. This phenomenon is characterized by an increase in metabolic activity due to waves of cellular depolarization that propagate through the brain, causing transient hyperemia followed by prolonged hypometabolism and hypoperfusion. Although it has been described after experimental ischemia (Busch *et al.* 1996; Hossmann 1996), its occurrence after

SAH has been questioned and needs to be determined (van den Bergh *et al.* 2002).

Other lines of evidence suggest that the hypometabolism could be due to a direct effect of the deposition of blood in the subarachnoid space. In accordance with this view, it has been reported that mitochondrial function is impaired in the acute phase after SAH (Marzatico *et al.* 1988; Marzatico *et al.* 1990).

Inflammation

The presence of elevated levels of cytokines, adhesion molecules and immune competent cells, as well as signs of complement activation in the CSF of SAH patients, suggest that inflammation is one of the numerous processes activated in the brain after SAH (Fassbender *et al.* 2001; Gaetani *et al.* 1998; Kasuya and Shimizu 1989; Kikuchi *et al.* 1995; Kwon and Jeon 2001; Mack *et al.* 2002; Mathiesen *et al.* 1993; Mathiesen *et al.* 1997; Mathiesen and Lefvert 1996; Nissen *et al.* 2001; Osuka *et al.* 1998a; Pellettieri *et al.* 1986; Polin *et al.* 1998; Takizawa *et al.* 2001). The few studies concerning the source of the inflammation after rupture of an aneurysm have concentrated on examining the cerebral arterial walls, where signs of an immune reaction have been found (Ryba *et al.* 1992). This was confirmed by experimental studies which also indicated that inflammation may play a role in the development of vasospasm (Aihara *et al.* 2001; Bavbek *et al.* 1998; German *et al.* 1996; Handa *et al.* 1991a; Handa *et al.* 1991b; Handa *et al.* 1995; Osuka *et al.* 1998b; Sills *et al.* 1997; Zhang *et al.* 1994). However, little is known about the incidence, genesis and location of the inflammatory reaction after SAH and its contribution to brain damage.

The possible harmful role of an inflammatory reaction in the brain has been studied after various injuries. It is well known that ischemia and brain trauma trigger an inflammatory response in the CNS with the activation and proliferation of microglia and infiltration of monocytes/macrophages (Barone and Feuerstein 1999; del Zoppo *et al.* 2000; Morganti-Kossmann *et al.* 2002). This reaction is thought to mediate repair, but it can also be harmful to the surrounding tissue. On activation, immunocompetent cells secrete cytokines, potent molecular signals in the inflammation response. Proinflammatory cytokines, like IL-1β, IL-6 and TNF-α, further activate microglia, astrocytes and other immune cell, which, in turn, generate cytokines and other biologically active molecules

(Barone and Feuerstein 1999). Thus, activated cells upregulate the inducible form of nitric oxide synthase (iNOS) and cyclo-oxygenase 2 that generate reactive oxygen species and other inflammation-related products that are toxic to neurons (Iadecola and Alexander 2001). Proinflammatory cytokines also upregulate locally the expression of adhesion molecules, such as ICAM-1, in endothelial cells and integrins in leukocytes, that mediate the leukocyte-endothelium adhesion process (rolling and adhesion of leukocytes to the arterial wall, followed by their transmigration to the target tissue) (Frijns and Kappelle 2002). Enhanced leukocyte adhesion to the endothelium may cause plugging of the microvessels, worsen perfusion and thereby contribute to brain damage (Kochanek and Hallenbeck 1992; Schmid-Schonbein 1987). Other possible harmful effects of inflammation include an increase in metabolic demands (IL-1β is a known pyrogen) (Kluger *et al.* 1995) and the formation of edema (Petty and Lo 2002).

Several experimental studies have evidenced an adverse effect of inflammation on the CNS. For example, systemic depletion of neutrophils reduced the size of ischemic infarcts (Matsuo *et al.* 1994). The brain damage after ischemia and trauma is exacerbated by the administration of IL-1β or TNF-α, while it is reduced by the inhibition of these cytokines (Allan and Rothwell 2001). An absence or antagonism of adhesion molecules reduces the size of infarcts after ischemia, and attenuates vasospasm after SAH (Bavbek *et al.* 1998; Zhang *et al.* 1994). Inhibition or knockout of iNOS or cyclo-oxygenase 2 also causes less ischemic damage in the brain (Iadecola and Alexander 2001).

However, the view that an inflammation negatively affects the brain after a stroke is being reexamined since recent studies have shown beneficial effects of some inflammatory mediators,- e. g., mice lacking TNF-α receptors sustained exacerbated damage after ischemia (Bruce *et al.* 1996; Gary *et al.* 1998). Some studies showed that IL-6 had a protective effect on brain damage (Loddick *et al.* 1998). Moreover, TNF-α and IL-1β may protect against a subsequent ischemic injury (ischemic precondition) (Nawashiro *et al.* 1997; Ohtsuki *et al.* 1996). These apparently conflicting data may in fact be due to timing; the production of inflammatory mediators could be toxic when produced early after the injury, but protective later on (Scherbel *et al.* 1999). The ongoing debate on the role of inflammation in brain damage after injury indicates complex interactions between the

many factors involved in the inflammatory process. More data are obviously needed to understand the harmful and beneficial effects of inflammation on the brain.

Excitotoxicity

Glutamate is the main excitatory neurotransmitter in the brain. During normal neurotransmission, it is liberated in the synaptic cleft, acts on specific receptors, and is rapidly eliminated from the extracellular space. However, during some pathological conditions, its extracellular levels may remain elevated and become toxic to the cells (Doble 1999; Sattler and Tymianski 2001).

Glutamate excitotoxicity probably contributes to brain damage after SAH: a 5-fold increase in the extracellular levels of glutamate was reported in the rat brain immediately after SAH (Bederson *et al.* 1998), and an elevated glutamate concentration in the brain parenchyma is common during the first few days after SAH in patients who have a bad outcome (Nilsson *et al.* 1996; Persson *et al.* 1996; Staub *et al.* 2000).

Glutamate acts via 4 receptors: the ionic receptors amino-hydroxy-methyl-isoxalone propionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA), and the metabotropic receptor. Because of its high conductance to Ca^{++} and, to a lesser extent, to Na^{+}, activation of the NMDA receptor (NMDAr) is thought to be a main mediator of the excitotoxic effects of glutamate (Doble 1999). Ca^{++} is a potent second messenger and its intracellular levels are kept about 10.000 times lower than the extracellular ones. Thus, a pathological stimulation of the NMDAr could easily lead to cellular Ca^{++} overload that triggers a cascade of events that may damage the cell by the activation of proteases, lipases, DNases and the generation of free radicals. Moreover, the enhanced influx of Na^{+} through the NMDA receptor is accompanied by the accumulation of water that may cause edema, thereby negatively affecting cerebral perfusion.

The NMDAr are heteromeric proteins composed by the combination of 3 subunits: NR1 subunit, which has 8 splice variants, a family of 4 NR2 subunits (NR2A, NR2B, NR2C and NR2D) and 2 NR3 subunits (NR3A and NR3B) (Cull-Candy *et al.* 2001). The receptors seem to be composed of many NR1 subunits together with at least 1 NR2 subunit holding the binding site to glutamate, while the NR3 subunits can co-assemble with NR1/2 complexes. Each receptor subunit has a characteristic

developmental and regional distribution, and the differential assembly of specific polipeptides determines the physiology and pharmacology of the receptor in a way that is not fully understood. Interestingly, different types of brain injuries, including ischemia, can alter NMDAr subunit expression (Hsu *et al.* 1998; Small *et al.* 1997; Zhang *et al.* 1997). At present, the contribution of each subunit to the receptor's functional properties is a field of intense investigation.

Due to its importance in neurotoxicity, the blocking of NMDAr seems to be a straightforward therapeutic approach. Antagonism of its action produces an effective protection, among others, against ischemia, trauma and oxidative damage (Lynch and Guttmann 2002). However, clinical trials in stroke and traumatic brain injury with NMDAr antagonists have been aborted mainly because of serious side effects (Martinez-Vila and Sieira 2001). The goal now is to find more selective NMDAr antagonists, which might overcome their unwanted actions. Specific subunits have been proposed as likely targets.

Other causes of damage

The presence of lysed blood in the subarachnoid space causes the induction of stress genes and cell death (Harada *et al.* 1997; Klinge *et al.* 1999; Matz *et al.* 1996a; Matz *et al.* 1996b; Turner *et al.* 1998; Turner *et al.* 1999). Among the blood products, hemoglobin (Hb) released from lysed red cells has been proposed as a direct mediator of cell injury because of its toxic effects on nervous tissue: exposure of cultures of neurons to Hb induced their death in a dose-dependent manner (Regan and Panter 1993), while intracortical injection of Hb was associated with epileptogenesis (Ohyagi and Goto 1994). One of the mechanisms whereby Hb might be toxic is the release of free radicals and iron during its degradation, leading to lipid peroxidation and DNA damage (Braughler *et al.* 1986; Imlay *et al.* 1988). Moreover, oxyhemoglobin (OxyHb), the reduced form of Hb, may also cause vasospasm because it avidly binds the potent vascular relaxant nitric oxide (NO), and induces the formation of the vasoconstrictor endothelin (Gladwin *et al.* 2003; Lin *et al.* 2001). Ionic potassium, another component of lysed blood, potentiates the damaging effect of OxyHb (Nishiye *et al.* 1989).

After SAH, many mechanisms other than Hb degradation may induce the formation of free radicals. For

example, ischemic episodes activate several Ca^{++}-dependent enzymes, such as neuronal NOS, phospholipase A2 and calpain, which can increase the generation of free radicals (Dirnagl *et al.* 1999). Moreover, the presence of blood activates immune cells that produce substantial amounts of these reactive molecules, and blood products, like Fe^{++}, stimulate the formation of these radicals (Jackowski *et al.* 1990; Sadrzadeh *et al.* 1987).

Finally, the balance between vasoconstrictor and vasodilator forces seems to change after SAH, which favours the occurrence of vasospasm. For example, after SAH, the levels of the strong vasoconstrictor endothelins increase and the expression of their receptors is augmented in the brain (Hansen-Schwartz *et al.* 2003; Zimmermann and Seifert 1998), while the production of the potent relaxant NO in the endothelium is reduced (Hino *et al.* 1996; Kasuya *et al.* 1995).

Mechanisms of cell death: necrosis versus apoptosis

The prevalent view is that cells die in two ways: necrosis or apoptosis. However, other mixed variants may exist between these extremes (MacManus and Buchan 2000; Martin 2001). Thus the same insult may cause both types of cell death depending on its severity, - i. e., the greater the severity, the higher the probability of necrosis (Bonfoco *et al.* 1995; Lennon *et al.* 1991). The traditional view links the damage by a stroke to necrotic and quickly evolving cell death, but increasing evidence suggests that the mechanisms of injury are long-lasting and involve both necrosis and apoptosis (Li *et al.* 1998; MacManus and Buchan 2000; Raghupathi *et al.* 2000).

The distinction between necrosis and apoptosis relies mostly on morphological criteria (Figure 1), but their molecular and biochemical pathways are now being clarified. Necrosis is associated with passive degeneration of the cell due to severe loss of homeostasis. It is characterized by nuclear pyknosis, swelling and disruption of the cellular membrane. The cellular components are released into the extracellular space, which affect cells in the vicinity and induce an inflammatory reaction. On the other hand, apoptotic cell death is an active process that requires the induction of an internal and very organized suicidal programme. Its morphological features include chromatin condensation and cytoplasmic shrinkage followed by fragmentation of the cell in apoptotic bodies (pieces of nucleus surrounded by cytoplasm) that

are phagocytized without affecting the surrounding tissue (Charriaut-Marlangue and Ben-Ari 1995; Li *et al.* 1995). In this process, the cell membrane and organelles are apparently intact. Since many days can separate the harmful episode and the development of apoptotic features in a cell programed to die, it seems probable that, unlike a necrotic event, treatment that targets apoptotic pathways may be effective in a broad time window.

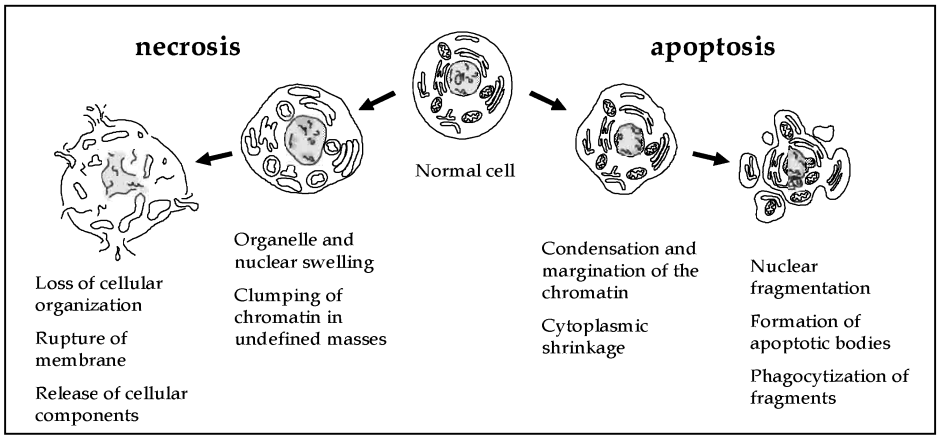

Figure 1- Diagram showing the morphological events during necrotic and apoptotic cell death.

The discovery and characterization of genes that control cell death in the development of the nervous system in *C. elegans* have led to the identification of its homologues in mammals and in a vertiginous advance in the understanding of the underlying molecular mechanisms of apoptosis (Yuan and Horvitz 1992). The cysteine proteases, called caspases, are key regulators of apoptosis (Bredesen 2000). Humans and mice have been shown to have 11 different caspases and most (if not all) are present in the brain (Troy and Salvesen 2002). Activation of initiator caspases (e. g. caspases 8 and 9) induces a cascade of proteolytic and positive feedback loops of reactions causing activation of downstream effector caspases (e. g. caspases 3, 6 and 7) that ensure cell death. In particular, activation of caspase 3 has been implicated in brain damage after various injuries, including ischemia and trauma (Springer *et al.* 2001). Among its targets, the cleavage of the inhibitor of the caspase-activated deoxyribonuclease activates an endonuclease which cleaves the DNA into the characteristic internucleosome fragments that are seen during apoptosis (Enari *et al.* 1998; Liu *et al.* 1997).

Due to its importance in cell life/death decisions, caspase activities are closely regulated. For example, the Bcl-2 family, consisting of antiapoptotic (Bcl-2, Bcl-xl and Boo) and proapoptotic members (Bax, Bcl-xs, Bad, Bid, Bak, Bik, Bim and Mtd), seems to exercise substantial control of caspase activities (Borner 2003). Bcl-2, the best characterized antiapoptotic member, is thought to protect neurons exposed to excitotoxicity, growth factor, serum or glucose deprivation, Ca^{++} overload and free radicals (Garcia *et al.* 1992; Kane *et al.* 1993; Zhong *et al.* 1993a; Zhong *et al.* 1993b). In particulart, overexpression of Bcl-2 leads to a reduction in infarct size after in vivo ischemia and brain trauma (Graham *et al.* 2000; Raghupathi *et al.* 2000). On the other hand, upregulation of proapototic members,- e. g., Bax, is associated with damage by a stroke, and its absence or downregulation protects neurons from apoptotic death induced by deprivation of growth factors (Deckwerth *et al.* 1996; Gillardon *et al.* 1996; Hara *et al.* 1996; Krajewski *et al.* 1995). The *bcl-2* family genes control apoptosis through the so-called intrinsic or mitochondrial pathway of the apoptotic program (Hengartner 2000). Bcl-2 antiapoptotic members are integral mitochondrial proteins that stabilize the mitochondria preventing the efflux of cytochrome *c*. In response to injury, proapoptotic proteins translocate from the cytosol to the mitochondria, where they induce mitochondrial membrane permeabilization and release of cytochrome *c*. Cytosolic cytochrome *c* permits the formation of the apoptosome complex, composed of cytochrome *c*, Apaf-1 and caspase 9 zymogen that activates caspase 9. After activation of caspase 9, downstream effector caspases, such as caspase 3, are activated. The involvement of the intrinsic apoptotic pathway in ischemia and trauma has been clearly shown by several authors (Graham *et al.* 2000; MacManus and Buchan 2000; Raghupathi *et al.* 2000).

Another well-defined apoptotic pathway is the extrinsic or death receptor pathway that starts by the binding of death ligands (e. g. Fas, TNF) to cytoplasmic membrane death receptors (Ashkenazi and Dixit 1998). This result in clustering of receptors, recruitment of an adaptor molecule (e. g. FADD) and activation of the initiator caspase 8 which, in turn, activates executioner caspases – e. g., 3 and 7. Evidence is accumulating hat this pathway is also active after ischemia and trauma (Beer *et al.* 2000; Martin-Villalba *et al.* 2001; Martin-Villalba *et al.* 1999; Qiu *et al.* 2002; Rosenbaum *et al.* 2000).

Hardly any studies have been done on the occurrence of apoptosis after SAH, but some data suggest that it may contribute to brain damage after SAH. Thus, cells with apoptotic characteristics have been found in the hippocampus of patients dying after SAH, and the injection of blood products into the subarachnoid space results in the occurrence of cells with apoptotic morphology, DNA fragmentation in a pattern consistent with apoptosis and increased levels of cytoplasmic cytocrome *c* in the rat brain (Matz *et al.* 2000; Matz *et al.* 2001; Nau *et al.* 2002).

AIMS OF STUDY

The general aim of this thesis was to provide basic knowledge of the pathophysiology of SAH. We therefore investigated the occurrence and significance of various harmful mechanisms that probably contribute to the brain damage after SAH.

Specific aims

To develop a suitable, simple and reproducible animal model for studying the pathophysiology of SAH (Papers I, II and III)

To study the effect of the amount of subarachnoid blood and the acute changes in ICP, CPP and CBF on the outcome after experimental SAH, and to determine possible causal relationships between them (Paper II).

To evaluate the occurrence and importance of changes in cerebral metabolism and their relation to CBF immediately after experimental SAH (Paper III).

To determine whether SAH causes delayed cell death, to describe the types of cells affected and the incidence of apoptosis during the days following SAH (Paper IV).

To study the inflammatory reaction in the brain after SAH, the factors involved in its genesis and its effect on brain damage (Paper V).

To investigate whether SAH causes early changes in the mRNA levels of the NMDA receptor subunits, and determine whether they are related to the subsequent cell death (Paper VI).

To study the contribution of acute ischemic events to the subsequent brain damage after SAH (Papers IV-VI).

MATERIALS AND METHODS

Animals (Papers I-VI)

Male Sprague-Dawley rats (B & K Universal AB, Sollentuna, Sweden), weighting 300-400 g were used in the experiments. They were maintained in a 12-hour light/dark cycle with free access to food and water. The experiments were approved by the Northern Committee for Animal Experimentation, Stockholm, Sweden.

Anesthesia and catheterization (Papers I-VI)

The animals were endotracheally intubated with a polyethylene catheter (OD = 2.08 mm) during halothane anesthesia (Fluothane®, Zeneca, Gothenburg, Sweden), and artificially ventilated with 70% N_2O and 30% O_2. During surgery, the anesthesia was kept with 1% halothane in the inspired mixture. The body temperature was maintained at 37.5° ± 0.5° C with an automatic heating pad (LSI Letica Scientific Instruments, Barcelona, Spain). The femoral arteries were cannulated in acute experiments (animals sacrificed within 90 minutes of SAH) to measure blood gases, blood pH, hematocrit and mean arterial blood pressure, while the tail artery was used in long-term experiments (animals sacrificed 3 hours or later after SAH). One femoral vein (in acute experiments) or a tail vein (in long-term experiments) was cannulated for the infusion of drugs.

The animals were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with the mouthpiece at 0 degree. Surgery to place monitor probes and to prepare for SAH was performed. Fentanyl (Leptanal®, Janssen-Cilag AB, Sollentuna, Sweden; 15 µg as a bolus followed by 25 µg/hr intravenously) was given instead of halothane. After 30-60 minutes of equilibration, SAH was induced.

Induction of SAH (Papers I-VI)

We used a slight modification of the **perforation SAH** model as described by Bederson *et al.* and Veelken *et al.* (Bederson *et al.* 1995; Veelken *et al.* 1995) (Papers II-VI). The bifurcation of the right common carotid artery was exposed and the external carotid

artery clamped, using an aneurysmal clip. The legs of the clip had an indentation and were covered by a soft catheter. An obliquely cut 3.0-monofilament suture (Prolene, Ethicon, Inc, Johnson & Johnson Int., Brussels, Belgium) was advanced via the external carotid artery into the internal carotid artery until a resistance was felt about 18-20 mm from the bifurcation of the common carotid artery. The modified clip permitted the suture to pass without causing bleeding. The suture was advanced for about 3 mm more until it perforate the internal carotid artery at its intracranial bifurcation into the anterior and middle cerebral arteries. It was withdrawn after about 15 seconds. The suture was kept intravascularly for about 90 seconds where it did not interfere with CBF, as judged by laser-Doppler flowmetry (LDF). In sham operated rats, the suture was advanced into the internal carotid artery until the resistance was felt and withdrawn after 15 seconds.

In the **prechiasmatic SAH** model (Papers I-V), a needle with a rounded tip and a side hole (Withcare Spinal Set 0.41 x 90 mm, Becton Dickinson, Madrid, Spain) was inserted stereotaxically into the prechiasmatic cistern. The needle was tilted 30° anteriorly and placed 7.5 mm anterior to the bregma in the midline. With the aperture pointing to the right; it was lowered until the tip reached the base of the skull 2-3 mm anterior to the chiasma, about 10 mm from the brain's surface. The burr hole was sealed with bone wax before inserting the needle. Two hundred (Papers I-V), 250 or 300 µl (Paper I) of autologous blood was injected manually with the aim of keeping the ICP at the level of the MABP. The injection of 200 µl of blood was given in about 12 seconds. The control animals received saline.

In the **cisterna magna SAH** model (Papers II and III), the animals were tilted 30° with the head down, and 300 µl of autologous blood was injected during 15 seconds via a catheter placed in the cisterna magna (see below). The control animals received saline.

ICP monitoring (Papers I-V)

ICP was measured by a polyethylene catheter (OD = 1.22 mm, ID = 0.76 mm) placed in the cisterna magna. The tip of the catheter was heated to form a collar. A small longitudinal incision was made in the atlanto-occipital membrane, including the arachnoid membrane, and the collar was inserted into the incision

like a button into a buttonhole. Histoacryl® (B. Braun Surgical Gmbh, Melsungen, Germany) was used to prevent cerebrospinal fluid (CSF) from leaking. ICP was monitored every 2 seconds, starting 15 minutes before and until 15, 60 or 90 minutes after SAH. CPP was calculated as MABP minus ICP. The average of the values during 10 minutes before SAH was referred to as the baseline. The data were analyzed at baseline, maximal increase, 2.5, 5, 10, 15, and when possible, at 45, 60 and 90 minutes.

In the cisterna magna SAH group, an additional catheter was implanted in the cisterna magna for the administration of blood.

Measurements of CBF (Papers II-VI)

CBF was measured by laser-Doppler flowmetry (LDF) and autoradiographic CBF (aCBF) technique, two methods that provide complementary information. LDF permit serial measurements in the same animals relative to a baseline and therefore, no quantitative data are obtained. Moreover, only a small amount of tissue around the tip of the probe can be sampled (about 1 mm^3). On the other hand, quantitative measurements in various areas of the brain simultaneously, but at only one time point in each animal can be made with aCBF.

To measure CBF by **LDF** (Papers II-VI), we placed LDF probes (MT B500-1, Perimed AB, Järfälla, Sweden) over both frontal cortices (3 mm anterior to the coronal suture and 2 mm lateral to the midline) on the epidural surface, away from large vessels, and fixed them to the bone with Histoacryl®. The signals were monitored by a laser-Doppler monitor (Periflux 4001 Master, Perimed AB). LDF values from both sides were monitored every 2 seconds starting 15 minutes before and until 15, 60 or 90 minutes after SAH. The values were expressed as a percentage of baseline, calculated as an average of the 10 minute values before SAH. The data were analyzed at baseline, maximal reduction, 2.5, 5, 10, 15 and, when possible, at 45, 60 and 90 minutes. In some studies (Papers IV-VI and unpublished results), we determined how long LDF values were below 30% of baseline within 60 minutes of SAH.

We measured the regional **aCBF** (Paper III) 15 or 90 minutes after SAH with Sakurada *et al.*'s method (Sakurada *et al.* 1978), as modified by Gjedde *et al.* (Gjedde *et al.* 1980). In brief, 100 µCi of ^{14}C iodo-antipyrine (American Radiolabel Chemicals, Inc, St.

Louis, USA) was given as an intravenously bolus. Arterial blood was withdrawn over 20 seconds using a constant velocity withdrawal pump for mechanical integration of the tracer concentration. At the end of the 20 seconds, KCl (6 mmol/kg) was given intravenously to stop the blood circulation. The brains were quickly dissected and frozen. The radioactivity content in the blood samples was determined by β-scintillation counting; for details, see Delgado *et al.*, 1986a. The brains were coronally sectioned in 20 µm slices. The sections were dried on a 60° C surface for 10 minutes, and exposed together with ^{14}C standards (Amersham Life Science, Amersham, England) to imaging plates (BAS, SR, Fuji Photo Film CO., Ltd., Tokyo, Japan) for 17 hours. The plates were scanned with a phosphorous imaging analyzer (Phosphor Imager BAS 1000, Fuji Photo Film CO., Ltd., Tokyo, Japan). The ^{14}C content was determined in the following regions: frontal, parietal, temporal and occipital cortices, striatum, septum, hippocampus, thalamus, hypothalamus, superior colliculus, inferior colliculus, cerebellar cortex, facial nucleus, cochlear nucleus, vestibular nucleus, trigeminal nucleus and corpus callosum.

We calculated the regional aCBF (ml/100g/min) using Gjedde *et al.*'s equation (Gjedde *et al.* 1980):

$$f^{bl} = \frac{C_{Br}(T)}{E(T)\int_0^T Ca(t)dt}$$

where f^{bl} is the blood flow per unit mass, $C_{Br}(T)$ the isotope content, E(T) the next extraction fraction of the isotope in the time from t = 0 to t = T, t = the variable time, T = the experimental time and $C_a(t)$ is the arterial blood concentration of the isotope at time t.

Measurements of the arterio-venous difference in oxygen ($AVDO_2$) and cerebral metabolic rate of oxygen ($CMRO_2$) (Paper III)

$AVDO_2$ and cortical $CMRO_2$ were calculated 15 or 90 minutes after SAH. Immediately before the aCBF measurement, a cerebral venous blood sample was taken from the sagittal sinus just above the sinus confluence. For this purpose, the bone was thinned by an air drill and the tip of a tapered and obliquely cut and heparinized capillary tube was introduced into the sinus to take a 100 µl blood sample. Venous blood loss was prevented by covering

the sinus with a hemostatic absorbable gelatin sponge (Spongostan ®, Ferrosan, Denmark). At the same time, we took an arterial blood sample from a femoral artery. $AVDO_2$ (µmol O_2/ml) was determined as the difference in oxygen content between the arterial and that in sinus blood. $CMRO_2$ (µmol/g/min) was calculated as the mean cortical aCBF multiplied by $AVDO_2$.

Monitoring of parenchymal O_2 tension (Paper III)

To measure the availability of O_2 in the brain paremchyma, we placed a p(ti)O_2 microcatheter (Clarck type, Licox cmp® GMS, Kiel, Germany) connected to a pO_2 monitor (Licox cmp®) into the left striatum, according to the following coordinates: 0.5 mm posterior to the coronal suture, 3.2 mm lateral to the midline and 6 mm below from the brain's surface. It was fixed to the bone with Histoacryl® (B. Braun Surgical Gmbh, Melsungen, Germany). Parenchymal O_2 tension was monitored every 10 seconds starting 15 minutes before and until 90 minutes after SAH. The average of the values during 10 minutes before SAH was referred to as the baseline. The data were analyzed at baseline, maximal reduction, 2.5, 5, 10, 15, 45 and 90 minutes, and expressed as a percentage of the baseline to eliminate the difference in probe sensitivity due to repeated use.

Microdialysis (Paper III)

The extracellular levels of glucose, lactate and pyruvate after SAH were measured by microdialysis. A guide cannula (CMA/12, CMA/Microdyalsis AB, Stockholm, Sweden) was inserted into the right striatum, using the coordinates of 0.5 mm posterior to the coronal suture, 3.2 mm lateral to the midline and 3.5 mm below the brain's surface. The cannula was fixed to a screw placed in the skull with methylmethacrylate cement. A microdialysis probe having a membrane length of 4 mm (CMA/2, CMA/Microdialysis), perfused with Ringer's solution (148 mM Na^+, 3 mM K^+, 2,7 mM Ca^{++} and 154 mM Cl^-) at a flux of 2 µl/min, was introduced into the guide cannula. After 60 minutes of stabilization, the samples were collected at 15-minute intervals, starting 15 minutes before SAH, and kept at -20° C until analysis. The levels of glucose, lactate and pyruvate in the perfusates were determined, using an enzymatic analyzer (CMA 600,

CMA/Microdialysis). The methods of detection are shown in Figure 2. The sample immediately before the SAH was considered the baseline value and the results were expressed as percentage of the basal value to eliminate differences in probe recovery.

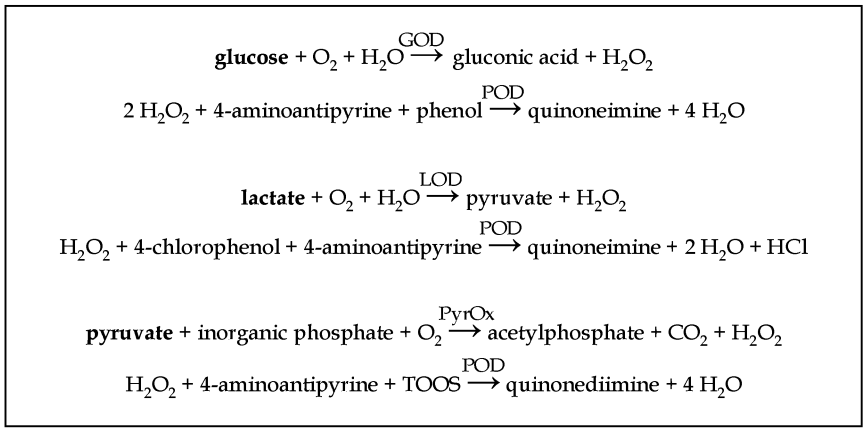

Figure 2- Colorimetric methods for the quantification of glucose, lactate and pyruvate in microdialysates. Glucose, lactate and pyruvate are enzymatically oxidized by glucose oxidase (GOD), lactate oxidase (LOD) and pyruvate oxidase (PyrOx), respectively. Peroxidase (POD) catalyzes the reaction between the formed hydrogen peroxide with 4-aminoantipyrine and pheno, 4-chlorophenol or N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (TOOS) that generates the colored substance quinoneimine or quinonediimine; its rate of formation is measured photometrically at 546 nm.

Quantification of the amount of blood in the subarachnoid space (Papers I and II)

The subarachnoid blood volume was measured 90 minutes following SAH. After collecting an arterial blood sample, we perfused the animals with 250 ml of saline solution to remove the blood from the cerebral vessels. The brains were dissected, while keeping the arachnoid membrane intact, and homogenized at 4° C in 15 ml of 60 mM potassium phosphate buffer with 5% triton X-100 and 100 IU/ml heparin. The samples were centrifuged (3500 rpm, 10 minutes, 4° C) and the supernatant collected. The sediments were resuspended in 10 ml buffer and centrifuged again. This procedure was repeated 3 times. The concentration of hemoglobin in the supernatant samples was measured by absorbance at 415 nm. The amount of blood in the subarachnoid

space was calculated from the degree of hemoglobin dilution in the supernatant samples in relation to the arterial samples.

Assessment of CSF volume (Paper II)

To measure the total CSF volume, we injected 10 µl of ^{125}I albumin, with a known radioactivity, into the cisterna magna in normal rats anesthetized with Hypnorm®/Dormicum®. Two hours later, a 10 µl sample was withdrawn from the cisterna magna and the radioactivity measured. We calculated the total CSF volume from the degree of dilution.

Histochemistry (Papers I, II, IV-VI)

The surviving animals were anesthetized with fentanyl and fluanisone (Hypnorm®, Janssen Pharmaceutica, Beerse, Belgium; 0.1 and 5 mg/kg, intraperitonealy) and midazolam (Dormicum®, Roche AB, Stockholm, Sweden; 1 mg/kg, intraperitonealy) and sacrificed by decapitation at 3 hours, 5 hours, 2 days or 7 days after SAH. The brains were removed, chilled in isopenthane and stored at –130 °C. Coronal sections (14 µm) were made at the chiasmal level (between –0.3 and +1.1 mm in relation to the bregma) and the hippocampal level (between 3.3 and 3.8 mm behind the bregma).

Hematoxylin-eosin and cresyl violet staining (Papers I, II and VI)

To study cell death, we stained sections with hematoxylin-eosin (Paper II) or cresyl violet (Papers I and VI). They were dried at room temperature and fixed in 4% paraformaldehyde for 10 minutes, and stained with hemtaoxylin (Hematoxylin III according to Gill, Merck, Dernstadt, Germany) for 5 seconds, following by washing and staining with eosin (Certistain®, eosin for microscopy, Merck, Dernstadt, Germany) for 5 seconds and dipped in ethanol 70%-HCl (99:1). In other cases, they were incubated with 0.5% cresyl violet (Certistain®, cresyl violet for microscopy, Merck, Dernstadt, Germany) for 30 minutes. After washing, the sections were dehydrated in increasing concentrations of ethanol and mounted with DPX.

In sections stained with cresyl violet, we estimated neuronal death in the hippocampal formation (Paper VI): the

neuronal layers in CA1, CA3 and dentate gyrus were delineated and the grey values were measured with NIH Image software. Specific values were obtained by subtracting the background.

TUNEL staining (Papers IV-VI)

To assess DNA fragmentation, sections were processed for TdT-mediated dUTP nick end labeling (TUNEL), using an *in situ* cell death detection kit (Roche Diagnostics GmbH, Mannheim, Germany). In brief, the sections were dried, rehydrated in PBS and fixed in paraformaldehyde at room temperature for 10 minutes (with the exception of sections co-stained for active caspase 3 where acetone at –20 °C was used for fixation). After permiabilizing for 5 minutes with ethanol 95%-acetic acid (2:1) at –20 °C, they were incubated with a mixture of TdT and fluorescein-conjugated dUTP for 1 hour at 37 °C. The reaction was stopped by PBS washing. Glycerol/PBS (1:1) was used for mounting. As a negative control, the enzyme was omitted in the incubation mixture. The fluorescein labeling was assessed by fluorescence microscopy.

In Paper IV, we did a semi-quantification of the degree of TUNEL staining,- i. e., the relative density of TUNEL-positive cells was compared among the animals and rated as - = less than 10 TUNEL-positive cells, + = between 10 and 500 TUNEL-positive cells, and ++ = more than 500 TUNEL-positive cells.

Fluoro-Jade staining (Paper VI and unpublished results)

Fluoro-Jade staining was used to visualize the degenerating neurons. We employed a slight modification of the original method (Schmued and Hopkins 2000). The sections were dried, rehydrated and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Then they were incubated with 0.00002% of Fluoro-Jade (Histochem, Jefferson, AR, USA) in 0.1% acetic acid during shaking. After washing, they were dried on a 50° hot plate for 3 minutes, dipped in xylene and coverslipped with DPX. The Fluoro-Jade labeling was assessed with fluorescence microscopy.

Immunohistochemistry staining (Papers IV-V)

We determined the presence of specific proteins by staining their immunoreactivity in brain sections after perforation and prechiasmatic SAH.

Single-labeling (Paper V): The avidin-biotin complex (ABC) technique was used to detect inflammatory markers. In brief, the sections were dried for 1 hour at room temperature and rehydrated in PBS. They were fixed during 10 minutes in paraformaldehyde at room temperature or acetone at –20 °C. Then they were submerged in 95% ethanol for 5 minutes at –20 °C followed by quenching with 0.3% H_2O_2 for 30 minutes at room temperature. To prevent nonspecific conjugate binding, the sections were incubated with 1% bovine serum albumin, 0.1% sodium azide, 0.3% Triton X-100 and avidin block solution (Vector Laboratories Inc, Burlingame, CA, USA). The primary antibodies (Table 1) diluted in 1% bovine serum albumin, 0.1% sodium azide, 0.3% Triton X-100 and biotin block solution (Vector Laboratories Inc, Burlingame, CA, USA), were applied overnight at 4 °C. Then the sections were incubated for 1 hour with the corresponding biotinylated secondary antibodies, followed by incubation with ABC (Vectastain Elite ABC Kit, Vector Laboratories Inc, Burlingame, CA, USA) for 1 hour. The immunoreactivity was visualized by developing with di-aminobenzidine (DAB Substrate Kit for Peroxidase, Vector Laboratories Inc, Burlingame, CA, USA) for 5 minutes. The sections were counterstained with hematoxyline (Hematoxylin III according to Gill) for 5 seconds, dehydrated in increasing concentrations of ethanol and mounted with DPX. Omission of the primary antibodies was used in negative controls.

For each inflammation-related marker, the degree of immunoreactivity was rated from 0 = no upregulation, 1 = mild upregulation, 2 = moderate upregulation or 3 = severe upregulation. The degree of inflammation in each animal was calculated as the sum of the score for each inflammatory marker.

Double-labeling with TUNEL (Paper IV): The cell type or the presence of apoptotic-related proteins in cells with DNA fragmentation was determined with TUNEL and indirect immunofluorescence labeling. The sections were first processed for TUNEL, as described above, followed by incubation with PBS containing 10% normal goat serum, 1% bovine serum albumin, 0.1% sodium azide and 0.3% Triton X-100 to prevent nonspecific

conjugate binding. The sections were then exposed overnight at 4 °C to the primary antibodies. The specificity, dilution and source of the antibodies used are listed in Table 1. After washing, they were incubated with indocarbocyanine (Cy-3)-conjugated secondary antibodies to the corresponding species for 1 hour. To visualize all

Table 1- Primary antibodies used in these studies

Antibody	Specificity	Type	Host	Dilution	Source
Inflammatory markers					
ICAM-1	Intercellular adhesion molecule 1	Mono	Mouse	1:1000	Genzyme
OX6	I-A antigen (MHC-II)	Mono	Mouse	1:2000	Serotec
ED1	Monocytes/macrophages and reactive microglia	Mono	Mouse	1:4000	Serotec
TNF-α	Tumor necrosis factor α	Poly	Rabbit	1:200	Serotec
IL-1β	Interleukin 1-β	Poly	Goat	1:100	R&D
IL-6	Interleukin 6	Poly	Goat	1:50	R&D
INOS	Iinducible nitric oxide synthase	Poly	Rabbit	1:800	Transd.
Nestin	Nestin	Poly	Rabbit	1:1000	U. Lendhal
Cellular markers					
NeuN	Neuronal nuclei protein	Mono	Mouse	1:500	Chemicon
GFAP	Glial fibrillary acidic protein (astrocytes)	Poly	Rabbit	1:1000	Dako
Transferrin	Transferrin (oligodendroglia)	Poly	Rabbit	1:1000	Nordic
ED1	Monocytes/macrophages and reactive microglia	Mono	Mouse	1:4000	Serotec
Apoptotic markers					
Bcl-2	Bcl-2	Poly	Rabbit	1:200	B-D
Bax	Bax	Poly	Rabbit	1:200	B-D
Active caspase 3	Active caspase 3	Poly	Rabbit	1:500	B-D

B-D, Becton Dekinson, San José, CA, USA; Chemicon, Chemicon International, Inc, Temecula, CA, USA; Dako, Dakopatts, Glostrup, Denmark; Genzyme, Genzyme Diagnostics, Cambridge, MA, USA; R&D, R&D Systems Europe Ltd., Abingdon, England; Nordic, Nordic Immunological Laboratories, Tilburg, The Netherlands; Serotec, Serotec Ltd, Oxford, England; Transd., Transduction Laboratories, Lexington, KY, USA; U. Lendhal, kindly provided by Professor Urban Lendhal, Institution for Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden. Poly, polyclonal; Mono, monoclonal

nuclei, Hoescht 33342 (Molecular Probes, Oregon, USA) was added to the last washing before coverslipped with glycerol-PBS (1:1). Omission of the primary antibodies was used in negative controls. Fluorescence microscopy was employed to visualize the fluorescein and Cy3 labeling.

We calculated the percentage of cells with DNA fragmentation that co-stained with the antibodies in each of the areas showing DNA fragmentation by counting the number of TUNEL-positive cells and double-stained cells in 3 fields at a magnification of 20 x for cellular markers, and in 4 fields at a magnification of 40 x for apoptotic markers The percentage of double-stained cells was calculated per area and animal.

***In situ* hybridization studies (Paper VI)**

The mRNA levels of the NMDAr subunits NR1, NR2A-D and NR3A-B were determined in the hippocampus 3-5 hours after perforation SAH by *in situ* hybridization. The following antisense probes (Medprobe, Oslo, Norway) were used:

NR1: 5'- CAGTGCATCT TCCTCCTCCT CCTCACTGTT CACCTTGAAT CGGCC -3'

NR2A: 5'- AGGCCCGTGG GGAGCTTTCC CTTTGGCTAA GTTTCTGTTG TATCC -3'

NR2B: 5'- AATTGCTTTG CCGATGGTGA AAGATGGGCC TCCTGGCTCT CTGCC -3'

NR2C: 5'- CTGACAGGGC TGAAGTACTC GAACATGAAG ACAGTGATGG CAACC -3'

NR2D: 5'- CGTGGCCAGG CTTCGGTTAT AGCCCACAGG ACTGAGGTAC TC -3'

NR3A: 5'- TGACTCAGAT TGGAAGTATT CCATACCATG AGCTGCTGGG GTCCC -3'

NR3B: 5'- AGGTTGCTCC TCCTTGGGGC CGCTGCACTC CTGCTCTGCC CTCTC -3'

These probes label all known splice variants of the respective rat gene, except for the NR3B probe which corresponds to the mouse gene.

In situ hybridization was done as previously described (Andersson *et al.* 2001). The oligonuclotides were labeled at the 3'-end with α[^{35}S]dATP (NEN), using terminal deoxyribonucleotidyl transferase (Amersham-Pharmacia Biotech, Uppsala, Sweden), purified and their radioactivity content determined. The sections were dried and hybridized overnight at 42 °C. After washing and dehydration, they were exposed to β-max hyperfilm (Amersham-Pharmacia Biotech) or Kodak BioMax MR for 1-2 weeks, after which the film was developed and fixed in Kodak LX24 and AL4.
The autoradiographic films were analyzed using the NIH Image software. The neuronal layers in CA1, CA3 and dentate gyrus were delineated and the grey values were measured. We determined the specific values by subtracting the background.

Statistical analysis (Papers I-VI)

The data were expressed as mean ± s.e.m.. For variables that were measured repeatedly, repeated-measures analysis of variance followed by the simple main effects test was used for between-group comparisons. For variables measured at one time with a normal distribution, one-way analysis of variance followed by the simple main effects test or LSD test were used to compare more than 2 groups, while for variables without a normal distribution, we used the Kruskal-Wallis test followed by the Dunn test. When 2 groups were compared, the t-test or Wilcoxon test were used for variables that were normally and not normally distributed, respectively, and when we compared the data from the same group of animals, the paired t-test or Wilcoxon's paired test was used. Correlations were assessed by regression analysis for normally distributed variables or by Spearman rank order correlation test for variables that were not normally distributed. Differences with $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

The prechiasmatic SAH model (Papers I-V)

As part of this thesis, a new experimental model of SAH was developed that induces SAH in rats by injecting blood into the prechiasmatic cistern (Paper I). Blood was deposited throughout the basal cisterns of the brain, and, to a lesser extent, over the cerebral hemispheres and cerebellum, thereby resembling the commonest findings in spontaneous clinical SAH that is the rupture of aneurysms in the anterior circulation (Kassell *et al.* 1990; Velthuis *et al.* 1998). The rate of the injection of blood was controlled by maintaining the ICP at MABP levels, simulating bleeding from an artery. It also resembled clinical SAH because it induced changes in CBF, as shown by LDF and aCBF measurements (Papers II and III). As in SAH patients (Carpenter *et al.* 1991; Grubb *et al.* 1977; Hayashi *et al.* 2000; Jakobsen *et al.* 1991; Kawamura *et al.* 1992; Martin *et al.* 1984; Voldby *et al.* 1985b), the prechiasmatic SAH model reduced metabolism in the brain (decrease in $CMRO_2$) and caused inflammation (increase in immunoreactivity to various inflammatory markers) (Papers III and V). Routine histological, TUNEL and Fluoro-Jade stains indicated that, as expected after SAH, it also induced cell death in the brain (Papers II, IV and unpublished results). For research purposes it has several advantages: the amount of blood deposited in the subarachnoid space was very reproducible; after the injection of 200 µl of blood, 180-190 µl were usually recovered (Paper I). Mortality rate after such an amount was acceptable,- i. e. about 25% within 7 days; the injection of 300 µl of blood increased the number of deaths to 100% (Paper I). Two hundred µl of blood injected into the prechiasmatic cistern (corresponding to about 50% of the total CSF volume) also induced a significant reduction in CBF and pathological findings (Paper II). It therefore seems to be an appropriate amount for studies of the pathophysiology of SAH. Finally, the model is easy to perform and saline-injected animals are adequate controls. A recent report from an independent laboratory showed that the model was useful to investigate receptor alterations in the cerebral arteries after SAH (Hansen-Schwartz *et al.* 2003), supporting the view that the prechiasmatic model is valuable for studies of the cerebral changes after SAH. It has the disadvantage of not including the

damage to the arterial wall. However, at present, there are no indications that this is an important factor in the etiology of the disease.

In Papers 2 and 3, we compared the two commonest used models with the prechiasmatic SAH model. A summary of the findings is shown in Table 2. The perforation SAH model simulated the mechanism of aneurysmal rupture, but the subarachnoid blood volume varied greatly. In agreement with other reports, about 50% of the animals died within 7 days. The incidence of histological damage was low in the survivors. After cisterna magna SAH, the amount of blood in the cerebral subarachnoid space was surprisingly lower than the amount injected and varied considerably. The escape of blood into the spinal canal probably accounted for these findings. Another disadvantage of this model is that the distribution of blood resembled only that in the patients with hemorrhages into the posterior fossa, a minority of the SAH cases. Moreover, the CBF normalized within 15 minutes, no changes in metabolism were detected and the incidence of pathological findings was low.

Table 2- Comparison of some pathophysiological and outcome-related parameters after perforation, prechiasmatic and cisterna magna SAH.

	Perforation SAH	**Prechiasmatic SAH**	**Cisterna magna SAH**
Amount of subarachnoid blood	40-480 µl	190 µl in 85% of the animals	40-190 µl
Reduction in acute CBF	Severe	Moderate	Mild
Changes in metabolism	Reduction in $CMRO_2$, anaerobic metabolism	Reduction in $CMRO_2$	No changes
Survival of animals with pathology	11-33%	37-62%	28%
Mortality rate	44%	25%	0%

CBF, cerebral blood flow; $CMRO_2$, cerebral metabolic rate of oxygen; SAH, subarachnoid hemorrhage

If one considers the distribution and amount of blood in the subarachnoid space, CBF and metabolic changes, pathological consequences and mortality rate, the perforation SAH model

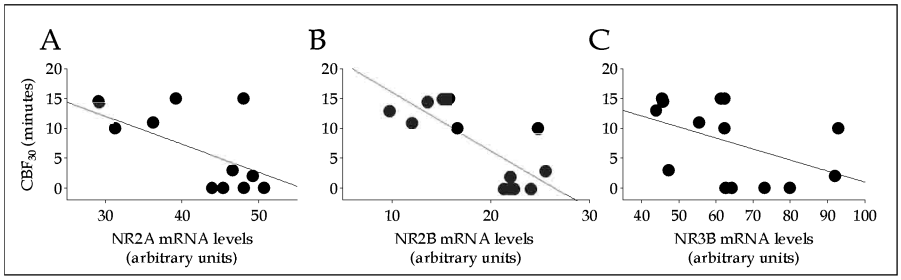

Figure 5- Effects of the acute reduction in CBF on the expression of NMDAr subunits. The correlations are shown between the duration of LDF below 30% of the baseline (CBF_{30}) within 15 minutes of SAH and the mRNA levels of the NR2A (A), NR2B (B) and NR3B (C) subunits in the hippocampal CA1 region at 3-5 hours after SAH. All correlations were significant ($P < 0.05$, Spearman rank order correlation test).

duration of CBF below 30% of baseline was also significantly correlated with the degree of cerebral inflammation (Paper V) and changes in the mRNA levels of NMDAr subunits (Figure 5; paper VI). It is noteworthy that the animals which died after SAH had longer durations of CBF values below 30% of baseline (Figure 6). Although the links between CBF and other signs of brain damage and outcome may merely indicate an association rather than a causal effect, it is more likely that the immediate reduction in CBF triggers a cascade of biochemical and molecular events that cause acute injury, but also secondary damage. However, in some cases, changes in metabolism, inflammation and cell damage occurred in the absence of substantial acute CBF reductions and consequently, other harmful mechanisms may also be partly responsible for the final injury after SAH.

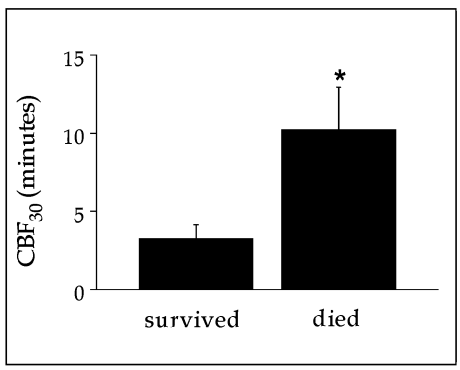

Figure 6- Acute reductions in CBF and mortality rate. The figure shows the duration of LDF below 30% of the baseline (CBF_{30}) within 60 minutes of SAH in the animals that died (n = 27) and survived (n = 39) after perforation and prechiasmatic SAH. Values are mean ± s.e.m. The animals that did not survived had significantly longer CBF_{30} than those that did (* = $P < 0.05$, Mann-Whitney U-test).

The mechanisms whereby SAH leads to acute reductions in CBF remain to be determined. It has been suggested that the increase in ICP during the bleeding causes a reduction in CPP, and consequently, in CBF (Grote and Hassler 1988; Nornes 1973; 1978; Weaver and Fisher 1994). However, experimental evidence indicates that the formal can not fully explain the changes in CBF (Bederson *et al.* 1995; Dorsch *et al.* 1989; Jackowski *et al.* 1990; Umansky *et al.* 1983). Our data also indicated that an increase in ICP is not the main factor leading to a reduction in CBF (Papers II and III): animals injected with saline and blood had similar increases in ICP and decreases in CPP, but only the SAH animals showed a long-lasting reduction in CBF. CPP values approached normal levels after SAH, several minutes before CBF did. Moreover, ICP and CPP showed a weak correlation with LDF values only at their maximal levels (immediately after SAH) but not later. Finally, and most importantly, some of the animals that were given injections with blood into the prechiasmatic cistern showed only a moderate increase in ICP as a result of CSF leakage, but had LDF patterns that resembled those in animals with a marked increase in ICP. Not only did our data not support the view that the early ICP and CPP changes play a main role causing the acute hypoperfusion, but they also showed that they could not have significantly contributed to the outcome: acute ICP and CPP chnages were not related to cell death, inflammation or the mortality rate after SAH.

A more likely cause of the acute hypoperfusion is vasoconstriction of arteries and arterioles. Acute vasospasm of the main arteries has been seen angiographically and histologically in rats, and some findings indicate that the small vessels are prone to constriction in the acute phase of experimental SAH (Bederson *et al.* 1998; Delgado *et al.* 1985; Park *et al.* 2001). Our studies did not directly address this issue, but the global reduction in CBF observed was compatible with widespread diffuse vasoconstriction as the factor responsible for hypoperfusion (Paper III).

A reduction in metabolism may also reduce CBF (Magistretti 1997). Its possible contribution to the acute hypoperfusion after SAH is discussed below.

Progressive cell death after other acute brain injuries- e. g., ischemia and trauma- has been carefully documented (Du *et al.* 1996; Kirino 1982; Pulsinelli *et al.* 1982; Raghupathi *et al.* 2000), but the data as regards SAH are restricted to the observation of cells with DNA fragmentation in the dentate gyrus of patients dying a few days after SAH (Nau *et al.* 2002). Experimental studies evaluating DNA fragmentation after the injection of lysed blood into the subarachnoid space shown the presence of dying cells at 24 hours (Matz *et al.* 1996b), but no later times were assessed. An indirect indication of cell injury during the days following experimental SAH or injection of blood products into the subarachnoid space is the induction of stress proteins in the brain, as reported by several authors (Harada *et al.* 1997; Klinge *et al.* 1999; Matz *et al.* 1996a; Matz *et al.* 1996b; Matz *et al.* 1996c; Turner *et al.* 1998; Turner *et al.* 1999). Our data provide evidence that cells continue to die in various regions of the brain for at least 7 days after SAH.

We used double labelling with TUNEL and specific cellular markers to assess which cell types were damaged during the days following SAH. The results indicated that most of the dying cells at 2 and 7 days were neurons (<80%), but we were surprised to find a marked reduction in the immunoreactivity to astrocytic and oligodendroglial antigens. This suggested that these cell types probably been severely damaged or had died before, which does not accord with the current view that neurons are particularly vulnerable to many injuries, including ischemia (Chen *et al.* 1997; Li *et al.* 1995; Li *et al.* 1998; Pulsinelli 1985). However, several recent publications have shown situations where glial cells are more susceptible than neurons to ischemia (Garcia *et al.* 1993; Lukaszevicz *et al.* 2002; Pantoni *et al.* 1996; Petito *et al.* 1998).

We then analyzed the occurrence of apoptosis in the cell death process after SAH. More than 90% of the cells with DNA fragmentation at 7 days after SAH had morphological characteristics associated with apoptosis: condensed chromatin and/or apoptotic bodies. Moreover, molecular changes consistent with the turning on of the apoptotic machinery in degenerating cells were seen, as indicated by the colocalization of TUNEL with increases in the levels of Bax and active caspase 3. The increase in the Bax levels suggested the probable participation of the mitochondrial pathway in the ongoing apoptotic process. Our

findings are in agreement with the few previous studies in this field which have shown cells with apoptotic features in the dentate gyrus of patients dying of SAH (Nau *et al.* 2002) and TUNEL-positive cells with apoptotic morphology and increases in cytosolic cytochrome *c* levels, and the presence of electrophoretic DNA laddering 24 hours after the injection of lysed blood into the subarachnoid space (Matz *et al.* 1996b; Matz *et al.* 2000; Matz *et al.* 2001). Our data and previous reports indicate that apoptosis may play a main role in delayed brain damage after SAH.

We have also studied the expression of the antiapoptotic protein Bcl-2. Its levels were upregulated in areas where DNA fragmentation was seen, which would suggest that an attempt to counteract the death signals took place. The effect of Bcl-2 protection, however, seemed limited since only a few cells showed increase in Bcl-2 expression and colocalization of TUNEL with upregulation of Bcl-2 was also noted.

Inflammation after SAH: a likely contributor to brain injury (Paper V)

A battery of antibodies against various markers of an inflammatory process was used to study the occurrence and distribution of the inflammatory reaction after SAH. We found ICAM-1 upregulation in several areas of the brain at 2 and 7 days after SAH, in agreement with other reports on ICAM-1 upregulation in large vessels, which would suggest the occurrence of a vascular component of the inflammation process (Aihara *et al.* 2001; Handa *et al.* 1995). The inflammatory reaction, however, was not restricted to a vascular phase, and parenchymal inflammation was also seen by the presence of cells expressing MHC II molecules (a marker for antigen presenting cells), ED1 antigen (a marker for activated microglia and monocytes/macrophages) and nestin (a marker for activated astrocytes), upregulation of the enzyme iNOS and the induction of the potent inflammatory mediator TNF-α. The severity of the inflammation was similar at 2 and 7 days, but larger areas tended to be affected at 7 days.

Our data did not support a large production of IL-1β and IL-6 in the brain after SAH. Clinical studies have consistently shown an increased in the concentration of IL-6 in the CSF after SAH (Fassbender *et al.* 2001; Gaetani *et al.* 1998; Kikuchi *et al.* 1995; Kwon and Jeon 2001; Mathiesen *et al.* 1993; Osuka *et al.* 1998a;

Takizawa *et al.* 2001). As regards IL-1β, the results have been contradictory, with some authors reporting high values in the CSF of patients only within 24 hours after SAH, and others finding higher or only slightly elevated concentrations during the following days after the bleeding or even undetectable values (Fassbender *et al.* 2001; Kwon and Jeon 2001; Osuka *et al.* 1998a; Takizawa *et al.* 2001). Thus more studies are needed to determine whether species differences, cytokine sources other than the brain, or differences in the severity of SAH account for the discrepancies between our observations and those in patients, especially concerning IL-6.

The degree of the inflammatory reaction varied from one animal to another and in several animals, we found no signs of inflammation. However, the level of immune activation seen in about 1/3 of the animals could certainly have had an adverse effect on the tissue. It is note worthy that animals with DNA fragmentation also had inflammation, and a marked overlapping was observed between the distribution of inflammatory markers and cell damage in individual animals, which would support the hypothesis that the inflammatory reaction triggered by SAH could contribute significantly to the final brain damage in many cases. However, the opposite may have occurred, and necrotic cells could have triggered the inflammatory cascade. However, since more than 90% of the cells dying at 7 days after SAH had apoptotic features (Paper V), it seems unlikely that this factor plays an important role in the genesis of inflammation after SAH.

As mentioned above, there was a good correlation between the degree of inflammation and the acute reduction in CBF: animals with a marked CBF reduction immediately after SAH showed the strongest inflammatory reactions. Much data support the view that ischemia triggers inflammation in the brain (Barone and Feuerstein 1999; del Zoppo *et al.* 2000) and therefore seems likely that the acute CBF reduction after SAH was partly responsible for the inflammatory response seen during the following days. The location of blood also appeared to be of importance in the inflammation after SAH, because areas in the proximity of the subarachnoid blood clots were commonly affected. The extravasated blood may cause a direct inflammatory reaction via various mechanisms. For example, the presence of subarachnoid blood causes a dramatic increase in subarachnoid macrophages that remove the blood elements (Jackowski *et al.* 1990). Moreover, the exposure of femoral arteries to blood clots

induces ICAM-1 upregulation in the arterial wall (Sills *et al.* 1997). The platelets of the extravasated blood may become activated by collagen in the extracellular matrix and generate inflammatory mediators. Finally, many of the reactions triggered by components of the blood and elements of its degradation may also disturb the blood brain barrier (Petty and Lo 2002).

Changes in NMDA receptor subunit mRNA expression- are they involved in cell death? (Paper VI)

The acute effects of SAH on the expression of the NMDAr subunits was studied by *in situ* hybridization in the hippocampus, an area that had previously been shown to be damaged after experimental SAH (Klinge *et al.* 1999; Matz *et al.* 1996c; Veelken *et al.* 1995) and is particularly sensitive to ischemia (Kirino 1982; Pulsinelli *et al.* 1982). Our findings indicated that SAH caused a moderate, but significant, reduction in the mRNA levels of the NR2A, NR2B and NR3B subunits at 3-5 hours after SAH when the acute CBF had fallen below 30% of baseline for more than 9 minutes, which would suggest ischemia as a probably significant cause. On the other hand, no changes were seen in the NR1 subunit mRNA levels. In accordance with previous studies showing that NR2C, NR2D and NR3A subunits are expressed in very low levels in the adult hippocampus (Ishii *et al.* 1993; Wong *et al.* 2002), we found their mRNA concentrations below the detection limits.

No other studies have investigated the effects of SAH on the NMDAr and the available data from studies on cerebral ischemia are contradictory. In agreement with our results, transient global cerebral ischemia in rodents has been reported to have no effect on NR1 subunit expression (Gass *et al.* 1993; Pellegrini-Giampietro *et al.* 1994; Sugimoto *et al.* 1994; Takagi *et al.* 1999) and to reduce NR2A and NR2B subunit mRNA levels in the hippocampus within 24 hours (Hsu *et al.* 1998; Zhang *et al.* 1997). However, decreases and large increases in NR1 mRNA hippocampal levels, and no changes or increases in NR2A and NR2B mRNA hippocampal levels have been also described (Friedman *et al.* 2001; Heurteaux *et al.* 1994; Kang *et al.* 2001; Won *et al.* 2001). To our knowledge, there are no studies on the effects of acute brain injury on the NR3B subunit that we can compare with our findings.

The functional implications of the changes observed remain to be determined. If the changes in the mRNA level were reflected by the protein levels, a reduction in the NR2A-B and NR3B subunits took place immediately after SAH. NR2A-B subunits contain the glutamate binding site needed for activation of the receptor. Therefore, a reduction in their levels is likely to reduce receptor activation and Ca^{++} influx (Laube *et al.* 1997). On the other hand, incorporation of the NR3B subunits in heterodimers has been reported to reduce the receptor conductance and hence, a reduction in the level of this subunit would be expected to increase permeability to Ca^{++} (Chatterton *et al.* 2002; Matsuda *et al.* 2002; Nishi *et al.* 2001). Functional studies in rodents on the hippocampal NMDAr shortly after global ischemia have shown increases and decreases in the receptor activity (Crepel *et al.* 1998; Hori and Carpenter 1994; Hori *et al.* 1991; Hsu *et al.* 1998; Zhang *et al.* 1997). The functional significances of changes in the subunit expression are still not understood. However, the subunit composition of the NMDAr may be a novel variable that affects the SAH pathophysiology.

When SAH caused an acute reduction in CBF 30% below the baseline for more than 9 minutes, cell injury, assessed by cresyl violet, Fluoro-Jade and TUNEL staining, was not seen at 3-5 hours, but selective cell damage in CA1 and CA3 was detected at 2-7 days after SAH. The fact that the mRNA subunit changes preceded cell death raises the question whether they were involved in the degeneration. Although we found a correlation in CA1 between acute mRNA subunit changes on the one hand, and delayed cell death on the other, not such a correlation was shown in CA3 or in the dentate gyrus. In CA3, little or no changes in the mRNA subunit levels were seen despite the occurrence of delayed cell death. In the dentate gyrus, there were changes in the mRNA subunits levels, but no delayed cell death. Thus, our data did not support a direct causal relationship between changes in NMDAr subunit mRNA levels at this stage and cellular degeneration. Although the changes in NMDAr may have played a role in cell death, other factors must have been involved in the degeneration process.

Global versus local mechanisms in the SAH injury (Paper II-VI)

It has been suggested that the brain injury after SAH is determined by local harmful causes, such as the proximity to the blood clot or the territorial distribution of arteries affected by vasospasm. On the other hand, global phenomena, like neuronal, metabolic or humoral mechanisms, have been also proposed as the main causes. Both views are supported by a number of studies. Global and focal hemodynamic and metabolic changes have been described in SAH patients and after experimental SAH (Brouwers *et al.* 1992; Carpenter *et al.* 1991; d'Avella *et al.* 1996; Delgado *et al.* 1986a; Delgado-Zygmunt *et al.* 1993; Gewirtz *et al.* 1999; Glenn *et al.* 2002; Grubb *et al.* 1977; Heilbrun *et al.* 1972; Ishii 1979; Martin *et al.* 1984; Mickey *et al.* 1984; Yonas *et al.* 1989). Moreover, SAH or injections of blood products in the subarachnoid space, induce stress proteins and cause cell death throughout the brain, but particularly near the injection site (Harada *et al.* 1997; Matz *et al.* 1996a; Matz *et al.* 1996b; Matz *et al.* 2000; Matz *et al.* 1996c; Turner *et al.* 1998; Turner *et al.* 1999). Consistent with a role for global harmful effects, neuronal systems are implicated in different aspects of the pathophysiology of SAH (Delgado *et al.* 1986b; Shiokawa *et al.* 1992b; Shiokawa *et al.* 1993; Svendgaard *et al.* 1992).

Our data have shown evidence that both local and global mechanisms participate in inducing damage after SAH. It seems that the injury was greater in areas of the brain where local factors were superimposed on global mechanisms. The evidence for local effects included the presence of more signs of inflammation, DNA fragmentation and Fluoro-Jade staining after SAH in brain sections close to the injection or the site of perforation than those in remote regions. Moreover, after perforation SAH, the right hemisphere and after prechiasmatic SAH, the interhemispheric region were especially susceptible to inflammation and signs of cellular damage, consistent with effects related to the proximity of blood (Papers IV and V). In some animals, the affected areas resembled the territorial distribution of arteries that would have been expected to be especially exposed to blood clots. On the other hand, aCBF measurements showed that the reduction in CBF affected the whole brain (Paper III). Therefore, the changes in CBF immediately after SAH seemed to be mainly governed by global mechanisms. Finally, markers of inflammation, DNA fragmentation and changes in

NMDAr subunit mRNA levels were detected in areas far from the deposition of blood (Papers IV, V and VI).

GENERAL DISCUSSION AND MAIN CONCLUSIONS

The devastating outcome of SAH has prompted researchers to try to identify the mechanisms involved and to assess various types of therapy. The effects of rebleedings have been reduced considerably by improvements in microsurgery and endovascular occlusion. However, the initial hemorrhage has already triggered to a series of cerebral biochemical reactions that result in a high morbidity and mortality. Most studies have aimed at identifing one major factor that determines the outcome. Delayed vasospasm has been seen to play a main role in local reductions of CBF and subsequent ischemic infarction (Fisher 1975; Fisher *et al.* 1977). On the other hand, some investigators have described the occurrence of other equally destructive processes, including metabolic derangements, vascular inflammation, hydrocephalus and electrolyte disturbances (Carpenter *et al.* 1991; Claassen *et al.* 2002; Grubb *et al.* 1977; Hasan *et al.* 1990; Lin *et al.* 1999; Ryba *et al.* 1992). Our data add to the body of knowledge on the mechanisms underlying the pathophysiology of SAH. Acute events, specially the reduction in CBF, seemed to be a main determinant of the final outcome. Evidence of delayed cell death by apoptosis and the occurrence of cerebral inflammation improve understanding of the effects of SAH that need to be prevented. The changes in the expression of the NMDAr subunits add a novel variable that may affect neuronal death after SAH.

The use of SAH models also permits general speculations. It was at first surprising that the outcomes of experimental SAH were as varied as those of clinical SAH. After prechiasmatic SAH, a well-controlled experimental condition, about the same number of animals survived with no detectable damage, survived, but had cerebral damage, or died. The distribution of the outcomes did not accord with the existence of a single main cause, but rather with multiple causes in which their interrelationships and dynamics had to be taken into account to explain the ultimate damage (Figure 8). Our evaluation of the SAH models showed that they induced changes that bore a closer resemblance to clinical SAH than might have been expected, which would suggest that they are of considerable value in studying the intricacy of the complex processes involved in the pathophysiology of SAH and assessing therapy for patients with this condition.

and thus the inflammatory reaction may have been a significant cause of the brain damage after SAH.

- Severe SAH caused an acute reduction in the expression of genes for the NMDAr subunits NR2A, NR2B and NR3B in the hippocampal formation. Although these changes precede cell death, no causal relationship was found between the mRNA reductions and subsequent cell death in all the hippocampal neuronal layers.

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