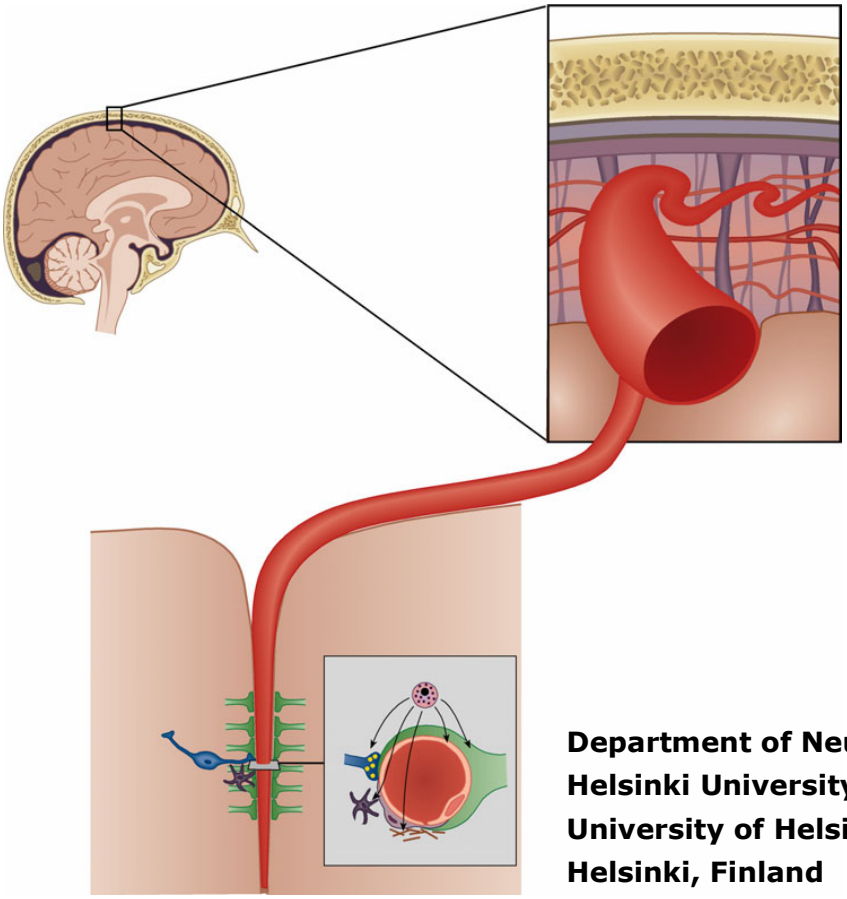
THE ROLE OF MAST CELLS IN ISCHEMIC AND HEMORRHAGIC BRAIN INJURY

Daniel Štrbian



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ACADEMIC DISSERTATION

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To Niki, Karla, David, my parents, and my brother

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals:

I Strbian D, Karjalainen-Lindsberg M-L, Tatlisumak T, Lindsberg PJ. Cerebral mast cells regulate early ischemic brain swelling and neutrophil accumulation. J Cereb Blood Flow Metab 2006; 26:605-612.

II Strbian D, Karjalainen-Lindsberg M-L, Kovanen PT, Tatlisumak T, Lindsberg PJ. Mast cell stabilization reduces hemorrhage formation and mortality after administration of thrombolytics in experimental ischemic stroke. Circulation 2007; 116:411-418.

III Strbian D, Tatlisumak T, Ramadan UA, Lindsberg PJ. Mast cell blocking reduces brain edema and hematoma volume and improves outcome after experimental intracerebral hemorrhage. J Cereb Blood Flow Metab 2007; 27:795-802.

In addition, some unpublished data are presented.

ABBREVIATIONS

ACA	anterior cerebral artery
ADC	apparent diffusion coefficient
ATP	adenosine triphosphate
BBB	blood-brain barrier
CBF	cerebral blood flow
CBV	cerebral blood volume
CCA	common carotid artery
$CMRO_2$	cerebral metabolic rate of oxygen
CPP	cerebral perfusion pressure
CSF	cerebrospinal fluid
CT	computed tomography
DWI	diffusion-weighted imaging
EB	Evans blue
EC	endothelial cell
ECA	external carotid artery
ECM	extracellular matrix
ET	endothelin
HF	hemorrhage formation
HS	hypertonic saline
ICA	internal carotid artery
ICAM	intercellular adhesion molecule
ICH	intracerebral hemorrhage
ICP	intracranial pressure
icv	intracerebroventricular
IL	interleukin
iv	intravenous
LPR	late-phase reaction
LT	leukotrienes
MABP	mean arterial blood pressure
MC	mast cell
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
NGF	nerve growth factor
NO	nitric oxide
OEF	oxygen extraction fraction
PAF	platelet-activating factor
PET	positron emission tomography
PCA	posterior cerebral artery
PG	prostaglandins
PMNL	polymorphonuclear leukocytes
RI	reperfusion injury
ROS	reactive oxygen species
SAH	subarachnoid hemorrhage
SCF	stem-cell factor
TIMP	tissue inhibitor of metalloproteinases
TNF	tumor necrosis factor
TPA	tissue plasminogen activator
TTC	2,3,5-triphenyltetrazolium chloride
T_2/T_2*-WI	T_2/T_2*-weighted magnetic resonance imaging
VEGF	vascular endothelial growth factor
WT	wild-type

ABSTRACT

Stroke, ischemic or hemorrhagic, belongs among the foremost causes of death and disability worldwide. Massive **brain swelling** is the leading cause of death in large hemispheric strokes and is only modestly alleviated by the limited conservative treatments available today. Quite invasive surgical decompressive craniectomy is a promising option in selected patients.

Aimed at fast restoration of circulation, thrombolysis with tissue plasminogen activator (TPA) is the only approved therapy in acute ischemic stroke, but fear of **TPA-mediated hemorrhage** is often a reason for withholding this otherwise beneficial treatment. In addition, recanalization of the occluded artery (spontaneously or with thrombolysis) may cause **reperfusion injury** (depending on severity and duration of the preceding ischemia) by promoting brain edema, hemorrhage, and inflammatory cell infiltration. A dominant event underlying these phenomena seems to be disruption of the **blood-brain barrier** (BBB).

In contrast to ischemic stroke, no widely approved clinical therapy exists for **intracerebral hemorrhage** (ICH), which is associated with poor outcome mainly due to the mass effect of enlarging hematoma and associated brain swelling.
Obviously, novel treatment strategies for brain swelling associated with large hemispheric strokes, TPA-associated hemorrhage, and spontaneous ICH are urgently needed.

Mast cells (MCs) are perivascularly located resident inflammatory cells which contain potent vasoactive, proteolytic, and fibrinolytic substances encapsulated in their cytoplasmic granules. Experiments from our laboratory showed MC density and their state of granulation to be altered early following focal transient cerebral ischemia, and degranulating MCs were associated with perivascular edema and hemorrhage. This led to the hypothesis that MCs might be involved in regulation of BBB permeability, brain swelling, and neutrophil infiltration, as well as in hazardous TPA-mediated cerebral hematomas after stroke. Since the mass effect from increasing edema and hematoma volume are associated with poor outcome in ICH, the efficacy of MC-modulating strategies was tested also in this catastrophic condition.

To examine the above hypothesis, the first question was whether MCs play a role in modulating BBB permeability, brain swelling, and neutrophil infiltration in a rat model of transient middle cerebral artery occlusion (MCAO) (I). Thereafter, in the same model, subsequent studies examined the potential role of MCs in TPA-mediated deleterious effects, and tested the effect of TPA on MCs in vitro (II). Finally, the role of MCs was studied in an autologous blood injection model of ICH (III).

(I) Pharmacological MC stabilization led to significantly reduced ischemic brain swelling (40%) and BBB leakage (50%), whereas pharmacologically potentiated MC degranulation

raised these by 90% and 50%, respectively. Pharmacological MC stabilization also revealed a 40% reduction in neutrophil infiltration. Moreover, genetic MC deficiency was associated with an almost 60% reduction in brain swelling, 50% reduction in BBB leakage, and 50% less neutrophil infiltration, compared with control figures.

(II) Experiments with TPA showed strong TPA-induced MC degranulation in vitro. In vivo experiments with post-ischemic TPA administration demonstrated 70- to 100-fold increases in hemorrhage formation (HF) compared with controls' HF. HF was significantly reduced by pharmacological MC stabilization at 3 (95%), 6 (75%), and 24 hours (95%) of follow-up. Genetic MC deficiency again supported the role of MCs, leading to 90% reduction in HF at 6 and 24 hours. Pharmacological MC stabilization and genetic MC deficiency were also associated with significant reduction in brain swelling and in neutrophil infiltration. Importantly, these effects translated into a significantly better neurological outcome and lower mortality after 24 hours.

(III) Finally, in experiments utilizing the ICH model, pharmacological MC stabilization resulted in significantly less brain swelling, diminished growth in hematoma volume, better neurological scores, and decreased mortality. Pharmacologically potentiated MC degranulation produced the opposite effects. Genetic MC deficiency revealed a beneficial effect similar to that found with pharmacological MC stabilization.

In sum, the role of MCs in these clinically relevant scenarios is supported by a series of experiments performed both in vitro and in vivo. That not only genetic MC deficiency but also drugs targeting MCs could modulate these parameters (translated into better outcome and decreased mortality), suggests a potential therapeutic approach in a number of highly prevalent cerebral insults in which extensive tissue injury is followed by dangerous brain swelling and inflammatory cell infiltration. Furthermore, these experiments could hint at a novel therapy to improve the safety of thrombolytics, and a potential cellular target for those seeking novel forms of treatment for ICH.

1 INTRODUCTION

Worldwide, **ischemic stroke** is one of the leading causes of disability and death. The major cause of death after a large hemispheric stroke is space-occupying **brain swelling**.[1] Brain swelling occurs in about two-thirds of patients within 6 hours of ischemic stroke onset.[2] The two main detrimental effects of swelling are (1) mass effect with compression of normal brain structures (herniation), and (2) decreased blood flow in the ischemic penumbra. Both effects damage the penumbra's metabolism and facilitate its transformation into an infarction. **Blood-brain barrier** (BBB) damage increases tissue water content; the entry of serum proteins into brain tissue magnifies vasogenic edema and further swelling. Thus far, only limited conservative anti-edema treatment strategies exist. Invasive surgical decompressive craniectomy offers some benefit to selected patients, and experiments with hypothermia also seem promising. However, the possibilities to fight massive brain swelling are far from sufficient.

Thrombolysis with tissue plasminogen activator (TPA) is the only approved pharmacological therapy for acute ischemic stroke, aiming to provide early recanalization of an occluded cerebral artery. For every 1000 patients treated with TPA within 3 hours after symptom onset, more than 140 will avoid disability, and the therapeutic window may extend to 4.5 hours.[3] A major issue regarding this treatment is the risk for clinically relevant **parenchymal hemorrhage**, the etiology of which is not yet fully understood. In pooled analysis of placebo-controlled trials of thrombolysis, 6% of stroke patients developed a substantial brain hemorrhage, as compared to 1% in the placebo group.[3] Furthermore, TPA-treated patients with acute myocardial infarction have had a 1.1%,[4] and with pulmonary embolism a 3%[5] risk for cerebral hemorrhage.

Spontaneous recanalization or medical thrombolysis can evoke **reperfusion injury** (RI), which can worsen BBB leakage, leading to extravasation of plasma and of erythrocytes (formation of edema and of hemorrhage) and inflammatory cell infiltration.

Intracerebral hemorrhage (ICH) accounts for 15% of all strokes in Western countries, and the percentage is even higher (20-30%) in Asian and black populations.[6] ICH is associated with high mortality and disability, with only 40 to 50% of the patients surviving through the first year,[7,8] many of them with chronic disability. Poor outcome results from direct tissue damage, especially in deep brain structures such as the basal ganglia, and the mass effect of the growing hematoma. In addition to the mass effect, the hematoma itself induces a number of early secondary changes in the surrounding tissue, including neuronal and glial cell death due to apoptosis[9-11] as well as inflammation and vasogenic edema caused by disruption of the BBB.[12,13] The compressive effect of perihematomal edema may contribute to secondary perihematomal ischemic injury, although its being ischemic is controversial. The mass effect of the growing hematoma and edema leads to displacement and disruption of brain structures and often to increased intracranial pressure (ICP). Currently, no approved effective acute medical treatment exists, and surgical evaluation of ICH was recently found non-beneficial.[14]

Mast cells (MCs) are tissue-based stationary effector cells that form a first-line defense against various challenges. They have been found in a variety of locations in the nervous system in different species, including humans,[15] and appear to be concentrated in relatively few locations, which include the diencephalic parenchyma, particularly the thalamus, cerebral cortex, and the meninges, positioned in close proximity to potential cellular targets of mediator/cytokine action.[16] Their metachromatic granules contain potent preformed vasoactive substances (e.g. histamine, bradykinin, leukotrienes (LT), and serotonin), proteolytic enzymes (e.g. chymase, tryptase, and cathepsin G), anticoagulants (heparin and chondroitin sulfate), and chemotactic factors (e.g. neutrophil and eosinophil chemotactic factor). MCs are well-known to be involved in allergic reactions but participate in various other conditions including inflammatory arthritis, coronary inflammation, interstitial cystitis, irritable bowel syndrome, multiple sclerosis, and migraine. Furthermore, they were found to play a role in angiogenesis, neoplasm formation, wound healing and tissue remodeling, blood clotting, tissue fibrosis, and ischemia-RI in the peripheral tissues.

In the pilot microscopic experiments, MC density and their state of granulation was altered early following focal transient cerebral ischemia and degranulating MCs were frequently found in association with edema and hemorrhage formation. These findings together with characteristic perivascular location of MCs, the enormous potential to store and release potent mediators, and the ability of their proteases to degrade the basal lamina constituents[17,18] led to hypothesis that MCs might be involved in the regulation of BBB permeability, brain swelling, neutrophil infiltration, as well as in the feared TPA-mediated cerebral hemorrhages after stroke. In line with the assumed role in development of edema and hemorrhage, MC blocking was hypothesized to be beneficial in experimental ICH.

2 REVIEW OF THE LITERATURE

2.1 Ischemic stroke

Ischemic stroke originates from an abrupt decrease in the blood supply to a region of the brain, and results in a corresponding loss of neurologic function. The most common life-threatening neurological disease, it is the third most common cause of death in developed countries, and the second most common in the world.[19] It is estimated that one sixth of all human beings will suffer at least one stroke during their lives.[20] Overall, stroke is killing more than 50/100,000 individuals annually in the USA, where its incidence is approximately 250/100,000 per year, costing around $40 billion annually.[21] In Finland, stroke mortality is around 40/100,000 per year, stroke incidence about 270/100,000 per year, and costs approximately 6.1% of the whole health-care budget.[19] Ischemic stroke accounts for 80 to 85% of all cases of stroke.

Stroke is not only the leading cause of death, but also of disability and long-term hospitalization, being second to Alzheimer's disease as a cause of dementia.[21] The earlier, rather nihilistic, view that acute ischemic stroke is untreatable has been replaced with some level of optimism based on the clinical use of thrombolysis and on some studies with neuroprotectants, although experience with clinical trials of the latter still offers no reason for much enthusiasm. However, multidisciplinary hospital treatment approaches, i.e., stroke unit therapy, have also been effective in reducing death and disability after stroke.

Ischemic stroke is most commonly caused by atherosclerosis of extra- and intracranial arteries, embolism of cardiac origin, and small artery disease (lacunar stroke). Furthermore, ischemic stroke can be caused by vasculitides and dissections. Risk factors for stroke include: advanced age, male gender, genetic predisposition, arterial hypertension, ischemic heart disease, hypercholesterolemia, thrombotic disorders (e.g., protein C-, protein S-, AT3-, and APC-deficiencies, lupus anticoagulans, elevated FVIII levels, FV-Leiden and FII-prothrombinogen mutation), diabetes mellitus, and smoking. In addition, excessive alcohol consumption, diet, physical inactivity, and obesity all play a role.

2.1.1 Pathophysiology of cerebral ischemia

2.1.1.1 Cerebral blood flow and ischemic cascade

The brain represents only 2% of body weight but requires 15% of heart minute volume and 20% of the body's oxygen supply. The brain's energy source is glucose, the metabolism of which is 95% aerobic and 5% anaerobic; the latter can, however, be of short duration only. The brain has no stores of energy, and there exists a tight balance between oxygen and glucose supply. Should **cerebral blood flow** (CBF) in any area of

the brain be insufficient or absent, the subsequent loss of these two essential substances for oxidative phosphorylation disrupts the energy balance in brain cells and an **ischemic cascade** will be launched. This chain of spatial and temporal events following CBF reduction and energy failure includes free oxygen radical (e.g., hydrogen peroxide, hydroxyl radicals, and superoxide) formation, elevation of intracellular Ca^{2+} levels, excitotoxicity, and spreading depression, accompanied by ion changes, depolarization of cell membranes, and activation of enzymes with lytic activities, followed by the BBB disruption and inflammation.[22]

Once the supply of energy substrates ceases or significantly decreases, lactate builds up via anaerobic glycolysis, free fatty acids are formed, and pH decreases, all potentially stimulating free radical formation.[23] Energy-requiring calcium pumps fail with falling adenosine triphosphate (ATP) levels, resulting in perturbation of the Na^+/K^+-ATP-ase and Ca^{2+}/H-ATP-ase pumps; the Na^+-Ca^{2+} transporter is reversed. Even if sodium- and calcium-exchange pumps remove some of the Ca^{2+}, three Na^+ ions still enter for every two Ca^{2+} ions removed.

The ionic changes described (elevation of intracellular Na^+, Ca^{2+}, Cl^-, and elevation of extracellular K^+ levels) lead to osmotic accumulation of water molecules inside the cell, called cytotoxic edema. Membrane depolarization and failure triggers the release of neurotoxic glutamate, with further increase in Ca^{2+} entry via glutamate receptors.[24] NMDA receptor-mediated glutamate neurotoxicity after cerebral ischemia may be at least partially mediated by excessive production of nitric oxide (NO).[25]

Other sources of elevated intracellular levels of Ca^{2+} include: Ca^{2+} release from the endoplasmic reticulum, mitochondria, synaptic vesicles, and from Ca^{2+}-binding proteins. Calcium activates a number of enzymes, such as protein kinase C, phospholipase A2, phospholipase C, cyclooxygenase, calcium-dependent NO-synthase, calpain, and various proteases and endonucleases affecting cellular integrity.

Subsequently, formation of LT and free radicals (reactive oxygen, ROS, and nitrogen species) is launched. Free radicals react irreversibly with such cellular components as proteins, double bonds of phospholipids, and nuclear DNA[26]; they cause lipid peroxidation, membrane damage, dysregulation of cellular processes, and mutations of the genome. Oxygen radicals also serve as signaling molecules influencing inflammation and apoptosis.[24] Both Ca^{2+}- and free radical-mediated events are multiplied in association with reperfusion-mediated injury (see below).

Crucial for the ultimate fate of the brain tissue is the severity and duration of the compromised CBF, calculated as cerebral perfusion pressure (CPP) divided by vessel resistance. CPP in turn is the difference between mean arterial blood pressure (MABP) and ICP. In humans, normal CBF value is approximately 50 ml per 100 g of brain tissue per minute. In the region with impaired blood supply, acidosis ensues, leading to local vasodilatation, hence reducing vessel resistance. The meaning of such **autoregulation** is

to maintain the CBF at sufficient levels. Ischemia caused by a drop in CBF to a third or a quarter can still be reversible; representing the ischemic threshold. On the other hand, CBF reduction below 10 ml / 100 g / min lasting for several minutes can already cause tissue damage, infarction (a histologic finding derived from a brain region that has suffered ischemic injury).

Experimental work[27] shows that 45 to 90 minutes of middle cerebral artery occlusion (MCAO) leads to swelling of astrocytes and endothelial cells (ECs), microvascular plugging by erythrocytes and polymorphonuclear leukocytes (PMNL) in regions with the lowest CBF, with minimal changes to the neuronal perikarya. Already 1 to 3 hours later, astrocytic changes spread to the cortex, and swollen and pale neurons appear in the striatum. Eosinophilic neurons (markers of necrosis) do not appear in the core of the ischemic lesion until more than 12 hours after MCAO and until 24 to 48 hours in the ischemic cortex. It is thus important, when acute treatment strategies are considered, to focus on salvageable ischemic tissue that is not irreversibly injured.

2.1.1.2 The penumbra concept

The region with CBF values below 10 to 15 ml / 100 g / minute represents the **ischemic core**; it undergoes rapid, anoxic cell death within minutes of ischemia onset with rapid (1 hour or less) evolution into irreversible injury. Surrounding the core are the regions with CBF reduced to 15 to 35 ml / 100 g / minute, showing much slower (hours) evolution. These regions are potentially salvageable, depending on the duration and magnitude of perfusion deficit, collateral blood flow, temperature, glucose levels, and acidosis.[28] Such tissue surrounding the ischemic core is called the **ischemic penumbra**. The penumbra is considered potentially salvageable tissue-at-risk and differs from the core in terms of the process of cellular injury and cell death. While **necrosis** is more dominant in the core tissue, both necrosis and **apoptosis** are involved in penumbral cell death, and apoptosis alone seems to be involved in cell death further away from the core.[29]

Apoptosis can be triggered by a number of processes, including excitotoxicity, free-radical formation (damaging cellular lipids, proteins and nucleic acids), inflammation, mitochondrial and DNA damage, and cytochrome c release from mitochondria.[29] Apoptotic cell morphology differs from the necrotic type. Cellular necrosis includes cellular swelling, loosening of cellular architecture by cytoskeletal disruption, breakdown of cellular organelles, and denaturation of cytoplasmic proteins followed by an inflammatory response. In contrast, apoptosis is characterized by cellular shrinkage down to 60% and marked condensation of chromatin in the nucleus. In addition, apoptotic features include membrane blebbing, and fragmentation of the cell by separation of the protuberances to form multiple small membrane-bound bodies that contain intact organelles or dense clumps (apoptotic bodies) or both.[29,30] Apoptotic cells are rapidly removed by macrophage- and microglia-mediated phagocytosis without eliciting an inflammatory reaction, contrary to the PMNL-mediated phagocytosis of necrotic cells.[30]

Recently, it was suggested that both necrosis and apoptosis are two extreme poles of cellular death after ischemic stroke, "aponecrosis".[26] Even if the features of apoptosis are wellknown from experimental work, few studies demonstrate apoptotic features in human stroke.[30-32] As mentioned, apoptosis seems to occur after milder ischemic injury, particularly in association with the penumbra.[29]

The process of recruiting the penumbral tissue into the core and of infarct propagation was, experimentally, found to involve recurrent waves of depolarization (an energy-consuming process called spreading depression), which start within the core and extend outwards to surrounding tissue.[33,34] Each wave of depolarization increases infarct volume by 20% during the first 3 hours after vessel occlusion.[34] The most likely source for the depolarization is the elevated extracellular K^+ level and increase in the glutamate release at the boundaries between the ischemic core and the penumbra.[33]

The penumbra can be identified by imaging techniques and some studies suggest its existence for up to 48 hours in individual patients.[35,36] In **magnetic resonance imaging** (MRI), the penumbra corresponds to a mismatch between perfusion- and diffusion-weighted imaging (DWI).[28,37] Hossmann and coworkers[38] found that 7 hours after onset of ischemia, the DWI-based infarct region was almost identical to the severe ATP-decline region. With different durations of ischemia, those authors found the DWI changes already to be maximal at 105 minutes, but the severe ATP-decline region was noticeably smaller by that time; ATP decline corresponded well with CBF decline.

Utilizing **positron emission tomography** (PET), Heiss and coworkers[28] described the penumbra in terms of hypoperfusion (reduced CBF), preserved cerebral metabolic rate of oxygen ($CMRO_2$), and increased oxygen extraction fraction (OEF). Altogether, they defined four different patterns of PET findings: autoregulation (increased cerebral blood volume (CBV) to maintain CBF), oligemia (decreased CBF and increased OEF to maintain $CMRO_2$), ischemia (decreased CBF and decreased $CMRO_2$ despite increased OEF), and irreversible injury (very low CBF and $CMRO_2$). In that work, pattern 3 was widely present at 1 hour after MCAO, at 4 hours pattern 4 was common in the central zone and pattern 3 peripherally, and at 24 hours everywhere had nearly completely pattern 4 (highly correlating with postmortem infarct size).

When $CMRO_2$ below 1.5 ml / 100 g / minute was used to define severely ischemic tissue in a PET MCAO study in primates,[39] no significant change was found in the volume of tissue below this value in the ischemic hemisphere over the initial 7 hours, but the volume of such tissue significantly increased at 24 hours and was even larger at 14 days, suggesting that evolution of focal ischemic injury continues in primates over a longer time period. Similar results from a human study[40] suggest that many stroke patients have potentially salvageable ischemic tissue within 24 hours after onset. In another PET study,[41] CBF was calculated in 10 patients within 3 hours of onset of ischemia to define critically hypoperfused tissue, penumbral tissue, and sufficiently perfused tissue. After 2 to 3 weeks, the authors calculated the relative contribution of these regions to final

infarct volume as defined by MRI. Almost 70% of the final infarct volume was caused by initially critically hypoperfused tissue, 18% by penumbral tissue, and 12% by initially sufficiently perfused tissue, demonstrating that acute critical CBF decline is the predominant cause of infarction. This in turn justifies a rather aggressive approach aiming at rapid recanalization of the occluded artery and reperfusion of ischemic brain tissue.

2.1.2 Neuroprotection and recanalization

2.1.2.1 Neuroprotection

Several successful experimental attempts aimed to reduce infarct size and outcome. In the clinical setting, however, apart from approved intravenous (iv) thrombolysis (see below), many of the phase I to III clinical trials did not reproduce the exciting laboratory findings for pharmacologic **neuroprotection**. Among the numerous drugs found to be clinically ineffective were NMDA antagonists, GABA agonists, free radical scavengers, anti-adhesion molecules, calcium antagonists, and agents that promote cell membrane stabilization (citicoline). The reason for the discrepancies between experimental and clinical data may lay in the susceptibility of animals to ischemia and to neuroprotectants, adverse effects of neuroprotectants, differences in pharmacokinetics, and in the time windows and delayed treatment in human trials.[24]

Thus, it is possible that glutamate pathway-related neuroprotectants can be effective when used within a shorter time window and, interestingly, a pilot trial of FAST MAG (The Field Administration of Stroke Therapy-Magnesium) with iv magnesium (acting presumably as an NMDA receptor blocker), was found beneficial.[42] In contrast, the parallel study did not confirm the positive results in terms of primary outcome, i.e., mortality and 90-day disability.[43] However, in the parallel study, most of the patients received treatment beyond 3 hours (up to 12 hours) of symptom onset, and the FAST-MAG investigators received permission to start a phase III trial with magnesium administration within 1 to 2 hours of symptom onset.

Another positive clinical trial was SAINT-I (Stroke-Acute Ischemic NXY Treatment I),[44] testing a free radical trapping agent NXY-057, which was found effective experimentally. This trial found improved primary outcome (90-day disability), but it did not significantly improve other outcome measures (neurological function evaluated by National Institutes of Health stroke scale score); therefore, results of the phase II trial (SAINT-II) were eagerly awaited. However, the SAINT-II trial was negative and did not meet its primary outcome in stroke-related disability.[45]

Based on preliminary evidence, early induction of hypothermia may prove beneficial in a clinical situation.[46] It seems likely that the neuroprotective approaches per se cannot be beneficial if blood circulation is not restored at least to some degree. They could play a

role in widening the therapeutic window for successful recanalization/reperfusion or in lessening cellular death (e.g., inhibiting apoptosis) following recanalization.

2.1.2.2 Recanalization and thrombolysis

Acute critical changes in CBF disturbances offer a major contribution to cumulative ischemic brain damage.[41] Thus—apart from the unrivaled "time window" for stroke treatment, prevention—the most suitable and beneficial moment to reverse the drastic consequences of stroke is in the hyperacute phase before the tissue-at-risk evolves into infarction. Recent meta-analysis of angiography- or transcranial Doppler-monitored studies in humans reported that **spontaneous recanalization** occurs in approximately one-quarter of patients within 24 hours, and in approximately half the patients up to 1 week after stroke.[47] **Therapeutic recanalization** is reported to have a success rate of 46% for intravenous fibrinolysis, 63% for intra-arterial and 67% for combined intravenous/intra-arterial thrombolysis, and 84% for mechanical thrombolysis.[47]

To date, the most effective treatment for acute ischemic stroke is early reperfusion of the ischemic brain to salvage the penumbral tissue, reduce final infarct size, and improve functional outcome. The only proven reperfusion therapy is the administration of **iv TPA** within 3 hours of symptom onset in selected acute ischemic stroke patients. For every 1000 patients treated with TPA within 3 hours after symptom onset, more than 140 will avoid disability, and the therapeutic window may extend to 4.5 hours.[3] In that pooled analysis (based on all major randomized placebo-controlled trials of TPA for acute stroke including more than 99% of all TPA-treated patients), TPA treatment reduced death and disability at 3 months compared with results for the placebo group. According to the investigators, it is disappearance of the ischemic penumbra that is responsible for onset-to-treatment-dependent beneficial outcome.

While 15% of ischemic stroke patients admitted to the Helsinki University Central Hospital receive thrombolysis,[48] fear of TPA-associated hemorrhage may in part explain why only a mean of 2 to 5% of ischemic stroke patients as of yet receive this beneficial treatment even in specialized stroke centers worldwide.[49,50] Despite the clear benefit of early thrombolysis, tight inclusion and exclusion criteria exist as to patients to be treated, since TPA treatment is associated with an almost 6% risk (compared with 1% with placebo) of clinically relevant **parenchymal hemorrhages**.[3] Such hemorrhages were not associated with onset-to-treatment time (3-6 hours vs < 3 hours), but rather with TPA-treatment per se and with age of patient.[3] Risk for any hemorrhage in that analysis was 11%, including asymptomatic intracranial hemorrhage in patients treated with TPA. Moreover, patients treated with TPA for acute myocardial infarction or pulmonary embolism were at a 1.1%[4] and 3%[5] risk for cerebral bleeding, respectively.

Hemorrhagic transformation is a frequent consequence of ischemic stroke even without thrombolysis. Up to 30 to 40% of all ischemic strokes[51,52] and 60% of embolic strokes[53]

undergo **spontaneous hemorrhagic conversion**, i.e., transformation of a bland (anemic) infarct into a hemorrhagic one. The two types of hemorrhagic transformation are **hemorrhagic infarction** (without clinical deterioration) and **parenchymal hemorrhage** (clinically relevant) (Table 1).

The percentage of parenchymal hemorrhages in different studies differs according to the criteria selected. Recently published and performed under European Union regulations, the SITS-MOST (Safe Implementation of Thrombolysis in Stroke-Monitoring Study) study[54] reports a proportion of 1.7% patients with symptomatic (deterioration in National Institutes of Health stroke scale score of ≥4) ICH at 24 hours. There, hemorrhage was defined as a dense blood clot exceeding 30% of the infarct volume with a significant space-occupying effect (parenchymal hemorrhage type 2, Table 1). However, the proportion of patients with symptomatic ICH increased to 7.3% using the criteria for the National Institutes of Neurological Disorders and Stroke trial and for the Cochrane. These included patients with any degree of hemorrhage on computed tomography (CT) combined with any neurological worsening.

The European Cooperative Acute Stroke Study investigators defined symptomatic ICH as any hemorrhage plus a neurological deterioration in a National Institutes of Health stroke scale score of ≥4. By this definition, the proportion of patients with symptomatic ICH in SITS-MOST was 4.6%. While it is most likely that—including parenchymal hemorrhage type 2 (Table 1)—the SITS-MOST will detect major symptomatic ICH, the Canadian counterpart of SITS-MOST recently reported that parenchymal hemorrhage type 1 and hemorrhagic infarction type 2 (Table 1) were also significant predictors of poor outcome.[55] Despite variation in reported TPA-mediated hemorrhagic events, the SITS-MOST registry justifies administration of TPA for selected acute ischemic stroke patients.

Table 1. Definitions of cerebral bleeding

National Institutes of Neurological Disorders and Stroke	
HI	acute infarction with punctuate or variable hypodensity/hyperdensity, with an indistinct border within the vascular territory
PH	typical homogeneous, hyperdense lesion with a sharp border with or without edema or mass effect
European Cooperative Acute Stroke Study	
HI	petechial infarction without space-occupying effect
HI1	small petechiae
HI2	more confluent petechiae
PH	hemorrhage with mass effect
PH1	< 30% of infarcted area with mild space-occupying effect
PH2	> 30% of infarcted area with significant space-occupying effect

HI, hemorrhagic infarction; **PH,** parenchymal hemorrhage

If no recanalization is achievable, potential unfavorable actions of TPA may overwhelm the purported beneficial effects of the treatment. Hemorrhages and vascular RI (the BBB disruption, brain swelling, and neutrophil infiltration) can devastate prognosis even following successful clot lysis. TPA is a **pleiotropic** molecule. It is a fibrin-specific activator for conversion of the precursor molecule plasminogen to active plasmin. Plasmin, in turn, dissolves fibrin-based clots. In addition to its role in clot lysis, TPA may also possess signaling and protease action in the vasculature.[50] Such effects can mediate formation of hemorrhages in the brain parenchyma.

Some data suggest, that TPA and plasmin are potentially neurotoxic, should they reach the extracellular space[56]; if TPA is administered promptly after ischemia onset, the BBB remains intact, and exogenous TPA remains within the vascular space and is eliminated within minutes due to its short half-life in circulation. Experiments with TPA-deficient mice[57] showed increased TPA-associated neuronal damage in the hippocampus. Concern was, however, raised about that study, since under normal conditions, endogenous TPA does not lead to degeneration of the hippocampus.[58]

It seems that the beneficial effects of TPA-induced early thrombolysis and restoration of CBF are overwhelmingly greater than the potential detrimental effects such as neurotoxicity (cleavage of the NMDA R1 subunit, amplification of intracellular Ca^{2+} conductance), BBB disruption, edema, and hemorrhage (matrix metalloproteinases, MMPs), and inflammatory exacerbation of ischemic insults (accumulation of PMNL and free radicals).[56,59] Interestingly, the protease actions of TPA are involved in extracellular matrix (ECM) and parenchymal matrix modifications, which may mediate neuronal precursor migration as well as neurite and axonal extension[60,61] and thus affect neuronal plasticity during recovery from stroke.[50]

Indeed, the effects of TPA in the setting of cerebral ischemia are manifold, difficult to predict, and dependent on the stage of evolution of the imminent infarction as well as on the success in opening the occluded cerebral artery.

2.1.2.3 Reperfusion injury

In ischemic stroke, once the recanalization of the occluded artery has occurred either spontaneously or following a therapeutic intervention, the ensuing reperfusion delivers oxygen and glucose to the tissue and removes waste substances that have accumulated during ischemia. On the other hand, during reperfusion of the previously ischemic tissue, functionally impaired mitochondria are unable to use superfluous delivery of oxygen in electron transport, which may result in reduction in cellular ATP levels, production of ROS, activation of apoptosis, and initiation of inflammatory processes.[22]

Such a cascade of events can lead to deterioration of the tissue state; hence the term **reperfusion injury**.[62,63] Besides free radicals, several other substances have been

suggested to play a role in the cascade of RI, including excitatory amino acids, free fatty acids, cytokines, expression of endothelial adhesion molecules, Ca^{2+} influx, and protein kinases.[22,62-64] A critical step is the remodeling of the microvascular structure. Free oxygen radicals may cause peroxidation of lipids in the cell membranes, resulting in the failure of membrane ATP-ase, changes in cellular homeostasis, and elevation in the concentration of free fatty acids.[64]

The existence and nature of RI has been a subject of debate. Recanalization does not always lead to noticeable RI and, importantly, recanalization does not automatically mean adequate tissue reperfusion.[65] Effective reperfusion may not occur despite recanalization because of complex phenomena leading to **"no-reflow"** such as migration of emboli, secondary thrombosis, hemorrhage, edema, swelling of the intima, microvascular plugging by PMNL and platelets, and tissue factor-mediated coagulation system activation in the microvessels.[66]

Despite some controversies about RI (see below), a time window may exist, during which reperfusion is clearly beneficial, with only the late reperfusion being associated with RI. Early reperfusion (up to 90 minutes after MCAO) was beneficial in studies in rats,[67] with later reperfusion being harmful, causing BBB disruption, formation of vasogenic edema and hemorrhage, neutrophil infiltration, and larger infarcts.[62,68-71] The existence of such a time window for reperfusion to occur is very important from the clinical point of view, especially with respect to thrombolytic treatment and possible "anti RI" treatment strategies.

Controversy exists as to hyperperfusion and RI as judged by differences between the human and experimental data including rodents,[69] cats,[72] or nonhuman primates.[73] In human studies by Marchal and coworkers,[74-76] early post-ischemic hyperperfusion was not seen to be deleterious for tissue survival; in one of their studies, they found almost no overlap between hyperperfusion as seen in initial PET and infarct area seen on final CT.[76] However, patients with hemorrhage were excluded from this study; hence it is unknown whether hyperperfusion contributed to hemorrhage. Furthermore, normal CT rules out pan-necrosis, but not selective neuronal loss.[77] In their studies, Marchal and coworkers[74-76] suggested that when observed 5 to 18 hours after stroke onset, focal hyperperfusion without already extensive irreversible tissue damage invariably predicts minute (or absent) infarcts and excellent spontaneous recanalization/reperfusion, which presumably occurs sufficiently early to prevent ischemic tissue from evolving into infarction.

Heiss and coworkers[78] found hyperperfusion sometimes to be associated with incomplete infarction or selective neuronal loss and confirmed[72] the findings of Marchal and coworkers[74-76] that early hyperperfusion is beneficial and is a marker of good outcome. Hyperperfusion does not always occur after recanalization, however. In one study of 14 patients, only 2 showed an area of hyperperfusion in relation to the recanalization noted in their angiograms.[79] Furthermore, even though Olsen and coworkers[80] noticed frequent

hyperperfusion 1 to 4 days after symptom onset, still only 4 of their 12 patients showed hyperperfusion within 48 hours and only 1 patient within 10 hours.

Von Kummer and coworkers[65] questioned the role of RI in the clinical setting even in cases of delayed recanalization (8-24 hours after symptom onset) and presumed that it was the collateral blood supply that kept the brain tissue viable more than 8 hours after MCAO in those who showed delayed recanalization. Similarly, Marchal and coworkers suggested that reperfusion does not contribute much to tissue injury and that RI develops only in the case of already irreversibly injured tissue, which would no longer benefit from recanalization/reperfusion anyway, nor from possible RI treatment.

Still, the results of Marchal and coworkers can be interpreted in a way that early, not late, reperfusion is beneficial, fitting with the assumed existence of the time window for intervention. Indeed, Marchal and coworkers admit that RI may develop with late hyperperfusion and that selective neuronal loss occurs in areas with early post-ischemic hyperperfusion.

Furthermore, Warach and Lawrence[81] reported RI to be exacerbated by thrombolysis in human ischemic stroke and suggested that early BBB disruption in humans may be a crucial target for adjunctive therapy to reduce the complications of RI/thrombolysis, to broaden the therapeutic window, and to improve clinical outcome (Figure 1). Surely, further research is needed to find how wide this time window is and how it could potentially be modified. Von Kummer and coworkers[65] suggested two critical periods for the time window, the first of which may already be closed by the time the patient is admitted, as it is influenced by the extent of primary ischemia. The second period seems to be open for an uncertain length of time, being influenced by collateral blood flow and cerebral oxygen supply.

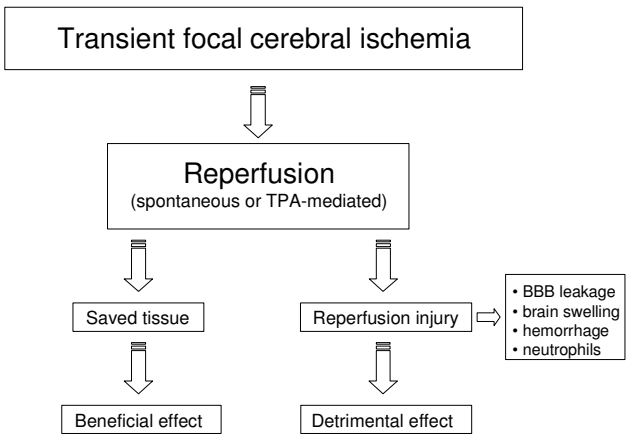

Figure 1. Reperfusion injury

It seems that more important than level of hyperperfusion after recanalization is the severity of CBF drop during the preceding ischemia together with the duration of ischemia. Aronowski and coworkers[62] found that reperfusion of long-lasting severe ischemia in rats (480 and 1080 minutes) no longer led to RI (perhaps the changes caused by ischemia were already maximally detrimental), and it is generally accepted that reperfusion after very severe ischemia (no residual CBF) does not lead to RI. Clearly, reperfusion presents a dilemma, as on the one hand it can reduce lesion volume and edema formation, but on the other hand, it may promote BBB damage, with subsequent formation of vasogenic edema and hemorrhage and neutrophil infiltration.

2.1.3 Blood-brain barrier and basal lamina disruption

The microenvironment of the CNS and integrity of the cerebral microvasculature is secured by two barriers: **the BBB** and **basal lamina** (Figure 2, bottom left and right). The former is composed of tight **interendothelial-cell junctions** of capillary and postcapillary venules. Tight junctions form a continuous network of parallel intramembrane strands of protein connected to the internal actin cytoskeleton.[82] Several integral transmembrane proteins exist (claudin, occludin, and junction adhesion molecule) as well as cytoplasmic accessory proteins that belong to the zona occludens family and others such as AF6 and cingulin.

The second barrier, the basal lamina, is a specialized part of the ECM that connects the EC to the adjoining cell layers and the smooth muscle of the media. The constituents of the basal lamina include the **matrix proteins** laminin, collagen type IV (both connected by entactin), fibronectin, thrombospondin, various proteoglycans, and heparan sulfates.[71,83] The basal lamina is connected to the endothelium by fibronectin and laminin.

Continuous disappearance of antigens of the three main constituents of the basal lamina (laminin, fibronectin, and collagen type IV) occurs very early after ischemia onset, when the basal lamina loses its integrity.[84,85] Some evidence exists (see below) that **proteases**, most importantly MMPs and PAs, are involved in the disruption of the ECM, which leads to changes in the vessel wall, and may loosen the matrix around cells. Recently, a role for another type of proteases (cathepsins) in degrading matrix components was demonstrated.[86]

MMPs comprise a large family of proteolytic enzymes, zinc endopeptidases, which are secreted in an inactive form and require mediation by other proteases. They are divided into five groups, the collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-7, MMP-10, and MMP-11), elastases (MMP-12), and the membrane-type MMPs (MMP-14, which activates pro-MMP-2).[87] Tissue inhibitors of metalloproteinases (TIMPs) are endogenous regulators of MMP activity.[87] Gelatinase A

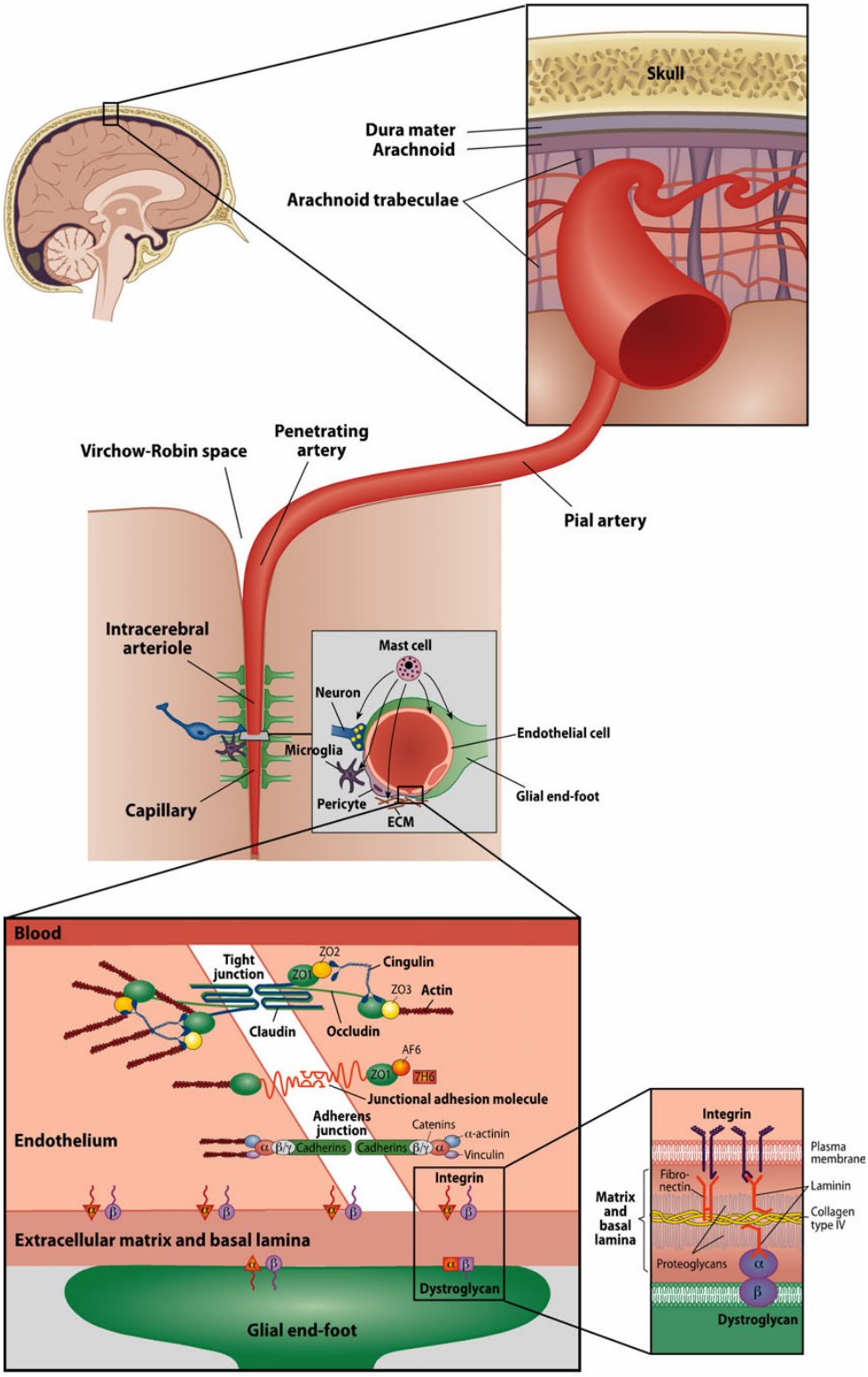

Figure 2. Schematic illustration of the neurovascular unit (middle) and the components of the blood-brain barrier (bottom left) and the basal lamina (bottom right). ECM=extracellular matrix, ZO=Zona occludens, AF6=afadin, 7H6=tight junction associated phosphoprotein. (Bottom left image adopted in part from del Zoppo, GJ et al. Arterioscler Thromb Vasc Biol 2006; 26:1966-1975 and Huber JD et al. Trends Neurosci 2001; 24:719-725.

(MMP-2) and B (MMP-9) are the largest members of the MMP family and are to date the only gelatinases identified.[88]

Activated MMPs can degrade most protein constituents of the neurovascular matrix: collagen, elastin, fibronectin, vitronectin, and gelatine.[89] Experimental data show involvement of the gelatinases MMP-2 and MMP-9 in degradation of components of the basal lamina followed by BBB disruption and development of edema and hemorrhage.[71,84,90-94] Such digestion of the endothelial basal lamina has occurred as early as 2 hours after transient ischemia.[84] Clinical data revealed some level of a positive correlation between degree of **brain edema** and level of MMP-9 in the cerebrospinal fluid (CSF).[95]

Furthermore, MMP-mediated disruption of the tight junction proteins occludin and claudin occurs, and a MMP inhibitor has reduced BBB disruption 3 hours after transient focal cerebral ischemia.[94,96] These results suggest rather early BBB disruption after transient MCAO. Fujimura and coworkers[97] found activated MMP-9 to appear as early as 3 hours after 60 min of transient MCAO, and a significant increase in pro-MMP-9 occurred in a time-dependent manner during reperfusion, the latter was not caused by the reduction in TIMP-1.

Neutralizing free oxygen radicals (their main source being arachidonic acid released from cell membrane phospholipids) by spin traps or scavenger enzymes (superoxide dismutase, catalase) reduces BBB disruption.[98,99] Furthermore, mice lacking superoxide dismutase are highly susceptible to focal cerebral ischemia-reperfusion with more vasogenic edema and higher mortality than in their wild-type (WT) littermates.[100] Free radicals may influence MMPs, either through activation of the latent forms or by mRNA induction.[89]

Besides having detrimental consequences, MMP activity and BBB disruption can also play a physiologic role in neovascularization, angiogenesis, and reconstruction-neurogenesis.[1,101,102] MMPs can be induced in a variety of cells in the CNS, including ECs,[103] astrocytes,[104] oligodendrocytes,[105] microglia,[106] neurons,[107] and MCs.[88] MCs are also a major source of MMP-1,[108] MMP-2,[109] MMP-3,[110] and MMP-9.[109,111,112]

Multiple levels of cell-cell and cell-matrix interactions influencing cerebral microvasculature integrity led to a proposal of a **neurovascular unit**,[26] including endothelium, basal lamina, ECM, neurons, astrocytes fibers and end feet, pericytes, and microglia (Figure 2, middle). Matrix adhesion receptors, e.g., the integrins and dystroglycan, are expressed in the microvasculature, by neurons, and by the glial cells, as recently reviewed.[113,114] Integrins are cell-surface transmembrane αβ heterodimers that recognize matrix ligands,[115] whereas dystroglycan is a single αβ heterodimeric transmembrane receptor which forms a link between the intracellular cytoskeleton and the ECM.[116] Integrins $\alpha_1\beta_1$ and $\alpha_6\beta_4$ disappear rapidly—within 2 hours of ischemia.[85,117] Complex interactions within the neurovascular unit suggest the need for a multilevel

approach for therapeutically targeting the loss of microvascular integrity shown by basal lamina dissolution and BBB disruption and its consequences such as formation of edema and hemorrhage, and inflammatory cell infiltration.

2.1.3.1 Ischemic brain edema: characteristics and treatment

Evolution of cerebral infarction is invariably associated with development of some degree of **brain edema**. By definition, brain edema represents abnormal accumulation of fluid within the brain parenchyma, leading to volumetric enlargement of the tissue.[118] Space-occupying brain swelling is one of the major determinants of patient survival beyond the first few hours after a major stroke, being the leading cause of death after the large hemispheric middle cerebral artery (MCA) strokes,[1,24] which account for 15 to 20% of all MCA strokes.[119] In addition, brain swelling is a major prognostic factor in cerebellar strokes.

Early ischemic brain swelling is already present in two-thirds of patients during the first 3 to 6 hours,[2] becomes evident within the first 24 hours, and peaks at 72 hours.[1] Mortality from progressive edema in patients with large hemispheric strokes is approximately 80%.[119,120] Brain edema aggravates the ischemic process by its volumetric effect's causing local compression of the microcirculation, rise in ICP, dislocation of parts of the brain (herniation), and decreased CBF in the ischemic penumbra. All these effects damage the penumbral metabolism and facilitate its transformation into infarction.

Ischemic brain edema is a combination of two types of edema: **cytotoxic (cellular)** and **vasogenic**.[118] Cytotoxic edema evolves over minutes to hours and may be reversible, whereas the vasogenic phase occurs over hours to days, and is considered an irreversibly damaging process. **Cytotoxic edema** is characterized by swelling of all the cellular elements of the brain. It represents a shift of water from the extracellular to intracellular compartment, accompanied by shrinkage of the former. In the presence of acute cerebral ischemia, neurons, astrocytes, and ECs swell within minutes of hypoxia due to failure of ATP-dependent ion (Na^+ and Ca^{2+}) transport. With the rapid accumulation of Na^+ within cells, water follows to maintain osmotic equilibrium. Increased intracellular calcium activates phospholipases and the release of arachidonic acid, leading to the release of oxygen-derived free radicals and to cell death.

Should the ischemia take only a short time, the disturbed membrane potential may recover. However, cellular swelling and Ca^{2+} influx are considered to cause irreversible damage to the cell.[23] **Vasogenic edema** is characterized by an increase in extracellular fluid volume due to increased permeability of brain capillary ECs to macromolecules (e.g., albumin, IgG, and dextran). Normally, the entry of plasma protein-containing fluid into the extracellular space is limited by tight EC junctions, but after BBB disruption, water, Na^+, proteins, and blood constituents enter the extracellular space. The degree of

edema is, like the BBB disruption, influenced by the severity and duration of ischemia and reperfusion.[69,121]

The skull offers rather limited possibility to expand. An increase in volume of any intracranial structure (brain, CSF, and blood) will lead to an increase in **ICP** or shift one compartment of the brain, thus compressing others. Further increase in ICP promotes brain shifts and distortion (**herniation**), resulting in compression of neurons, nerve tracts, and cerebral arteries. Herniation usually occurs in the tentorium, the foramen magnum, and the falx. Continuously increased ICP causes long-lasting ischemia and irreversible damage to brain cells. When ICP rises, CPP and CBF are reduced by autoregulatory vasodilatation. When CPP drops below a critical threshold of about 50 to 60 mmHg, maximal vasodilatation takes place, and autoregulation fails. CBF is further compromised, and worsening ischemia aggravates edema.

Several treatment strategies have been utilized in targeting ischemic brain edema and elevated ICP. Clinically, osmotherapy (mannitol, hypertonic saline (HS), glycerol, and sorbitol), steroids, barbiturates, hyperventilation, elevated head position, tromethamine, indomethacin, and furosemide have all been used with variable success. Hypothermia and invasive decompressive craniectomy seem promising.

Osmotherapy has served for the management of patients with elevated ICP since the early 1960s, yet its use remains controversial. The rationale of its use comes from the existence of the intracellular and extracellular compartment, with the latter further divided into intravascular and interstitial compartments. Mechanisms of ICP reduction by osmotherapy include water extraction from the brain, raising of blood pressure (resulting in autoregulatory vasoconstriction and a fall in CBV), and lowering of serum viscosity (leading to reduced CBV).

Mannitol neither crosses the cell membrane nor the intact BBB, i.e., it remains intravascular and can extract intracellular and interstitial water. However, mannitol can cross the injured BBB, arousing concern that it might accumulate within injured tissue, causing brain edema and midline shifts (not confirmed by others[122]) and shrinkage of the non-infarcted brain tissue.[123] This would cause further expansion of the ischemic hemisphere and potentially aggravate the midline shift. However, Diringer and Zazulia[124] proposed that a) it is probably fiction that mannitol accumulates in the injured brain; b) it is probably fact that mannitol shrinks only the normal brain; c) it is fiction that mannitol increases midline shift; that osmolality can serve to monitor its administration; that mannitol should not be administered if osmolality exceeds 320 mOsm; and d) it is unknown whether HS and mannitol are equally effective. Evidence is generally lacking for the efficacy of mannitol, but still the American Heart Association recommendeds it to treat post-stroke edema. Nevertheless, Cochrane analysis[125] lacked sufficient evidence for a general recommendation as to mannitol use.

Several human studies have compared HS with mannitol, but it is difficult to reach clear conclusions, since osmotically equivalent doses (this means 23.4% saline and mannitol) of the two agents were rarely used, and HS was used in cases refractory to mannitol. Prophylactic use of osmotherapy before onset of elevated ICP has not been addressed.

The BBB has a higher osmotic reflection coefficient for Na^+ and Cl^- than for mannitol, so saline crosses into the interstitial space but is excluded from the intracellular compartment. In stroke patients, HS (7.5% + 100 ml hydroxyethyl starch) and 40 g mannitol reduced the increased ICP, the former approach significantly lowering ICP more rapidly and effectively (for 16 of 16 patients) and increased CPP significantly (although not as much as did mannitol).[126] In another study by the same group, HS (10%) was beneficial after failure of other medical therapies including mannitol.[127] However, almost no evidence exists for the use of HS in ischemic stroke, since systematic trials are lacking.

In a study with ultrasound monitoring of ICP therapy with mannitol iv, sorbitol iv, and glycerol po (50 g each), the pulsatility index was significantly lowered in all groups on both the infarcted and healthy side, with no significant differences between the substances or sides. Glycerol showed the longest duration of effect, and all substances raised minimal flow velocity (glycerol showing the greatest increase), i.e., edema reduction.[128] Although glycerol 10% was beneficial in some studies, Cochrane review[129] suggests just short-term benefit, no long-term, and does not support its use as standard therapy.

Regarding treatment with **steroids**, the classical categorization of cerebral edema into cytotoxic and vasogenic[118] is important. Whereas the clinical response to steroids of the cytotoxic edema type is uncertain, BBB damage with pathologically separated tight junctions, as well as with disturbed pinocytosis (as seen in brain tumors) is highly responsive. In a study comparing dexamethasone and a dexamethasone+mannitol combination with placebo,[130] no effect on ten-day survival rate occurred. In a recent review,[131] glucocorticoids were considered ineffective in treatment of ischemic edema, and Cochrane analysis does not support their use in ischemic stroke.[132]

However, most conducted trials included few patients and started treatment too late; of 22 published trials, only 7 were accepted for Cochrane analysis. Besides having inadequate numbers of patients, these trials had no uniformity of evaluation or assessment, and reached disparate conclusions. Still, some authors justify the use of steroids,[133] and around 20% of US or Chinese physicians routinely use corticosteroids for ischemic stroke patients.[132]

Steroids do, however, raise risk for infections, hyperglycemia, and muscle catabolism, and it is debated whether the widespread use of steroids in response to a marginal therapeutic gain would expose large numbers of stroke patients to the more serious hazard of steroid treatment and convert patients who would otherwise have died into

bedridden, miserable survivors; the majority would consider this worse than death. Some authors[134] thus propose that no large multicenter trial is justified.

Barbiturate coma reduced the increase in ICP, but seemed to have no positive effect on neurologic outcome.[135] That study included no control group, however, and was tested only after failure of osmotherapy and hyperventilation. **Indomethacin** has achieved short-term ICP reduction, but a continuous infusion was ineffective.[136]

Regarding more **recent strategies**, hypothermia reduces cerebral metabolic rate, BBB disruption, edema formation, free radical formation, release of excitatory aminoacids, inflammatory response, and apoptosis, and, importantly, improves clinical outcome.[46] Invasive decompressive craniectomy (< 48 hours) is recommended as the treatment of choice for patients aged 60 years or younger—with severe infarction of at least 50% of the MCA territory—reducing mortality and improving outcome.[137] However, with this approach, the chance of surviving in a condition requiring assistance from others increases >10 times.

Experimentally, besides these clinical strategies, an enormous number of other agents and approaches have been tested with promising results. These include albumin[138] (acting presumably by diminishing bulk flow through the disrupted BBB and ameliorating vasogenic edema), bradykinin receptor antagonists,[139] and arginin vasopressin receptor antagonists.[140] Furthermore, aquaporin-4 deletion,[141] tumor necrosis factor (TNF)-α neutralization,[142] interleukin (IL)-1 receptor antagonists,[143] MMP inhibition,[144] ET-A receptor antagonists,[145] free radical scavenging,[146] and many others to some degree help. Interestingly, the TPA inhibitor neuroserpin reduced ischemic edema in TPA-treated rats.[147,148]

2.1.3.2 Hemorrhage

The etiology of post-thrombolytic hemorrhage after TPA is not understood. However, the mechanisms that mediate hemorrhagic transformation are likely to be correlated with components of RI, especially the BBB and basal lamina disruption.[83] Several suggested triggers include oxidative stress and oxidative damage to membranes constituting the BBB, matrix proteolysis, and vascular response (section 2.1.3).

VEGF expression is induced by focal cerebral ischemia, and iv VEGF application significantly increases BBB disruption, hemorrhage, and lesion volumes in rats subjected to ischemia.[149] An interesting approach to ameliorate endothelium damage and TPA-associated hemorrhage was reported by the group of Eng Lo.[150] In that study, immunoliposomes, recognizing intracellular antigens (anticytoskeletal antiactin) exposed in ECs after membrane damage, were used to bind to and "reseal" the damaged membrane. Endothelium can be directly injured by ROS,[151] and this detrimental effect is blocked by antioxidants.[152] Moreover, free radical spin traps have reduced experimentally

both spontaneous hemorrhage and thrombolysis-mediated hemorrhage,[153,154] improving outcome.

Confirming the **overlapping pattern** of pathways involved in hemorrhagic transformation, ROS production is highly correlated with focal areas of gelatinase activity in microvessels within the ischemic mouse brain.[144] Gelatinases, in turn, can degrade constituents of the basal lamina, enhancing vascular permeability and erythrocyte extravasation with consequent hemorrhage. Studies from the del Zoppo group[71,84] show that loss of integrity of basal lamina constituents and its connection with surrounding structures leading to BBB disruption is associated with hemorrhagic transformation.

Extracellular **proteolysis** includes activity of the serine proteases, PAs, MMPs, and proteases secreted by activated PMNL. Plasmin, activated by TPA or urokinase type PA, activates MMP-9 and MMP-2.[155] Activation of MMPs contributes to the dissolution of the basal lamina and correlates with development of hemorrhage.[71,91] TPA-induced hemorrhage, in turn, is significantly reduced by MMP inhibition,[92,156] as is TPA-mediated mortality[157] in experimental ischemia-reperfusion.

PAs contribute to ECM degradation,[158] acting by plasmin generation or activation of MMPs. Plasmin augments capillary injury by acting with other proteolytic enzymes to disrupt the ECM, contributes to the activation of the membrane-type metalloproteinase involved in activating gelatinase A (MMP-2), and activates ECM-degrading enzyme stromelysin.[159] Furthermore, plasmin can activate MMP-1 and MMP-3, whereas plasmin inhibitors as well as antibodies to urokinase type PA inhibit activation of pro-MMP-2.[93,160]

There exists some body of **clinical evidence** showing that MMPs are involved in hemorrhagic transformation with or without thrombolysis,[161-163] with one study showing a clear correlation between pretreatment levels of MMP-9 and intracranial hemorrhagic complications.[163] Besides showing increased levels of MMP-2 and MMP-9, and, importantly, also significantly decreased levels of intact laminin and TIMP-2, the study by Horstman and coworkers[162] was the only one that analyzed the activity of MMPs. In that study, the active form of MMP-9 appeared in only 4 of 17 patients treated with TPA but in no patients receiving heparin or undergoing hypothermia.

Since most experimental and clinical studies report latent but not active forms of MMP-2 and MMP-9 as appearing after MCA occlusion, their role in matrix degradation was in question.[86] As one answer, a role for the cysteine proteases cathepsins B and L in degradation of the matrix component perlecan as early as 2 hours after MCA occlusion emerged experimentally.[86]

Apart from spin trap agents and MMP inhibitors, the glycoprotein IIb/IIIa platelet receptor antagonist reduces hemorrhage when coadmistrated with thrombolysis,[164] presumably influencing vessel reocclusion and the ”no-reflow” phenomenon.

2.1.3.3 Neutrophil infiltration and inflammatory response

Under normal conditions, the cerebral microvascular endothelium acts as a barrier to the immune system that limits the entry of neutrophils and other leukocytes into brain tissue. Neutrophils can be detected in the microvessels of the ischemic hemisphere as early as 30 minutes after the arterial occlusion, peaking at 12 hours.[165] They are the first inflammatory cells to arrive in the ischemic tissue between 1 to 6 hours after reperfusion,[101,165,166] peaking at 24 hours and still visible after 7 days.[165] In the clinical scenario, neutrophil counts peaks at 1 to 3 days postinfarction.[167] Neutrophils are believed to contribute to the secondary damage by causing capillary plugging, microvascular disruption, edema, and hemorrhage. This process involves cytokines, lipid-derived mediators, proteases, and free radicals.[168]

Circulating monocytes can be detected within the microvasculature of the ischemic hemisphere after 4 to 6 hours.[165] Experimentally, macrophages become the predominant cell type within a few days,[101] and clinically within 1 to 2 weeks of neutrophil infiltration.[167] Interestingly, experiments with bone marrow chimeric mice during transient focal cerebral ischemia suggest that resident microglial activation precedes macrophage infiltration, and that the vast majority of macrophages in the infarcted area are derived by differentiation from local microglia.[169]

Microvascular obstruction by platelets, fibrin, and neutrophils (the **"no-reflow"** phenomenon) can worsen the degree of ischemia,[70,170-172] and production of toxic mediators by activated inflammatory cells and injured neurons (cytokines, NO, superoxide, and prostanoids) can amplify tissue damage. Neutrophils may contribute to further disruption of the endothelium and the BBB by producing free oxygen radicals and proteolytic enzymes; infiltrating neutrophils release an active form of MMP-9.[173] Protease activity resulting from leukocytes binding to activated endothelium may lead to degradation of cadherin (Figure 2), a component of the endothelial cell-to-cell junction.[174] Furthermore, increased leukocyte migration alters the molecular organization of the tight junction complex, the reorganization of the actin cytoskeleton, and the BBB disruption.[175]

The precise role of neutrophils in ischemia-reperfusion is still debated. Some authors consider neutrophils to have nothing but a bystander's role in the ischemia-reperfusion scenario,[166,176,177] while others suggest a critical causative role for them.[178,179] Several lines of evidence support the latter view. First, correlation exists between the time course of neutrophil accumulation in the ischemic zone and the expansion of cerebral damage during reperfusion in rats,[180,181] in nonhuman primates,[70,182] and in humans.[183] Second, the beneficial effect of neutropenia on RI in experimental settings[184,185] and the deleterious effect of elevated neutrophil count on stroke outcome in humans[186] is known. The last line of evidence comes from works studying adhesion molecules and recruitment of neutrophils during reperfusion as well as from the effect of anti-adhesion therapeutic strategies.

The adhesion of neutrophils to the endothelium follows initial contact and rolling; thereafter, transmigration into the cerebral compartment takes place. The recruitment process involves interactions between three groups of adhesion molecules: selectins, the immunoglobulin gene superfamily, and integrins.[66,187] E-selectins occur in ECs, L-selectins in leukocytes, and P-selectins in both platelets and ECs; all of these are involved in the low–affinity rolling of the neutrophil to the endothelium.[66] The high-affinity binding involves the immunoglobulin gene superfamily members together with their ligands, which are members of the integrin family.[66,187] The immunoglobulin gene superfamily members are expressed in ECs, including intercellular adhesion molecule (ICAM)-1 and -2, vascular cell adhesion molecule-1 (CD106), platelet-endothelial cell adhesion molecule-1, and mucosal addressin.[188] Integrins, in turn, are expressed in the circulating neutrophils and not only participate in binding of the neutrophils to ECs, but also mediate their adhesion to the ECM components fibronectin and laminin.[188,189] The most common integrins share a subunit β_2, $\alpha_M\beta_2$ (recognizes ICAM-1), $\alpha_L\beta_2$ (interacts with ICAM-1 and -2),[190] or subunit $\beta1$.[191]

Studies with adhesion molecules in the experimental[192,193] and clinical[167] setting showed different mediators to be involved in the upregulation of the adhesion molecules during experimental cerebral ischemia-reperfusion in vivo or in vitro. Specifically, TNF-α and IL-1 influence the expression of selectins[194] and ICAM-1.[195] Furthermore, platelet-activating factor (PAF) upregulates integrins on neutrophils.[196] Therapeutic antiadhesive approaches lead to amelioration of inflammatory response after transient ischemia in rats[197,198] and nonhuman primates.[171] In addition, Bowes and coworkers[199] found treatment with the anti-ICAM-1 antibody to lengthen the therapeutic window for beneficial administration of TPA. Antiadhesive strategies, however, show no beneficial effect in models of permanent ischemia.[200]

Despite promising results from **anti-inflammatory strategy** in experimental stroke, clinical trials with a murine anti-human ICAM-1 antibody (enlimomab) and inhibition of neutrophils were negative.[201,202] To define the possible mechanism of this negative result in the enlimomab trial, Furuya and coworkers[203] treated rats with a murine anti-rat ICAM-1 antibody and reported production of host antibodies against the heterologous protein administered and activation of circulating neutrophils, complement, and microvasculature.

Furthermore, the nonhumanized murine IgG2a subtype antibody itself activates complement in human whole blood, which leads to inflammatory consequences such as increased expression of CD11b/CD18 adhesion molecules and radical production of neutrophils.[204] These responses may be responsible for the failure of the clinical enlimomab trial as well as for the body-temperature elevation and the excess of cerebral causes of death in that trial.[202]

Although, in their review, Emerich and coworkers,[166] who claim the bystander's role for neutrophils, found no clear cause-effect relationship between leukocyte recruitment and

the pathogenesis of ischemia, they call for additional experiments to shed light on this subject.

2.1.4 Models of focal cerebral ischemia

The clinical variability of stroke (causes, localization, duration, severity, and coexisting systemic diseases) requires large patient cohorts in clinical trials in order to avoid confounding effects of this diversity. Thus, experimental models serve as a tool of investigation under strictly controlled conditions, and can provide us with important and clinically relevant data concerning mechanisms of ischemic cerebral injury and development of novel drugs.

In general, the ideal animal model should be relevant to the clinical situation, be reproducible, have minor or no extracerebral side-effects, and be technically easy to perform. It is obvious that no single animal model simulates all aspects of ischemic stroke, since it is a heterogeneous, incidental condition that occurs spontaneously, often triggered, if not governed, by profound precipitating factors such as infection, trauma or serious other comorbidity.

It should be mentioned that the relevance of stroke models to human stroke has been debated, since many assumed neuroprotective compounds fail to show efficacy in humans, despite positive results in preclinical settings.[205,206] On the other hand, ischemic stroke models contribute largely to understanding of pathophysiologic mechanisms, e.g., excitotoxicity, free radical generation, RI, periinfarct depolarizations, inflammation, BBB injury, programmed cell death, and gene expression after ischemia. Furthermore, these models play a significant role in developing methods for investigating the brain; a good example is the development of novel MRI modalities such as perfusion- and diffusion-WI, which were tested in rodent models in the 1990's and were rapidly incorporated into clinical practice.[207] Once we identify all possible causes of unsuccessful translation of the positive experimental neuroprotective studies into clinical practice, it is more likely that diverse effective treatments for ischemic stroke will soon be revealed.

The rat is the animal most commonly used for studying cerebral ischemia, although larger animal species (cats, dogs, rabbits, and nonhuman primates) have also been used. What makes rodent models dominant is their low cost, easy transportation, storage, and feeding, relative homogeneity within strains (inbreeding), and resemblance of their cerebrovascular anatomy[208] and physiology to that of higher species. Small brain size is suitable for fixation procedures, for microscopic and macroscopic examination, and for biochemical analysis. It is easy to perform physiological monitoring and to replicate studies, with greater acceptability from ethical perspectives.

The anatomic distribution of the anterior cerebral artery (ACA), posterior cerebral artery (PCA), and the MCA in rats is analogous to that in humans.[208] Although in rats the PCA arises from the proximal intracranial portion of the internal carotid artery (ICA), the posterior communicating artery connects the terminal cerebellar branch of the basilar artery with the PCA. The blood supply to the rat thalamus and basal ganglia is also similar to that in humans.[209] Most stroke models are based on inducing ischemia in MCA territory to mimic a common clinical situation. Extracranial occlusion of a carotid or vertebral artery without further surgical intervention does not produce cerebral ischemia in the rat (except in spontaneously hypertensive rats) because the circle of Willis provides sufficient collateral blood supply via the nonaffected vessels.

2.1.4.1 Craniectomy-requiring early MCAO models

These models are quite invasive, expose the brain to the atmosphere, affect ICP and BBB function, and do not produce large and reproducible infarcts.[210] Robinson and coworkers[211] first described ligation of the distal portion of the MCA in rats, which did not provide reproducible infarcts and cause no striatal damage. Others[208] accessed the more proximal regions of the MCA via a subtemporal approach, which produced an infarct involving both the cortex and the striatum. This model was modified by using a ligature or a clip to occlude the MCA for achieving reperfusion, but inconsistent patterns of reperfusion occurred.[208]

2.1.4.2 Intraluminal suture MCAO model

The most popular experimental model to induce focal cerebral ischemia is the suture occlusion model of the MCA, first described by Koizumi and coworkers,[212] who used a 4-0 nylon monofilament occluder with its tip rounded near a flame and then coated with silicon. They permanently occluded the common carotid artery (CCA) and the external carotid artery (ECA). The monofilament was inserted through an arteriectomy of the ipsilateral CCA and moved forward approximately 17 mm beyond the carotid bifurcation into the ICA and upwards, occluding the origins of the ACA, MCA, and the posterior communicating artery. This model allows large, well-reproducible infarcts because the coated suture completely obstructs the MCA trunk and the major sources of collateral blood flow. Zea Longa and coworkers[213] developed a similar model in which they inserted a 4-0 monofilament suture (without silicon coating) through the ECA while keeping the ipsilateral CCA patent. The infarct sizes produced with the Zea Longa's model are smaller, with larger variability because of a higher and more variable residual blood flow into the ischemic regions.[214]

Both models allow reperfusion, and do not require extensive surgery. The infarcts in suture models include cortical, subcortical, and hippocampal portions having a large penumbra, which makes these models preferable for drug-efficacy studies. Furthermore,

the time of reperfusion can be successfully controlled by withdrawal of a suture at desired intervals, making it practical for studies of RI. The high mortality rate limits, however, long-term observation studies with these two models. The length of the inserted filament required for a successful MCAO depends on several factors including body weight, rat strain, and vendor. Required filament length ranged from 17 to 22 mm among studies.[212,213,215]

2.1.4.3 Thromboembolic models of the MCA

This model simulates human stroke reasonably well and allows for study of both thrombolytic therapies and neuroprotectives. In the older thromboembolic models,[216] a suspension of small thrombi was injected into the ICA while blood circulation was being allowed through the CCA. The thrombi usually lodged in small arteries in the MCA territory causing multiple small lesions not particularly relevant for human disease. Furthermore, early spontaneous recanalization was a disadvantage of this model. Later, a single macroclot (fibrin-rich embolus) was used for MCA trunk or major branch occlusion.[217] Even if this model closely mimics human stroke, it causes a large variation in infarct size due to differences in the exact sites of occlusion. Its low reproducibility hampers its general use. Recently, Busch and coworkers[218] reported considerable improvement by injecting multiple fibrin-rich clots into the ECA one after another; this led to a consistent reduction in CBF without spontaneous reperfusion within 3 hours after emboli injection.

2.1.4.4 Other, non-clot, embolic models

Different artificial embolic materials have served to induce ischemia. The lesion development is slow, increasing in size up to 24 hours postinjection. Microsphere model may provide a larger therapeutic window for drug testing in stroke, but the lesions are of a multifocal and heterogeneous nature.[219]

2.1.4.5 Photochemically induced focal ischemia

This relatively noninvasive model is not widely used. It involves systemic administration of the photosensitive dye rose bengal, and irradiation of specific areas of the brain with a focused light beam at a specific wavelength. A reaction between the light and the circulating dye generates free radicals, resulting in platelet aggregation and thrombosis.[220] Lesion site and size can be selected by the researcher. Lesion size and depth depend upon intensity of the irradiating beam, duration of irradiation, and dose of rose bengal. The typical infarct is a sharply circumscribed, bowl-shaped necrotic lesion occupying the full cortical thickness but sparing underlying structures. Early BBB breakdown and vasogenic edema give rise to progressive microvascular compression at

the lesion periphery. The main disadvantage is the absence of a penumbra region around the ischemic core; there is, instead, a ring of hyperperfusion.[221] However, model improvements seem to induce cortical ischemic lesions involving a penumbra-like lesion.[222] Another disadvantage of this model is the end-arterial occlusive character of the lesion, which makes it resistant to collateral-perfusion-enhancing approaches. Still, this model can aid in testing drugs for restorative function and evaluation of neuronal repair.

2.1.4.6 Endothelin-1-injection model

A restriction associated with current models of MCAO is the mechanical damage caused to the vessel by clip or occluder. A novel method of MCAO is infusion of ET-1 directly onto the exposed MCA[223] or adjacent to the MCA by stereotaxic injection.[224] Such an ET-1 infusion produces a dose-dependent narrowing/occlusion of the MCA. Although this model is less invasive and enables production of ischemia in a desired brain region, the dose-dependent action of ET-1 still reduces control of ischemia: of its severity and duration.

2.1.4.7 In-bore MCAO models for MRI studies

Since the time that MRI became a powerful and widely available tool for studying hyperacute brain ischemia, developing models to induce focal ischemia and reperfusion in the MRI scanner has become a necessity. This allows for collection of pre- and post-ischemic images that are spatially co-registered and later, comparison of preischemic findings to ischemic changes. Such in-bore occlusion models may be as successful as the classical MCAO models.[215]

2.1.4.8. Summary of the ischemic stroke models

To sum up, the thromboembolic model is most relevant to human stroke, but its disadvantage is varying rate of spontaneous recanalization (making controlled reperfusion impossible) and a higher variability in lesion volumes. Suture MCAO is easy to perform, allows absolute control over reperfusion, and makes lesion volumes more reproducible. Its disadvantages include: a risk for subarachnoid hemorrhage (SAH), higher mortality in long-term experiments, and hyperthermia in permanent ischemia. Although the craniectomy models allow control of the site of occlusion (thus the infarct size and mortality), they are rather invasive and depend on the surgeon's skills. The photothrombotic model is less invasive and allows induction of the lesion in the desired cortical region. However, this model is less relevant to human conditions, since it typically lacks the penumbral tissue zone. Application of ET-1 to different brain regions controls lesion location and volume, but offers only modest control over ischemia duration and intensity.

2.2 Intracerebral hemorrhage

ICH is caused by bleeding into the brain parenchyma. In Western countries, ICH accounts for 15% of all cases of stroke, and the rate is even higher (20-30%) in Asian and African populations.[6] **Nontraumatic** ICH can be divided into one type that arises from pre-existing macroscopic "ictohemorrhagic" vascular lesions (secondary ICH) and one that does not (primary ICH).[225] The former includes arteriovenous malformations, cavernous malformations, aneurysms, brain tumors, and dural fistulas; the latter, accounting for approximately 85% of all cases,[226] is caused by micro-aneurysm-associated (Charcot Bouchard aneurysms) hypertension and amyloid angiopathy.[225] Other rare conditions include cerebral venous thrombosis, cerebral endometriosis, convulsions, and pregnancy toxemia.

Arterial hypertension is the most important risk factor for a nontraumatic primary ICH, present in approximately 50 to 70% of such patients.[226] Even borderline isolated hypertension is associated with increased risk for ICH.[227] Other factors include excessive use of alcohol, smoking, serum cholesterol levels less than 4.1 mmol/l (although a less reliable factor), and the presence of ε2 and ε4 alleles of the apolipoprotein E gene as related to amyloid angiopathy-associated ICH. Deficiencies in coagulation factors I, VII, VIII, IX, XIII, and von Willebrand factor are associated with ICH, as well. In addition, advanced age, male gender, pregnancy, the delivery and postpartum period, African or Japanese race, and use of anticoagulants, antiplatelet drugs, and cocaine also play a role.[226,228]

Physicians treating ICH patients possess no tool comparable to TPA in ischemic stroke. On the contrary, hemostatic therapy, considered the counterpart to emergency TPA treatment, was not found beneficial (see below). Currently, no widely approved effective acute medical treatment exists, and surgical evaluation of ICH was recently found not to be beneficial.[14]

Thus, ICH is associated with high mortality and disability, with 60 to 70% of the patients surviving through the first month and only 40 to 50% through the first year,[7,8] many of them with chronic disability. An even worse (95%) 3-month mortality is associated with those patients having large, deep hemorrhages associated with coma.[228] Poor outcome results from direct tissue damage, especially in deep brain structures (associated with disruption of the internal capsule) and the mass effect of the growing hematoma and edema. These factors contribute to expansive brain swelling and lead to displacement and disruption of brain structures and often to increased ICP.

While white matter and cortical hemorrhages are more likely due to amyloid angiopathy, arterial hypertension is usually deemed the underlying cause of ICH in areas that are supplied by the vessels arising from the high pressure of the circle of Willis (Figure 3), leading to hemorrhages in the basal ganglia (35-44% of cases), thalamus (10-25%),

cerebellum (5-10%), pons (5-9%), and neocortex (19-25%).[6,228]. Accordingly, ICH can be divided into supratentorial and posterior fossa hemorrhages.

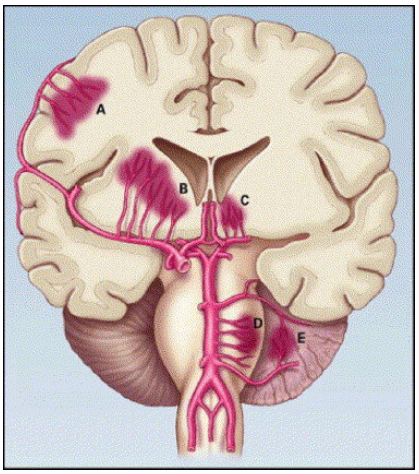

Figure 3. Common sites of intracerebral hemorrhage. A) Cerebral lobes - originating from penetrating cortical branches of the anterior, middle, or posterior cerebral arteries. B) Basal ganglia - originating from ascending lenticulostriate branches of the middle cerebral artery. C) Thalamus - originating from ascending thalamogeniculate branches of the posterior cerebral artery. D) Pons - originating from paramedian branches of the basilar artery. E) Cerebellum - originating from penetrating branches of the posterior inferior, anterior inferior, or superior cerebellar arteries. Reproduced from Qureshi A et al. N Engl J Med 2001; 344:1450-1460 with the written permission of the copyright holders.

2.2.1 Pathophysiology of intracerebral hemorrhage

Nontraumatic intraparenchymal hemorrhage without pre-existing ictohemorrhagic vascular lesions arises from the rupture of the small penetrating arteries that originate in the ACA, MCA, PCA, and in the basilar artery, all affected by degenerative changes associated with **hypertensive arteriolosclerosis or amyloid angiopathy**. Following the hemorrhagic event, direct tissue destruction and dissection of blood along tissue planes occurs, followed by edema formation and ICP increase. Delayed damage can be mediated by toxins associated with blood breakdown products, thrombin, and leukocyte infiltration. The role of secondary ischemia after ICH has been a subject of debate.

2.2.1.1 Hematoma growth

ICH is not a single-bleed event that stops quickly once it has started. In a prospective study,[229] early **hematoma growth** occurred in 38% of 103 patients within 20 hours after baseline imaging (scanned primarily within 3 hours of symptom onset), with two-thirds of the patients already showing hematoma growth 1 hour following baseline imaging. Hematoma expansion is suggested to arise from continuous bleeding from the primary source and from the mechanical disruption of surrounding vessels,[230] with acute hypertension and a local coagulation deficit possibly contributing to the expansion.[231,232]

Hematoma growth is an important predictor of outcome after primary ICH,[233] and its clinical importance is supported by a recent meta-analysis.[234] The mass effect of the

growing hematoma may result in increased ICP, transtentorial herniation, and dramatic reduction in CBF. In addition to the mass effect, the hematoma itself induces a number of secondary changes in the surrounding tissue, including neuronal and glial cell death due to necrosis and apoptosis,[9-11] inflammation (see below), and vasogenic edema caused by disruption of the BBB.[12,13]

2.2.1.2. Is there a perihematomal penumbra?

The mass effect of the ICH is thought to induce secondary ischemic injury due to direct mechanical compression of the blood vessels surrounding the hematoma and vasoconstrictor substances in blood; to this end, the term "**perihematomal penumbra**" was proposed based on a number of experimental studies in rats[235-237] and in primates,[238] as well as in clinical studies.[239,240]

Debate is ongoing, however, as to whether such a perihematomal penumbra exists. No evidence of perihematomal ischemia was found by other researchers in experimental[241,242] and clinical studies using MRI.[243-245] Butcher and coworkers[245] reported self-limited perihematomal oligemia, which normalized 3 to 5 days after symptom onset, and reported that the relative apparent diffusion coefficient (ADC) (ratio of ADC perihematomal / contralateral homologous regions) independently predicted absolute and relative edema volume, demonstrating its correlation with the rate of diffusion. This is the case for edema derived from extravasated plasma, but not acute ischemia-associated cytotoxic edema.

MRI-based results may, however, be misleading, since perihematomal measurement of diffusion may be influenced both by reduced ADC due to ischemia-related cytotoxic edema and, conversely, by increased ADC due to hemorrhage-related extracellular vasogenic edema.[246] Moreover, hemoglobin and its degradation products may interfere with analysis of MRI data.[246]

To continue the debate, PET studies (analyzing $CMRO_2$, OEF, and CBF) produced findings not consistent with ischemia and showed preserved autoregulation. This suggests that hypoperfusion surrounding ICH reflects reduced metabolic rate rather than ischemia.[247-249] Similarly, Herweh and coworkers[250] found in a human perfusion CT study no evidence of perihematomal penumbra. Diaschisis (remote autoregulatory hypoperfusion due to reduced oxygen demand), caused by inflammatory tissue damage and mass effect of the growing hematoma and clot-related vasogenic edema, is a probable reason for the commonly observed perihematomal perfusion alterations, as suggested earlier by the same group.[243]

In addition, Sook Kim-Han and coworkers[251] recently reported mitochondrial impairment and not ischemia to be responsible for the reduced metabolic demand. However, Nilsson and coworkers[252] found in a human study a perihematomal penumbra zone with

biochemical characteristics similar to that of the penumbra surrounding traumatic brain contusions. Such penumbra shows a relatively rapid (24–48 hours) normalization after surgical evacuation of the hematoma. Still, the changes observed in traumatic brain injury patients are suggested to be of mitochondrial dysfunction origin rather than being ischemia,[251] as supported by some others.[253] Furthermore, ROS produced by impaired mitochondria are likely to play a role in a progressive decline in mitochondrial respiration, and interact with metals released from the blood, which leads to oxidative damage.[251]

Because it seems that ischemia does not play a considerable role in the pathogenesis of perihematomal tissue damage, **other mechanisms** are proposed to play a role. These include excitotoxicity,[13,254] inflammatory changes with activation of leukocytes and platelets leading to production of inflammatory mediators (IL-1, IL-6, ICAM, TNF-α, and VEGF),[254-257] and involvement of complement[258] and MMPs.[12,259] In addition, a role for thrombin, fibrinogen, TPA,[12,260,261] and clotting factors[262] together with blood degradation products[263] has been proposed. Studies in humans support the role of cytokines (IL-6 and TNF-α)[254,257,264] and MMPs (especially MMP-3, MMP-9, and presumably MMP-12)[254,265,266] in the pathophysiology of ICH. Last but not least, hyperglycemia exacerbates perihematomal cell death and brain edema.[267]

2.2.1.3 ICH-induced edema

Gebel and coworkers[268] found **absolute edema** to increase by 37% from baseline to a 1-hour CT scan and to double during the first 24 hours, whereas **relative edema** (absolute edema volume divided by hematoma volume) increased by 75% during the first 24 hours. Relative edema, representing vasogenic edema (shown as increased relative ADC), is associated with outcome in some[233] but not in all[243] human studies. Such elevation of perihematomal ADC is significantly associated with hematoma volume.[269] Importantly, only a minimal association exists between relative edema volume and hematoma volume, confirming that hematoma volume does not confound relative edema volume measurement.[268]

Several studies address the role of the **blood** together with its **degradation products** and the coagulation cascade in association with ICH-induced brain edema. Contact between blood and tissue factor activates the extrinsic coagulation system,[270] leads to thrombin formation, and converts the blood into coagulum. Tissue factor was found in the cortex, basal ganglia, cerebellum, and cervical spinal cord of the baboon.[271] Lee and coworkers[272] studied the role of **thrombin** by injecting various solutions into the basal ganglia of rats. Evaluated 24 hours after injection, only whole blood but neither concentrated blood cells, nor serum from clotted blood, nor plasma from unclotted blood induced brain edema. However, adding prothrombinase to plasma led to edema formation similar to whole blood-mediated one; hirudin (a thrombin inhibitor) reduced it.

In further experiments, the same group found that infusion of packed erythrocytes led to delayed edema formation 72 hours later, whereas infusion of already lysed erythrocytes led to edema formation within 24 hours.[242] The latter finding suggests hemoglobin involvement. The role of thrombin and the coagulation cascade was studied in another study by the same group[273] when they injected blood, artificial clot (composed of styrene microspheres, fibrinogen, and thrombin), and separately the components of the artificial clot. Early edema was formed after clotting of the hematoma by exudation of the remaining serum proteins into the periphery of the hematoma.[258,268,274] Such hyperacute perihematomal edema was reduced by intrahematomal administration of TPA and prevented by an intrahematomal heparin injection.[275]

Based on extensive studies, the **temporal evolution** of experimental perihematomal edema has been described.[226,276] Hyperacute edema (< 24 hours) seems to be caused oncotically by serum proteins, glucose, and electrolytes. Acute edema (24-72 hours) may be caused by cellular toxicity (white blood cells and platelets), humoral toxicity (IL-1, IL-6, ICAM, TNF-α, prostaglandins (PG), LTs, VEGF, and complement), the coagulation cascade (thrombin, fibrinogen, and TPA), and excitotoxicity (glutamate). Finally, the late phase (> 72 hours) is suggested to involve blood degradation products (hemoglobin, iron, and biliverdin), NO, free radicals, apoptosis, and MMPs.

The BBB, the target of the various pathways leading to its disruption, seems to remain intact against large molecules for the first several hours,[274] with modest disruption at 12 and 24, but progressive disruption 48 hours later.[277] In our laboratory, we observed no BBB disruption, as judged by Evans blue (EB) albumin extravasation at 24 hours after ICH (unpublished data). Concordantly, Xi and coworkers[242] reported BBB disruption only 72 hours after packed erythrocyte injection, but only 24 hours after lysed erythrocyte infusion.

Differing experimental approaches to counteract the ICH-induced edema include MMP inhibitors,[87] inhibitors of monocytes/microglial activation leading to decrease in released cytokine levels,[278] inhibitors of complement activation,[258] NMDA-antagonists,[279,280] antioxidants,[281] erythropoietin,[282] and albumin.[283] Thrombin preconditioning reduces edema caused by erythrocytes and free iron.[284] Argatroban, an inhibitor of both fibrin-bound and free thrombin, reduces brain edema after ICH, presumably by interfering with thrombin-induced inflammatory responses.[285]

2.2.1.4 Inflammatory responses

After the hemorrhagic event, extracellular spaces of the brain are exposed to many blood components (hemoglobin, thrombin, plasmin, complement, fibrin degradation products, and leukocytes), which induce **inflammatory responses**.[255,276,286] Iron and iron-related compounds, including hemoglobin, catalyze hydroxyl radical production and lipid peroxidation, causing oxidative stress to the brain cells.[287] Interestingly, the only clinical

study addressing the role of free radicals in ICH[288] found no difference between ICH and control patients. However, the control tissue originated from peritumor and aneurysmal tissue, which may not represent an appropriate control.[255]

Clotting blood and damaged brain tissue liberate chemotactic factors, including thrombin, which is suggested to potentiate neutrophil infiltration.[289,290] Neutrophils, in turn, may contribute to the secondary tissue injury via processes involving the release of ROS, proteases,[289,291] and cytokines such as TNF-α and IL-6.[292] Although the primary role for activated microglia/macrophages after ICH is to clear the hematoma and tissue debris, they may contribute to further tissue damage[12] through release of cytokines, ROS, and NO.[11,255]

However, despite the increasing amount of evidence concerning the role of inflammatory machinery after ICH, what remains to be proven is whether the inflammation following ICH contributes directly to neuronal loss or is it merely an epiphenomenon. Some experimental data suggest a direct contribution based on the effects of various anti-inflammatory therapies.

To this end, different strategies were tested including antibodies against adhesion molecules,[197] anti-inflammatory actions of atorvastatin (decreased inducible NO-synthase expression and leukocytes and microglia infiltration),[293] cyclooxygenase-2 inhibitors,[294] targeting of microglial activation by minocycline[12] or tuftsin fragment 1-3,[278] MMP inhibitors,[259,295] overexpression of IL-1 receptor antagonist,[296] inhibition of TNF-α,[256] adenosine A2A receptor activation (presumably also by inhibition of TNF-α mRNA expression),[297] heme oxygenases,[281] deferoxamine,[298] and free radical trapping.[255,299] A clinical trial that primarily addressed the safety of the free radical-trapping agent NXY-059 in ICH patients found no benefit nor harm in terms of the 3-month functional outcome,[300] despite the positive results of an experimental study.[299]

2.2.2 Surgery or medical treatment for intracerebral hemorrhage?

Despite considerable efforts to dissect the pathophysiology of ICH, no other than supportive and symptomatic treatment modalities exist. These include proper control of the airways, breathing, and of circulation, blood pressure management, ICP monitoring, fluid balance, fever control, nutritional support, prophylaxis of gastrointestinal complications, seizures, and deep venous thrombosis.

2.2.2.1 Surgery

The rationale for surgical treatment in ICH is removal of the cause of the mass effect and reduction in toxic effects of the breakdown products of blood components. However,

surgical treatment of ICH failed to prove beneficial in a recent Surgical Trial in Intracerebral Hemorrhage (STICH).[14]

Unfortunately, the STICH trial did not differentiate basal ganglia-located ICH associated with intraventricular hemorrhage and hydrocephalus from superficially-located lobar ICH, which shows better prognosis.[301] Another unfortunate aspect of the STICH trial was the patients' randomization according to the neurosurgeon's subjective consideration of the need for surgical intervention either initially or during the immediate follow-up. This resulted in randomization of only 221 patients with lobar ICH (out of total 1033 patients), because many neurosurgeons favor surgery for these patients despite the absence of meaningful evidence for such a decision. Lack of any evidence was actually the reason to start the STICH trial. The pre-specified subgroup analysis of this trial found a 29% relative benefit for early surgery in patients with the more superficial ICH reaching subcortically within 1 cm of the cortical surface[14]; such subgroup analysis would, if corrected for the pre-specified subgroups, miss statistical significance.

An ongoing STICH II trial will address the outcome of early surgery in lobar nonaneurysmal ICH (reaching to within 1 cm of the cortical surface) without intraventricular hemorrhage.[302] The European Stroke Initiative recommends surgery for superficial hematomas in the latest guideline[303]; falling CCP and rising ICP together with deteriorating consciousness level are also indications for surgical evacuation.[301] Other ongoing ICH trials address treatment of intraventricular hemorrhage with clot volume smaller then 30 ml (CLEAR IVH)[304] and minimal invasive surgery in deep ICH (MISTIE).[305]

2.2.2.2 Recombinant Factor VII

Due to early hematoma growth, hemostatic therapy in ICH was considered to be a counterpart of emergency TPA treatment for acute ischemic stroke. To this point, ε-aminocaproic acid, aprotinin, and tranexamic acid were tested, albeit unsuccessfully.[306-308] Recently, a phase II b trial of recombinant factor VII found a reduced rate of clot expansion and improved outcome in the active treatment arm of the trial,[309] but the phase III trial failed to confirm improvement in outcome at 90 days despite finding the same rate of reduced clot expansion (presented by Dr. Stephan Mayer at the 16th European Stroke Conference 2007, Glasgow, UK).

2.2.3 Models of intracerebral hemorrhage

The ideal model should show reproducible volumes of hemorrhage, induce hemorrhage with mechanism(s) mimicking the clinical situation reasonably well, have the possibility of inducing hemorrhage in different regions of the brain by minimal variations of the

model, be easy to perform with slight variations among experimenters, and should not be burdened with unacceptable cost. Naturally, no model can be 100% relevant to the clinical situation, and different models can simulate reasonably well some clinically relevant aspects of the condition studied, but their limitations should be considered when interpreting study results. Luckily, there are several models to choose from, each more or less fulfilling the criteria mentioned. It is indisputable that experimental hemorrhagic stroke models are able to reproduce important pathophysiologic events relevant to the human situation. Such models helped us substantially in understanding the mechanisms and pathophysiology of hemorrhagic stroke and developing novel drugs. This is rather important, since, currently, nothing other than basic care is approved for acute medical treatment for ICH. The most commonly used ICH models are autologous blood injection model and collagenase injection model in the rat. In addition, there exists a balloon inflation model and a model of avulsion of cerebral blood vessels. Outcome measures include mortality, neurological scoring, behavioral tests, dynamics of the hematoma and of the edema growth (using MRI), extent of ischemic injury and apoptosis, neuroinflammation, and changes in CBF and ICP.

2.2.3.1 Autologous whole blood-induced ICH

Blood from an animal, collected from an easily accessible artery, is injected into the desired brain region, the basal ganglia (similar to human ICH) being the most common site of injection. Blood can be injected into virtually any brain region; but injection into the cortex is often complicated by SAH.[310] As for the volume of injected blood, range of variation among projects is wide, but 50 µl is the volume used most commonly. This amount of blood, when injected over a sufficiently long period within the stereotactically determined target area, produces hematomas that do not usually leak into compartments different from those desired: not, for instance, into intraventricular, subarachnoidal, or subdural space.

Although some researchers do inject larger volumes (up to 100 µl), such an approach may elevate ICP and lead to complications due to systemic effects.[311] Experience from our own laboratory suggests that 50 µl of blood injected with a Hamilton syringe slowly over 5 minutes results in reasonably good reproducibility of hematoma volumes. It should be mentioned that faster injections of larger volumes lead to backflow of the injected blood along the needle track, the direct tissue damage in a larger area than desired, to interstitial blood drift along the corpus callosum, or even to rupture into the brain ventricles (intraventricular hemorrhage). For these reasons, we keep the injection time constant (2 µl per 12 sec) and, before injecting the blood, we slightly withdraw the Hamilton syringe by 0.5 mm, thus producing a small pouch. Such an approach usually prevents backflow of blood and resultant intraventricular hemorrhage. Other researchers[11,312] have used double injection, i.e., the desired volume of blood injected at slow rate in two phases with a 7-minute break in between. In the first phase, a relatively smaller volume of blood is injected. It is possible that such a break will, however, lead to

clotting of blood along the needle track. For the same purpose, we keep the needle inserted at the site of injection for another 3 to 5 minutes after blood injection.
Our model is associated with approximately 30% 24-hour mortality, depending on the animal strain used.

2.2.3.2 Collagenase-induced ICH

In this model, described by Rosenberg and coworkers,[313] ICH is achieved by injection of bacterial collagenase into the basal ganglia region. As in the previous model, collagenase can be injected into virtually any brain region. The injection leads to degradation of one of the most important determinants of the basal lamina: collagen type IV. This produces the BBB leak, which in turn leads to extravasation of erythrocytes, eventually producing a solid hematoma. One can change the injected amount of bacterial collagenase. Rosenberg and coworkers[313] injected a range of 0.1 to 1 units of bacterial collagenase diluted in 2 µl of saline, with 0.5 units achieving the desired conditions. The final size of the hematoma correlates well with the amount of injected bacterial collagenase.[314] A major modification by Del Bigio and coworkers,[289] was injecting 0.14 units diluted in 0.7 µl saline. Interestingly, these authors added 1.4 units of heparin, leading to rather rapid evolution of the hematoma. Such hematomas not only show reasonable reproducibility, but appear in with uniform shapes as well. To exclude the possibility of collagenase's affecting other than the brain region desired, an elegant approach was chosen by Mun-Bryce and coworkers,[315] in which the very tip of the collagenase injector was filled with 5 µl of saline.

2.2.3.3. Balloon inflation model

This is a less commonly used mechanical model of ICH described by Sinar and coworkers,[316] studying the mass effect of a hematoma and of its removal on ischemic brain injury. Since the inflation is confirmed by x-ray, the inflating material must be a contrast agent. Modifiable variables of this model are balloon volume and inflation duration, which makes this mechanical model quite reproducible.

2.2.3.4 Cortical blood vessel avulsion

This is a rather infrequently used simple model of cortical injury, in which the surface cortical blood vessels are stripped, whereafter, avulsion of the veins leads to cortical ICH.[317,318] This model causes not only hemorrhage, however, but ischemic infarction as well; a fact which makes reliable comparison with other models rather challenging.

2.2.3.5 Summary of intracerebral hemorrhage models

The autologous blood injection model mimics ICH reasonably well, but the duration of injection and time to reach desired hematoma volume is significantly shorter than for events leading to spontaneous ICH in human beings. Furthermore, this model lacks a bleeding blood vessel. In the collagenase model, the bleeding occurs as soon as 10 to 30 minutes after injection, but develops rather slowly into a full hematoma 4 to 24 hours later.[289,313] Brain edema reaches its maximum 24 hours after induction in the blood injection model, remaining stable for several days,[277] and causes measurable deterioration in neurological function.

In the collagenase model, cerebral edema has already developed within 4 hours after injection and is resolved 48 hours later, being temporarily associated with neurological outcome.[313] Certainly, collagenase injection is simpler to perform and is not complicated by backflow of the blood along the needle track. Moreover, the amount of collagenase injected corresponds well to the hematoma's final size.

However, the cause of hemorrhage in this model differs from the clinical situation, and collagenase itself causes a robust inflammatory reaction which may cause early degradation of the hematoma,[289] with neutrophils contributing to delayed ICH-induced deterioration. Furthermore, inflammation and cell death occur earlier and are more prolonged in the collagenase model than in other methods.[318] Although anatomically the brain injury is most appropriate in the collagenase model, it is rather artificial biologically.[318] The additional inflammatory changes differ the collagenase model from the clinical situation and from the blood injection model, which resembles the clinical scenario perhaps more faithfully.[318]

In the autologous blood injection model, neutrophil infiltration begins within 24 hours, peaks at 2 to 3 days, and disappears at between 3 to 7 days.[9,290] CD8a-positive, possibly natural killer T-lymphocytes became apparent at 48 hours and persisted until 1 week.[9] Microglial reaction was evident at 4 hours, was maximal at 48 to 72 hours, and persisted for 4 weeks.[9,11] The temporal pattern of neutrophil infiltration is similar, although much more robust, in the collagenase model.[289,318]

In the blood injection model, cell death is maximal at 42 to 72 hours, with apoptosis already present at 6 hours, peaking at 3 days and continuing for at least 2 weeks after induction.[9,319] On the other hand, the collagenase model shows apoptosis within the first 24 hours after injection, peaking at 3 days and lasting at least 4 weeks.[10] The mechanical balloon inflation model was found to cause cell death involving apoptosis 6 to 24 hours after deflation. [320]

2.3 Mast cells

2.3.1 Basic characteristics

MCs are well known to be effector cells of inflammation and immunity.[321,322] They were first recognized by Friedrich von Recklinghausen in 1863, and their current name comes from the German word **"Mastzellen"** (well-fed cells, feeding cells) used by Paul Ehrlich in 1878.[323] Ehrlich observed that their cytoplasm is filled with prominent **granules,** and he identified MCs by their metachromatic properties,[324] which depend on the presence and degree of sulfation of proteoglycans such as heparin and heparan. MC identification by their metachromasia permits detection of MCs also in the brain.[325,326] MCs are derived from the pluripotential stem cells of the bone marrow that produce all **hematopoietic** cells.[327,328]

The number and distribution of MCs vary among individuals, species, and between genders; the sources of this variability are not well known. MCs contain granules that store preformed effector molecules, mediators, which are released upon activation in a process called compound exocytosis or **degranulation**.[329] MCs can be activated by different factors, they are capable of releasing various mediators, and—due to their ideal position—they can respond to a wide selection of stimuli.[330]

Besides releasing the stored content, activation of MCs triggers de novo synthesis and secretion of other mediators, including LT, PG, cytokines, and chemotactic factors, which produce profound inflammatory and vasoactive effects on the local tissue milieu.

2.3.2 Differentiation, heterogeneity, location, and abundance

2.3.2.1 Differentiation

MCs leave the bone marrow as immature cells, committed precursors,[331] before they home into specific tissues and then undergo differentiation under the specific microenvironmental conditions of the **homing tissue**.[327,332]

Some authors report that MCs enter the brain as relatively mature cells, with numerous secretory granules, along penetrating blood vessels.[333] Others[326] note that immature MCs (containing only a minuscule amount of small granules) infiltrate the CNS and undergo in situ differentiation within the neuropil, a finding generally accepted.[327,332]

It is well established that MC differentiation is dependent upon expression of the **stem-cell factor** (SCF, c-kit ligand) in tissues and c-kit on precursor-cell surfaces.[334] However, Shanas and coworkers[335] showed experimentally that brain MCs lack expression of the c-kit receptor necessary for MC survival and suggested that the special microenvironment of the CNS may provide factors other than SCF that sustain MC survival. Two factors, IL-

3[336] and nerve growth factor (NGF),[335,337] were suggested. Still, Shanas and coworkers[335] admitted the possibility that the c-kit receptor is present, but not recognized by the antisera used, or that c-kit expression or translation of the mRNA or both may be down-regulated.

2.3.2.2 Heterogeneity

Since MC differentiation occurs within organotypic tissue compartments, and MCs acquire phenotypes specific to their local microenvironment, MCs have a noteworthy heterogeneity.[327] This phenotypic diversity of MC populations, supported by both human and experimental data, means they possibly express different functions in health and disease.[327,328] At least three types of mature MCs are identified in rodents: **serosal** (lung, peritoneum, skin), **mucosal** (nasal, gastrointestinal), and **brain** (dural, perivascular, parenchymal),[338] although some debate whether brain MCs constitute a distinct type.[330,338]

MCs may, however, have a wider range of phenotypic heterogeneity, as assessed by their morphology, histochemistry, response to different drugs and stimuli of activation, and the qualitative and quantitative content of mediators they can release.[339] Fundamentally, serosal (connective tissue) MCs contain rat MC protease-I, heparin, tryptase, and chymase, whereas mucosal MCs store rat MC protease-II, chondroitin sulfate, and tryptase.[334]

Since brain MCs contain not only histamine but also heparin and rat MC protease-I, they resemble connective tissue (serosal) MCs.[340] Others[341,342] have proposed parenchymal brain MCs to be of the classical connective tissue type, as, in the rat, is the case for MCs within the connective tissue coverings, i.e., meninges of the nervous system.[343,344] Interestingly, some authors[340] showed that brain MCs lack the FcεRI receptor, which binds IgE, suggesting that brain MCs do represent a distinct phenotype.[335] However, brain MCs showing functional FcεRI-bound IgE receptors were purified by others.[345]

Once acquired, the **MC phenotype is not permanent**, since mucosal MCs can develop into connective-tissue MCs under proper microenvironmental conditions of the local milieu, mostly depending on the unique framework of local growth factors, stage of development, and the presence or absence of immune activation.[334,339] Furthermore, adjacent astrocytes may influence the phenotype or the migration of MCs,[346] since astrocytes synthesize MC growth factors such as IL-3[347,348] and NGF[349]. Accordingly, changes in the expression of MC-stimulating factors such as SCF, NGF, and IL-3, -4, -9, and -10, all alter the content of stored mediators produced by MCs.[321] Finally, the palette of mediators may vary between species, e.g., serotonin, a significant mediator in rodents, is absent from the MCs of higher mammals.[339]

2.3.2.3 Location

MCs, resident cells in the brain,[327,330] are often observed in close proximity to neurons in different peripheral tissues.[339] That MCs are present within the nervous system has been known for over 100 years,[350] and since then, the presence of MCs in the vertebrate brain has been accepted.[330,338,339,351] MCs enter the CNS during development via **penetrating blood vessels** with which they remain associated,[333] as shown by electron microscopic studies demonstrating the predominantly perivascular location of MCs.[341,352]

The known association of MCs with the vascular bed (preferentially at branching points) during development is dependent on contact of the blood vessel with astroglial processes; such adhesion to the vascular wall during development is suggested to involve MC-expressed α4-integrins.[353] In addition to their proximity to ECs, MCs reside close to fibroblasts, epithelial cells, or nerves,[327] all of which are important targets for mediator/cytokine action.

In mammals, MCs are typically found in the **dura mater, leptomeninges, choroid plexus,** and **the thalamus**.[325,330,338,351] In the latter, they reside on the neuropil surface of the BBB.[341,351] They also occur in the olfactory bulb, hypothalamus, and mesencephalon,[342,343] and in the cerebral cortex,[15,330,338] often—again—at the branching points of cortical penetrating arterioles.

2.3.2.4 Mast cell abundance in the brain

Although for unknown reasons, alterations appear in abundance of MCs in the brain, which may be caused by changes in marker-dependent detection, by the rate of both precursor entry and their differentiation activity, and by MC proliferation.[326] It is generally accepted that MCs circulate as devoted precursors rather than as mature cells.

But what is also possible (due to the rate of increase of a mature MC population in the adult brain) is that mature MCs translocate from peripheral sources into the CNS.[354] In that study, migrated donor MCs represent 2 to 20% of the total MC population in the analyzed brain region one hour after injection, suggesting fast crossing of the BBB. In the same study, reconstructions of confocal images showed that MCs were localized deep in the basal lamina, in nests of glial processes. Furthermore, electron microscopic analysis showed that MCs indeed migrate into the CNS.[355]

Although fully differentiated MCs as well as their precursors have been considered to be able to divide,[356] no evidence of MC division was found through BrdU labeling,[357] supporting the hypothesis that increase in end-organ MC population is apparently due to migration from the periphery or entry of new precursors from the circulation. Since mature cells do not circulate in the blood, it is suggested that the source of the augmented population is likely to be via the pial sheath of the thalamic blood vessels.[333,351]

The mechanism of MC transmigration into the brain capillary endothelium and its basal lamina is unknown, but angiogenic factors do stimulate MC migration in the periphery.[358] Furthermore, MCs, like other immune cells, can adhere to the endothelium and exhibit P-selectin-dependent rolling activity.[359] MCs also contain many proteases (such as chymase, tryptase, gelatinases, and cathepsin G), which can enzymatically create a temporary pathway through the endothelial junctions and the ECM of the basal lamina. This is supported by the fact that peripheral MCs are able to migrate from a connective tissue across the basement membrane to access the epithelium.[360] Moreover, in vitro data show that both dormant and activated MCs can attach to and/or move across a variety of structures,[361,362] one of them laminin, for which MCs have receptors.[361,362] Laminin occurs in the basal lamina of brain ECs and can be produced by astrocytes as well.[363]

Glial cells may also promote further MC movement by secreting matrix molecules[364] or by the expression of chemotactic factors such as transforming growth factor-β1[365] or IL-3[347,348]; the former, an MC mediator itself,[330] belongs among potent chemoattractants, as well,[366] and is also produced by astrocytes.[365] Suggesting the existence of some kind of autoregulatory mechanism, resident MCs can recruit other MCs by secreting ATP[367] and NGF.[368]

2.3.3 Activation

MCs show both IgE-dependent explosive (anaphylactic) **degranulation** and a more controlled (piecemeal) secretory process in response to non-IgE-related stimuli.[369] Degranulation is a stereotyped cascade of stimulus-activated events, biochemical and morphologic, which results in the fusion of the cytoplasmic granule membranes with the plasma membrane (with extracellular release of granule-associated mediators). Since degranulation of MCs per se is not associated with changes in the normal ultrastructure of the surrounding neuropil and no other immune-system cells are present, this suggests that the secretion occurs ad hoc without ongoing inflammation or imminent tissue damage.[330,370] Alternatively, MCs can secrete mediators without overt degranulation,[352,371] through differential or selective release.[372]

2.3.3.1 FcεRI-mediated activation

FcεRI is expressed on the MC surface,[373] and when adjacent FcεRIs are bridged (by antigens interacting with receptor-bound IgE, or by antibodies directed against either receptor-bound IgE or the receptor itself), the cells are rapidly activated and release their mediators. Bridging of only a few hundred pairs of IgE molecules is sufficient to trigger histamine release.[374] Because so few MC FcεRI's must be bridged to initiate the degranulation response, MCs cells may be simultaneously sensitized with IgE antibodies

of various specificities; they can therefore react to stimulation by many different antigens, which constitutes the basis for the stereotypic 'bulk' response of MCs in IgE-dependent immune reactions and allergic disorders.[322]

2.3.3.2 Nonimmunologic direct activation

A wide range of biologic substances, including products of complement activation, acetylcholine, and PAF, but also mechanical trauma, ionization, changes in pH, and iodine contrast agents can provoke MC degranulation and the release of MC mediators.[322,339,375] The sensitivity of different populations of MCs to individual stimuli varies, however.[339,375] Moreover, these stimuli can induce a pattern of mediator release that differs from the one associated with FcεRI -dependent MC activation.

2.3.4 Mediators

MC granules are loaded with an armamentarium of bioactive molecules: mediators. These mediators are performed or newly synthesized or both, which is the case for certain cytokines. It should be mentioned that although MCs produce a variety of mediators, no single MC subtype synthesizes the whole possible repertoire.[330]

2.3.4.1 Preformed mediators

Preformed mediators stored in the cytoplasmic granules include biogenic amines, proteoglycans, serine proteases, carboxypeptidase A, and small amounts of sulfatases and exoglycosidases.

Histamine is well known for its effects in allergic skin reactions like itching and swelling. It is a potent vasodilator and also mediates an immediate increase in vascular permeability; hence it contributes to capillary leak and edema formation in peripheral tissues and epithelium.[376] MC-derived histamine regulates expression of selectins in ECs with consecutive rolling of leukocytes.[376] Studies in genetically MC-deficient and normal mice indicate that MCs account for nearly all the histamine content stored in normal tissues, with the exception of the glandular stomach and the central nervous system. In the latter, MCs contribute up to 90% of the histamine content in the thalamus and up to 50% of whole brain histamine levels.[377] Other biogenic amines stored in MCs are the neurotransmitters dopamine and serotonin.

Proteoglycans are major constituents of MCs,[326] and MCs are the only endogenous cellular source of heparin in mammals.[378,379] Depending on location and age, human MCs can produce proteoglycans of the chondroitin sulfate and heparin sulfate type within the same cell, or individual cells may make only one.[380] Proteoglycans have several biologic

functions both within and outside the cells. They bind histamine, neutral proteases, and carboxypeptidases, and they may contribute (especially serglycin) to the packaging and storage of these molecules within the granules.[381]

Heparin can inactivate the serine protease thrombin. Specific sulfated pentasaccharide units in heparin glycosaminoglycans can bind antithrombin III, a protein that circulates in the blood, inducing an allosteric change and to a great extent enhancing its anti-clotting activity.[378,379] Studies in genetically MC-deficient mice[382] show that the natural anticoagulant properties of the vasculature reflect the presence of the heparan sulfate proteoglycans—not the heparin proteoglycans—on the surface of the ECs that line blood vessels.[379,382] As suggested by Forsberg and coworkers,[383] endogenous heparin cannot play a physiological role in regulating blood coagulation, since it is absent from the blood. Heparin and heparans are also well established as molecules necessary for the activity of some growth factors such as the fibroblast growth factor family.

Neutral proteases represent another major protein component of MC granules, where tryptase is the major enzyme stored in the cytoplasmic granules of all human MCs[322]; hence its measurement in biologic fluids such as plasma, serum, and inflammatory exudates can aid in assessing MC activation.

Tryptase is a serine endopeptidase stored in the granules in active form, which is stabilized by its association with heparin and perhaps other proteoglycans.[322] In the rat, only connective tissue MCs contain tryptase.[384] Chymase, another serine protease stored in active form in the granules of some human MCs, seems to play a major role, together with tryptase, in the proteolytic activation of latent forms of MMP-1, MMP-2, and MMP-9,[18,385-387] as well as in the physiologic degradation of tissue fibronectin and thrombin.[388] Pro-MMP-9 is a substrate for chymase in vitro.[389]

MCs themselves can release MMPs, in specific the gelatinases A (MMP-2) and B (MMP-9),[109] although it is debated whether latent MMP-9 coexists with histamine and tryptase in the granules[111] or is produced de novo.[88] The latter is supported by the finding of the MMP-9 protein being first detected at 6 hours and peaking at 22 hours after MC activation.[88] Cathepsin G, also produced by neutrophils and monocytes/macrophages, is a MC-derived protease which cleaves many components of the extracellular and pericellular matrix including fibronectin and vitronectin.[390] Last but not least, another serine protease, TPA, belongs to the palette of MC mediators.[376]

2.3.4.2 Newly synthesized mediators

Some of the MC mediators are not stored but are produced de novo and secreted only upon appropriate stimulation of the cells.[375] Of particular importance are the **cyclooxygenase** and **lipoxygenase** metabolites of arachidonic acid, which have potent inflammatory activities and which may also play a role in modulating the release process

per se.[375] The major cyclooxygenase product of MCs is neuromodulator $PGD_{2,}$[391] and the major lipoxygenase products derived from MCs are the sulfidopeptide LTs: LTC_4 and its peptidolytic derivatives, LTD_4 and LTE_4. Human MCs can also produce LTB_4, albeit in much smaller quantities than PGD_2 or LTC_4.[375] PAF, another MC mediator, has substantial chemoattractant, vasoactive, and platelet-activating properties implicated in brain injury.[392]

2.3.4.3 Cytokines

Cytokines are a diverse group of glycoproteins synthesized and, typically, secreted by many cell types in response to their activation or injury. Cytokines can modulate both specific immune responses and immunologically nonspecific inflammation by alteration of the function or gene expression in responding cells. MCs are a source of a number of cytokines.[16,322] Much of the ability of certain cytokines (IL-1, TNF-α) to promote allergic inflammation is thought to reflect the ability of these agents to enhance the recruitment of leukocytes by inducing increased expression of adhesion molecules such as P-selectin and E-selectin, vascular cell adhesion molecule-1, and ICAM-1, on ECs.[393,394]

TNF-α represents a distinct type of MC mediator, since it is derived from both preformed and newly synthesized pools.[334] Being probably the only cell type containing preformed stores of TNF-α,[395] MCs are likely to represent a critical initial cellular source of TNF-α during inflammation.[322] Later during the inflammation response, TNF-α is produced by neutrophils, eosinophils, T and B cells, and macrophages.[16] TNF-α promotes leukocyte infiltration through its effects on ECs and leukocytes, as well as promotes inflammation, granuloma formation, angiogenesis, and tissue fibrosis; MCs are involved in all these processes.[334] It should be noted, however, that most studies investigate release of cytokines upon activation via the FcεRI, and little is known as to whether indirect activation of MC leads to similar effects.

2.3.5 Mast cell functions and related disorders

Originally, Ehrlich thought that MCs help to maintain the nutrition of connective tissues.[323] Nowadays, it is widely accepted that MCs serve the host by many different functions in health and disease. Some evidence indicates that MCs participate in bacterial recognition, followed by endocytosis, processing and presentation of bacterial antigen, immune cell recruitment, and elimination of parasites.[321,396,397] In addition, MCs are involved in blood clotting, wound repair, and tissue remodeling.[16,398,399]

2.3.5.1 Type-1 hypersensitivity

Type-1 hypersensitivity is an allergic reaction provoked by re-exposure to a specific type of antigen, an allergen. In peripheral tissues, MCs play well-established roles in mediating inflammation and allergic responses during the host response to various environmental challenges.[334] The allergic reaction is generally divided into the **acute** (immediate) response mediated largely by degranulation and the **late-phase** response (LPR) resulting from MC activation and synthesis of de novo mediators.

The acute response is the pathophysiologic hallmark of allergic rhinitis, allergic asthma, and anaphylaxis. An immediate hypersensitivity reaction is initiated on the surface of MCs by the interaction of antigen-specific IgE molecules with the relevant antigen. The physiologic effects are due to the biologic responses of target cells (e.g., ECs, smooth muscle cells, glandular cells, and leukocytes) to mediators released by activated MCs. Besides allergens, other stimuli like certain activated complement fragments (anaphylatoxins C3a and C5a), neutrophil lysosomal proteins, peptides and peptide hormones, venoms, radiocontrast agents, cold exposure, calcium ionophores, narcotics, and muscle relaxants, may lead to the acute response and rapid release of mediators from MCs independently of IgE.[375]

The LPR follows the acute response by 4 to 8 hours as persistent swelling and leukocyte infiltration. Many of the consequences of IgE-dependent reactions are caused by the actions of the leukocytes recruited during the LPR rather than the direct effects of the initial release of MC mediators.[394]

It has been suggested that "**MC-leukocyte-cytokine cascade**"[334,394] makes a critical contribution to the initiation and perpetuation of IgE-dependent allergic inflammation in the airways and at other sites. Specifically, what is proposed is that activation of MCs through the FcεRI initiates the response, in part through the release of TNF-α and other cytokines that can influence the recruitment and function of additional effector cells. These recruited cells can consequently promote further progression of the inflammatory response by providing additional sources of certain cytokines.

2.3.5.2 Demyelinating diseases

The role of MCs in brain pathology was thus far rather ignored, even though their presence in the nervous system was demonstrated more than a century ago, in 1890, when they appeared in brain infarcts and at the edge of multiple sclerosis plaques.[350] One hundred years later, MCs have been suggested to serve as a link between the immune, endocrine, and nervous systems, to play an important role in the access of lymphocytes and pathogens to the brain,[338] and to be involved in mediating changes in blood flow, neurotransmission, and local immune responses in the brain.[330]

Further research concerning MCs' role in autoimmune demyelinating diseases demonstrated the ability of their neutral proteases to degrade myelin.[400,401] MCs degranulate upon exposure to myelin basic protein and can induce peripheral[402] and central[403] demyelination. Furthermore, the MC-specific enzyme tryptase is significantly elevated in the CSF of multiple sclerosis patients.[404] It is of interest that the Theoharides group[405] performed a pilot open-label clinical trial using hydroxyzine (histamine-1 receptor antagonist) in multiple sclerosis, concluding that hydroxyzine could serve as an adjuvant therapy in multiple sclerosis. However, the small number of enrolled patients and the short duration of the study precluded any definitive conclusions.

2.3.5.3 Excitoxicity and cerebral ischemia

Recently, Patkai and coworkers[406] demonstrated that brain MCs may contribute to the exacerbation of neonatal excitotoxic brain lesions produced by IL-9, suggesting a role for MCs in neonates at risk for cerebral palsy. Regarding excitotoxicity, reports showed that CNS neurons can acquire MC products at least in three ways[370] and that MC modulation causes excitation or inhibition of thalamic neuronal activity.[407] Skaper and coworkers[408] demonstrated that histamine potentiated NMDA receptor-mediated excitotoxicity in cultured hippocampal neurons and suggested a role for MCs in conditions under which enhanced glutamatergic neurotransmission occurs in conjunction with tissue acidification, such as epilepsy and cerebral ischemia.

Supporting this hypothesis, degranulation of MCs appeared to be dependent on local pH, which influenced the dissociation rates of proteoglycan-associated mediators; for instance, histamine is released very rapidly, with tryptase and chymase released much more slowly.[322] Such a pH-dependent occurrence may be relevant to the local lactate acidosis that prevails under ischemic conditions.

Indeed, brain MCs are demonstrated to be involved in cerebral ischemic insults,[409-411] with a role for MCs in cerebral ischemia and hemorrhage (I-III). Later, Jin and coworkers[412] found a rapid increase in cerebral populations of MCs and in their activation in association with hypoxic-ischemic (HI) brain damage in the immature rat. Activated MCs were present in the pia mater and parenchyma as well, and, importantly, were found in those regions showing neuronal loss. Those in the latter region were significantly reduced by MC stabilization, administered either pre- or post-HI or post-HI only. Jin and coworkers[412] observed a very important phenomenon: that hypoxia alone did not lead to MC-dependent brain damage, even if it was associated with elevated MC count and with some level of MC degranulation. This suggests that MCs react rather differently to different stimuli and not necessarily always by full-blown degranulation.

2.3.5.4 Other conditions

MCs have been reported to be actively involved in a range of other conditions, including migraine, inflammatory arthritis, atopic dermatitis, coronary inflammation, interstitial cystitis, irritable bowel syndrome,[413] atherothrombosis,[414] angiogenesis, tissue damage, neoplasms, blood clotting, wound repair and tissue remodeling,[16,398,399] and are found in human cerebral arteries after aneurysm rupture.[415]

3 AIMS OF THE STUDY

The aims of the study included in this thesis were to test the hypothesis whether mast cells play a role in the pathophysiology of ischemic stroke and intracerebral hemorrhage by use of in vitro and in vivo experiments. The latter examined pharmacological modulation of mast cell degranulation by application both of stimulatory and of inhibitory interventions. In addition, genetically modified mast-cell-deficient rat strains allowed authentication of the hypothesis behind the studies.

Specific questions elucidating possible mast cell involvement included:

1. Do mast cells play a role in the regulation of **blood-brain barrier permeability** following transient focal brain ischemia?

2. Are mast cells involved in promoting further sinister consequences of spontaneous or **tissue plasminogen activator**-mediated ischemia-**reperfusion injury**, such as development of brain **edema** and **hemorrhage** and **neutrophil infiltration**?

3. Is there a role for mast cells in regulating the growth of hematoma and expansive brain edema in **intracerebral hemorrhage**?

4. How are these effects of mast cell modulation related to **neurological outcome** and mortality?

4 MATERIALS AND METHODS

All experiments were carried out in Biomedicum Helsinki. The animal research committee approved all studies. All experiments were blinded, and group assignments were randomized.

4.1 Anesthesia

Experimental animals were adult male **Wistar** rats (Harlan Nederland, Horst, The Netherlands), and **MC-deficient WsRc$^{Ws/Ws}$** rats (Japan SLC, Inc., Tokyo, Japan), 290 to 340 grams. Anesthesia was achieved by an intraperitoneal injection of **ketamin** hydrochloride (50 mg/kg, Ketalar, Parke-Davis, Detroit, MI, USA) and a subcutaneous injection of **medetomidine** hydrochloride (0.5 mg/kg, Domitor, Orion, Espoo, Finland).

4.2 Measurement/monitoring of physiological parameters

A polyethylene tube was inserted into the left femoral artery for **blood pressure** monitoring (Olli Blood Pressure Meter 533, Kone, Espoo, Finland) and for collecting blood samples for measurement of **arterial pH**, **blood gases**, and **blood glucose** (AVL OPTI, Roche, Basel, Switzerland), and another tube into the left femoral vein for drug or vehicle **infusion** or both. Rectal **temperature** was maintained at 37 °C during the surgery with a heating blanket and a heating lamp.

4.3 Focal cerebral ischemia model

Transient focal cerebral ischemia was induced by the **suture occlusion model**. The right CCA and the right ECA were exposed through a ventral midline neck incision. The proximal CCA and the origin of the ECA were ligated. A 4-0 nylon monofilament suture (Ethilon Nylon Suture, ETHICON Inc., Somerville, NJ, USA) with its tip rounded by heating near a flame and then coated with silicone (Bayer, Leverkusen, Germany) was inserted into the right CCA via an arteriectomy approximately 3 mm below the right carotid bifurcation and was advanced into the ICA approximately 17 mm above the carotid bifurcation. At that point, a slight resistance is felt, indicating that the occluder is lodged in the ACA, thus occluding the orifice of the MCA, the ACA, and the posterior communicating artery. This model mimics well large hemispheric strokes, which are complicated with malignant swelling.

Blood pressure and body temperature were continuously monitored and recorded every 30 minutes. **Reperfusion** was accomplished by withdrawing the suture occluder 60 (I) or 90 (II) minutes after MCA occlusion. **Sham-operated** animals underwent the same

procedure, except that the suture occluder was inserted only 10 millimeters above the carotid bifurcation and was withdrawn one minute later. At the end of the experiment, the femoral catheters were removed, operation wounds sutured, and the animals allowed free recovery in separate cages.

4.4 Cardiac perfusion and tissue handling

After various periods of time following reperfusion, the rats were re-anesthetized with an intraperitoneal injection of 120 mg of **pentobarbital** sodium (Mebunat, Orion, Turku, Finland), and cardiac perfusion was performed. Briefly, the chest was opened, a catheter was inserted into aorta via the left ventricle while the heart was still beating but respiration had ceased, and 200 mL of ice cold 0.9% saline was infused at 100 mmHg inflow pressure into the arterial vascular system. Simultaneously, the right atrium was incised open to allow all the blood to be drained.

After cardiac perfusion, the brains were quickly removed and dissected coronally into six 2-mm-thick slices with a standard brain-cutting matrix. Each third slice was cut into two 1-mm portions (rostral and caudal). The rostral part was embedded in Tissue-Tek (Sakura Finetek Inc., Tokyo, Japan), **snap-frozen** in liquid nitrogen, and kept thereafter at -80 °C until 15-µm sections were cut for BBB permeability analysis.

Then 5-µm sections were cut from the caudal site of all slices and stained with a) **hematoxylin-eosin**; b) **Toluidine blue**, a standard metachromatic histopathological technique to detect the heparin-containing granules present exclusively in MCs (I, Figure 1A,B); and c) **chloracetate esterase (Leder)**[416] staining to detect polymorphonuclear neutrophils. Toluidine blue is an aniline dye to which MCs display the property of metachromasia, due to the dye's binding to sulfated glycosaminoglycans in MC granules.[417] The remaining slices were incubated for 15 minutes in 2,3,5 **triphenyltetrazolium chloride (TTC)** at 37 °C, and subsequently immersion-fixed in 10% formaldehyde.

4.5 Laser-Doppler flowmetry

CBF was measured by the BF/F/0.5 bare-fiber flexible probe of the Oxy-Flow device (Oxford Optronix, Oxford, UK). The scalp was incised in the midline and the skull exposed. The skull was thinned by a dental drill at an area ipsilateral to the ischemia between 1.0 to 2.5 mm posterior and 6.0 to 1.5 mm lateral from the bregma. The probe was attached, within the thinned area, to the skull surface at a place of representative baseline CBF signal. The CBF signal was then obtained from the same place throughout the entire experiment.

4.6 Intracerebral hemorrhage model

For ICH, we used the **autologous whole blood injection model** described elsewhere.[272] The head of the animal was fixed into a **stereotaxic frame** (Stoelting, Wood Dale, IL, USA) and a midline scalp incision was made to disclose the calvarium of the skull. A burr hole (1 mm in diameter) was drilled on the right side of the cranium: 0.2 mm anterior and 3.0 mm lateral to the bregma. A 27-gauge needle attached to a **Hamilton syringe** was inserted into the core of the right basal ganglia (at a 6.0 mm depth from the skull surface) and subsequently lifted by 0.5 mm, creating a small cavity. Then, 50 µl of freshly collected homologous arterial blood was injected into the brain slowly over 5 minutes, after which the needle was kept in place for 3 minutes. The burr hole was sealed with bone wax, and the scalp was sutured.

Twenty-four hours later, the animals were terminated with an overdose (120 mg) of sodium barbiturate (Pentobarbital, 1 mL, intraperitoneally) and underwent cardiac perfusion with 200 ml of ice-cold saline (see above). Afterwards, the brains were harvested, cut through the site of the intracerebral blood injection into 2 blocks (in order to examine the hematoma at its largest epicenter), and photographed with a digital camera (Sony Mavica, Tokyo, Japan).

4.7 In vitro assay of TPA-mediated MC degranulation

Rat peritoneal MCs were obtained from Wistar rats by an experienced coworker as described.[418] A standard amount of $2x10^5$ MCs in 50 µl of phosphate-buffered saline were placed into Eppendorf tubes and preincubated for 10 min at 37 °C. Then, freshly prepared TPA solution (Actilyse®, Boehringer-Ingelheim, Germany) was added to give the final concentrations indicated (ranging from 0 to 50 µg/ml), and incubation was continued for 15 min to allow completion of MC degranulation. Immersing the tubes in ice-cold water stopped the reaction, and the cells were sedimented by centrifugation at 4 °C. The histamine concentration in the supernatant was determined fluorometrically according to Bergendorff and Uvnäs with modifications.[419] Histamine release by TPA was expressed as a percentage of the maximal histamine release induced by the standard MC secretagogue compound 48/80 (1 µg/ml) set as 100%.

4.8 Drug characteristics and administration

1. Sodium cromoglycate (Sigma-Aldrich, Steinheim, Germany), a clinically recognized inhibitor of MC degranulation, given intracerebroventricularly (icv) to overcome its presumed minimal crossing of the BBB.[420] Due to a short biological half-life (90 minutes), the icv administration of cromoglycate in the 24-hour group (II) was followed by continuous icv infusion of cromoglycate with Alzet® osmotic pumps (model 2001, 200 µl,

1 µl/hour) and Brain infusion kit 2 (both Durect Corporation, Cupertino, CA, USA) according to instructions.
In Study III, however, in addition to icv administration, an iv route was also used, based on a report that cromoglycate was effective in an acute stress model when given iv.[421] This implies that this drug may penetrate into the brain at some extent.
Cromoglycate was suggested to inhibit MC degranulation by regulating phosphorylation of an MC 78-kDa protein involved in the regulation of secretion.[422] This protein appears to be involved in signal transduction by regulating functional associations between cell surface and cytoskeleton.[422]

2. Compound 48/80 (Benzeneethanamine, 4-Methoxy-N-Methyl-(9CI)*N-(P-Methoxyphen-ethyl) Methylamine*, Sigma-Aldrich, Steinheim, Germany) is a condensation product of N-methyl-p-methoxyphenethylamine with formaldehyde and is a standard MC degranulating secretagogue.[341,423,424] It inhibits calmodulin and activates G proteins. Compound 48/80 degranulates brain MCs in mammals.[341,425]

3. TPA (Actilyse®, Boehringer-Ingelheim, Germany) is the only approved pharmacological therapy for acute ischemic stroke. Ten mg/kg of body weight is the standard dose for TPA thrombolysis in rats in studies focusing both on therapeutic efficacy and TPA-mediated hemorrhage.[147]

4. Drug administration A polyethylene tube was inserted into the left femoral vein for drug or vehicle infusions or both. For icv drug administration, the head of the animal was fixed in a stereotaxic frame (Stoelting), and a midline scalp incision was made to disclose the calvarium of the skull. A burr hole was drilled on the left side of the skull: 0.9 mm posterior and 1.6 mm lateral to the bregma. A 27-gauge needle attached to a Hamilton syringe was then inserted into the left lateral brain ventricle (at 3.4 mm depth from the skull surface), and drug or saline was slowly injected over 3 minutes.

4.9 Study protocols

In addition to the **pharmacological modulation of MCs**, **genetically modified MC-deficient WsRc$^{Ws/Ws}$ rats**[426,427] carrying a defective gene for **c-kit** (ligand for SCF required for MC differentiation) were used. In comparison to their WT littermates, **newborn** WsRc$^{Ws/Ws}$ rats are **MC deficient** and **anemic**. Anemia, however, is already ameliorated by the age of 10 weeks, whereas the MC deficiency increases; this has led to wide use of adult rats in specific investigations of MC function.[426]

The present studies used adult rats (13-14 weeks old). Table 2 shows their hematological (Coulter Counter T-660, Coulter Electronics, London, UK) and hemostatic markers (Coagulometer KC-40, Lemgo, Germany) obtained by a mechanical clot method from rat plasma with 3.8% sodium citrate addition, as provided by the supplier (Japan SLC, Inc., Tokyo, Japan).

Table 2. Hematological markers and coagulation parameters in 12- to 13-week-old MC-deficient rats (WsWs) and their wild-type littermates

Parameters	WsWs (n=10)	Wild-type (n=8)
RBC ($x10^4/mm^3$)	608.0 ± 10.2	765.0 ± 37.0
Ht (%)	41.3 ± 1.1	44.2 ± 0.4
Hb (g/dL)	14.8 ± 0.3	16.2 ± 0.2
WBC ($x10^2/mm^3$)	86.0 ± 7.3	93.0 ± 5.3
BP ($x\ 10^4/mm^3$)	99.2 ± 3.5	96.5 ± 2.7
MCV (fL)	66.5 ± 0.9	52.3 ± 0.4
MCH (pg)	25.5 ± 0.5	19.4 ± 0.2
MCHC (g/dL)	38.3 ± 0.3	37.1 ± 0.2
APTT (sec)	15.0 ± 0.8	16.5 ± 0.3
PT (sec)	17.6 ± 0.3	18.6 ± 0.4

RBC/WBC, Red/white blood cell; **Ht,** Hematocrit; **Hb,** Hemoglobin; **BP,** Blood platelets; **MCV,** Mean corpuscular volume; **MCH,** Mean corpuscular hemoglobin; **MCHC,** Mean corpuscular hemoglobin concentration; **APTT,** Activated partial thromboplastin time; **PT,** Prothrombin time

4.9.1 Study I

Pharmacological modulation: Three groups of rats that underwent transient MCA occlusion received cromoglycate or compound 48/80 or saline (Table 3). Furthermore, sham-operated animals were included (4 receiving each treatment) (Table 3). Three separated groups of animals that underwent transient MCA occlusion were included for laser-Doppler measurement: control (n=4), cromoglycate (n=5), and compound 48/80 (n=4).

Gene manipulation: Afterwards, I induced transient MCA occlusion in MC-deficient rats and in their WT littermates (Table 3).

Table 3. Study protocols in Study I

	Pharmacological modulation			Gene manipulation	
Group	Cromo/Sham	C48/80 / Sham	Control/Sham	WsWs	Wild-type
N	14 / 4	11 / 4	13 / 4	10	8
-5 min	**cromo** icv	saline icv	saline icv	-	-
0 min	MCAO / sham	MCAO / sham	MCAO / sham	MCAO	MCAO
57 min	saline iv	**C 48/80** iv	saline iv	-	-
60 min	R / -	R / -	R / -	R	R

cromo icv or saline icv, cromoglycate 750 µg (dissolved in saline to a final volume of 10 µl) or saline 10 µl, given intracerebroventricularly (icv); **C 48/80 iv or saline iv,** compound 48/80 0.5 mg/ml of saline (200 µl) or saline 200 µl given intravenously (iv); **R,** reperfusion; follow-up 3 hours after reperfusion

4.9.2 Study II

Pharmacological modulation: Several series were performed with different timing of experiments. The infusion protocol of TPA was identical to that used in humans (Table 4). First, with 6 hours' follow-up after induction of MCA occlusion, a model to evaluate TPA-induced hemorrhage formation (HF), inflammatory cell infiltration, and the potential effect of the timing of the TPA infusion (before or after reperfusion) was established (Experiment 1).

Then, with the same 6-hour follow-up, I focused on the effect of pharmacological MC stabilization with cromoglycate on TPA-induced HF (Experiment 2). To assess the effect of post-ischemic interval and the treatment effect on the HF and outcome, I performed experiments with pharmacological MC stabilization with a 3- and a 24-hour follow-up (Experiments 4 and 5).

Gene manipulation: To confirm the role of MCs, I investigated the effect of TPA in genetically modified MC-deficient rats and their WT littermates after 6- and 24-hour follow-up (Table 4, **Experiments 3 and 6**).

Table 4. Study protocols in Study II

	Experiment 1 (6 h)				**Experiment 2 (6 h)**		**Experiment 3 (6 h)**	
Group	Sham	Saline	Early TPA	Late TPA	Cromo/TPA	Saline/TPA	WsWs	Wild-type
N	7	7	11	7	10	12	6	4
-5 min	-	-	-	-	**cromo** icv	**saline** icv	-	-
0 min	sham	MCAO	MCAO	MCAO	MCAO	MCAO	MCAO	MCAO
85 min	saline	saline	**TPA**	saline	TPA	TPA	TPA	TPA
90 min	-	R	R	R	R	R	R	R
180 min	saline	saline	saline	**TPA**	-	-	-	-

	Experiment 4 (3 h)			**Experiment 5 (24 h)**			**Experiment 6 (24 h)**	
Group	Sal/Sal	Sal/TPA	Cromo/TPA	Sal/Sal	Sal/TPA	Cromo/TPA	WsWs	Wild-type
N	6	6	6	7	11	6	6	4
0 min	MCAO	MCAO	MCAO	MCAO	MCAO	MCAO	MCAO	MCAO
15 min	saline icv	saline icv	**cromo** icv	saline icv	saline icv	**cromo** icv	-	-
85 min	saline	**TPA**	**TPA**	saline	**TPA**	**TPA**	TPA	TPA
90 min	R	R	R	R	R	R	R	R

cromo icv or saline icv, cromoglycate 750 µg (dissolved in saline to a final volume of 10 µl) or saline 10 µl, given intracerebroventricularly (icv), and in the 24-hour groups followed by continuous icv infusion of the same concentration of cromoglycate or saline with ALZET® osmotic pumps; **saline (Sal) or TPA,** intravenous bolus (10%) of calculated dose of TPA (10 mg/kg of body weight dissolved in saline) or a corresponding amount of saline, followed by 60-minute infusion of the rest of TPA or corresponding amount of saline; **R,** reperfusion

4.9.3 Study III

Pharmacological modulation: Four groups of rats received cromoglycate (two groups), compound 48/80, or saline 5 minutes before induction of autologous blood injection into the basal ganglia (Table 5).

Gene manipulation: MC-deficient rats and their WT littermates underwent the induction of ICH without any pharmacological modulation (Table 5).

Table 5. Study protocols in Study III

	Pharmacological modulation				**Gene manipulation**	
Group	Control	Cromo icv	Cromo iv	C 48/80	WsWs	Wild-type
N	11	11	10	11	8	8
-5 min	saline icv	**cromo icv**	saline icv	saline icv	-	-
-5 min	saline iv	saline iv	**cromo iv**	**C 48/80 iv**	**-**	**-**
0 min	ICH	ICH	ICH	ICH	ICH	ICH

cromo icv or saline icv, cromoglycate 750 µg (dissolved in saline to a final volume of 10 µl) or saline 10 µl, given intracerebroventricularly (icv); **cromo iv or C 48/80 iv or saline iv,** cromoglycate (100 mg/kg of body weight dissolved in saline to a final volume of 200 µl), or compound 48/80 (0.5 mg/ml of saline, 200 µl), or saline 200 µl given intravenously (iv); **ICH,** intracerebral hemorrhage

4.10 Magnetic resonance imaging

MRI scanning was performed with a 4.7 Tesla scanner (PharmaScan, Bruker BioSpin, Ettlingen, Germany) using a linear birdcage radiofrequency coil with an inner diameter of 38 mm. Following shimming and scout images, coronal T_2^*-weighted images were acquired with a gradient echo sequence (repetition time = 277 ms, echo time = 8 ms, flip angle = 35 °, matrix size = 256 × 256, field of view = 40 × 40 mm, number of averages = 4, slice thickness = 1 mm). Afterwards, coronal T_2-weighted images (T_2-WI) were acquired by rapid acquisition with a relaxation enhancement sequence (repetition time = 5257 ms, effective echo time = 64 ms, echo train length = 16, matrix size = 256 × 256, field of view = 40 × 40 mm, number of averages = 2, slice thickness = 1 mm). The rats were placed in the MRI scanner within 10 minutes after ICH induction. The survivors were reanesthetized 24 hours later, and MRI data were collected using the same sequences. Baseline data corresponds to 40 to 50 minutes after ICH induction.

Core **body temperature during imaging** was maintained at 37 °C by use of a MRI-compatible heating pad and pump (Gaymar Industries, Orchard Park, NY, USA).

MR images correlated very well with histopathological changes in a collagenase model of ICH, and the evolving appearance of human ICH on T_2-WI images has been attributed to

the effects of hemoglobin degeneration together with changes in tissue water (edema).[289] T_2*-WI is preferentially used to visualize the hematoma in ICH experiments.[245]

4.11 Evaluation of the blood-brain barrier damage

To visualize BBB leakage, animals received a 2% solution of **EB** albumin (fluorescent dye, Sigma, 20 mg/mL dissolved in 1% albumin) into the femoral vein (0.3 mL/100 g) 20 minutes before cardiac perfusion. EB is a **standard method** of BBB permeability assessment.[428]

In the experiments with pharmacological modulation of MCs (I), the 15-μm brain sections were examined for distribution of characteristic red fluorescence of EB in brain parenchyma by **epifluorescent microscope** Axioplan 2 (Carl Zeiss, Hallbergmoos, Germany) and a fluorescence filter specific for EB (Chroma, Rockingham, VT, USA, excitation at λ = 620 nm, emission at λ = 680 nm). For evaluation of BBB damage, images of 5 pre-specified regions of interest (3 from cortex and 2 from basal ganglia) from the infarcted area, as well as a reference image from the healthy hemisphere, were captured with an AxioCAM HR digital camera (Carl Zeiss). Digital imaging fluorescence microscopy is reliable in measurement of tracer concentration in sectioned tissue.[429]

We quantified EB-fluorescent pixels with Image J analyzing software (NIH, Bethesda, MD, USA). The level of autofluorescence was based on that obtained from the healthy hemisphere. For each animal, we calculated the difference between the fluorescence signal in five selected regions of interest (represented by the characteristic red fluorescence) and the level of autofluorescence. Finally, signals from all five regions of interest were averaged.

Following the acquisition of an improved **fluorescence scanner** Typhoon 9400 (Amersham Biosciences, Buckinghamshire, UK), a slightly revised technique was adopted for the gene-manipulated animals in Study I, and for the entire Study II. With this scanner, the difference in average fluorescence signal intensity (Figure 4) between the entire infarcted area and the intact hemisphere could be measured by image analyzer software ImageQuant (Amersham).

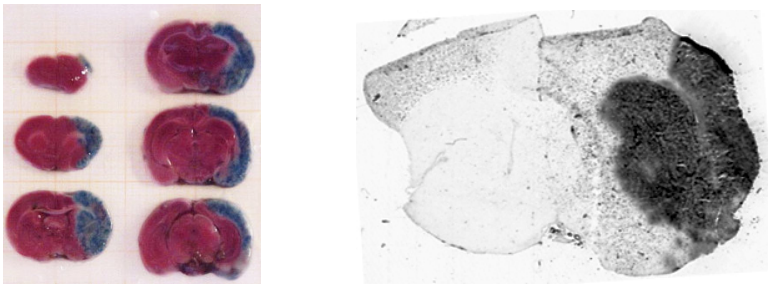

Figure 4. Extravasated Evans blue, digital imaging and fluorescence scanner

4.12 Histological evaluation

Light microscopy was performed by an experienced hematopathologist without prior knowledge of animal grouping with an Olympus BH-2 (Olympus, Tokyo, Japan). Findings were photographed with a Nikon Eclipse E600 microscope connected to a Nikon Coolpix 995 digital camera (Nikon, Tokyo, Japan).

The densities of intravascular and emigrated neutrophils were counted in systematically placed target areas in 5-µm chloracetate esterase-stained cross-sections through the area of maximal infarction. In each area, a total of 60 to 100 microscopic fields of 0.1 mm^2 were counted and averaged. Neutrophil number was counted in the temporoparietal infarction core and in the adjacent parasagittal infarct penumbra, as well as in the deep thalamic and basal ganglia.

4.13 Calculation of brain infarction and swelling

All (six) **TTC-stained** brain slices mounted on a scale were photographed (Figure 5) with a digital camera (Sony, Tokyo, Japan). TTC staining has served as a stain to detect ischemic infarction since 1958. This water-soluble salt is not a dye and is reduced in normal tissue by mitochondrial enzymes (specifically, succinate dehydrogenase) to a fat-soluble, light-sensitive compound called formazan that turns intact tissue deep red. The unstained area represents infarcted tissue.

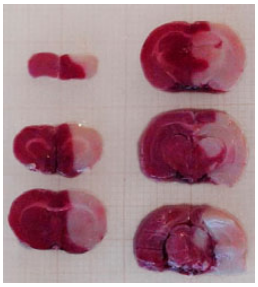

Figure 5. TTC staining, infarction

Infarction and corrected infarction volumes were calculated. Briefly, the areas of the infarcted tissue and the areas of both hemispheres were calculated for each brain slice. The **uncorrected infarct volume** was calculated by measuring the unstained area in each slice, multiplying it by slice thickness, and then summing all six slices. The **corrected infarct volume** was calculated to compensate for the effect of brain edema. The difference between the areas of the right and left hemisphere in a slice was considered edema and subtracted from the infarct area of that slice (corrected infarct area = uncorrected infarct area – [right hemisphere area – left hemisphere area]). The result was multiplied by slice thickness, and all six slices were summed to get total

corrected infarct volume. **Percentage of brain swelling** was derived from the volumetric growth of the ischemic hemisphere in comparison to the intact one (% of hemispheric expansion = [(right hemisphere volume / left hemisphere volume) – 1] * 100).

4.14 Calculation of hemorrhage formation

The areas of hemorrhage were calculated from the caudal site of all slices stained with **hematoxylin-eosin** (Figure 7D) by use of an Axioplan 2 microscope (Carl Zeiss, Hallbergmoos, Germany). All areas with HF were photographed with an AxioCam MRc digital camera (Carl Zeiss) connected to the microscope. Finally, total areas containing extravasated erythrocytes and representing intracerebral HF were calculated from digital images using Image J analysis software (NIH, Bethesda, MD, USA).

4.15 Calculation of intracerebral hemorrhage, brain swelling, and edema

Based on the specific signal intensity on T2*-weighted images, **hematoma** volume was calculated at 30 minutes and 24 hours (III, Figure 1). The boundaries of the hematoma were tracked manually (Paravision, Bruker BioSpin, Ettlingen, Germany), the surface area on each slice was multiplied by slice thickness, and the values were summed to yield total hematoma volume.

Since the hematoma volume was calculated from T2* images, these were used for calculation of **brain swelling** as well. The area of both hemispheres was first outlined and calculated on each slice (Paravision), and the areas were then multiplied by slice thickness, yielding the total volume of the two hemispheres. Afterwards, the percentage of hemispheric expansion (the volumetric increase of the ICH hemisphere compared to the intact one, was calculated (% of hemispheric expansion = [(right hemisphere volume / left hemisphere volume) – 1] x 100). The reliability of this approach was recently reported.[430]

The characteristic hyperintense area on T_2-WI, representing **brain edema** (III, Figure 4), was outlined on each slice and multiplied by slice thickness. The values were summed to yield total volume. Furthermore, I calculated T2 ratios by dividing the mean T2 signal intensity of the hyperintense areas by a reference point outside the brain tissue, a method adopted from Del Bigio and coworkers.[289] The T2 ratio is reported as the average of all hyperintense areas and separately as the average of the perihematomal hyperintense rims (III, Figure 5).

Postmortem, **hematoma** area was calculated at its maximal diameter from the digital images obtained after the second MRI round, 24 hours after ICH induction. The

hematoma area was outlined and measured with Image J software, and the percentage of brain swelling was calculated from the same digital images just as it was from MR images.

4.16 Mortality and neurological score

We scored neurological performance at 24 hours, before re-anesthesia, on a 6-point scale,[431] comprising **0**: normal; **1**: contralateral paw paresis; **2**: same as 1, plus decreased resistance to lateral push; **3**: same as 2, plus circling behavior; **4**: no spontaneous walking, plus depressed level of consciousness; **5**: death.

4.17 Statistical analysis

Data are presented as mean ± SE (I and II) and as mean ± SD (III). Normally distributed parametric data sets in multiple groups were compared with one-way ANOVA followed by the Holm-Sidak post-hoc test; an unpaired t-test was used in cases of two-group comparison. Neurological scores in multiple groups were compared with the Kruskal-Wallis ANOVA on ranks, followed by Dunn's post-hoc test; the Mann-Whitney rank sum test served for two-group comparison (neurological scores reported as medians and individually for each animal). Comparison of mortality versus control values was performed with Fisher's exact test. A two-tailed value of $P<0.05$ was considered significant.

5 RESULTS

5.1 Study I

This study addressed a role for MCs in regulation of BBB permeability, brain swelling, and neutrophil infiltration following transient (60 minutes) focal cerebral ischemia in the MCA occlusion model. Experiments involved both **pharmacological modulation of MCs** (either blocking or promoting their degranulation) and **gene-manipulated MC-deficient** animals.

No significant differences appeared among study groups in **physiological parameters** (MABP, temperature, pH, $PaCO_2$, PaO_2, glucose). After MCAO, a transient nonsignificant 7% reduction, compared with control figures, in MABP in the compound 48/80-treated group occurred at 100 min.

To confirm successful MCA occlusion and reperfusion, CBF was monitored by **laser-Doppler flowmetry** in three groups of animals: control, cromoglycate, and compound 48/80. No significant differences in CBF appeared among the study groups at monitored time points.

Corrected lesion volumes were similar in experiments with both pharmacological MC modulation and gene manipulation (P=0.33 and P=0.41, respectively), with no lesions in sham-operated animals, as calculated from TTC-stained brain sections.

Brain sections stained for **histopathological evaluation** revealed that already in the early post-ischemic phase, 4 hours after MCAO, ischemic neuronal necrosis was visible (hematoxylin-eosin staining). The sections stained with toluidine blue for detection of heparin in MC granules (MCs being the only cell type containing heparin[378,379]) showed that MCs were frequently visible in the vicinity of small cerebral cortical and thalamic penetrating vessels, often in widened Virchow-Robin spaces (Figure 2, middle), as described earlier.[341] In ischemic areas, extracellular granules on the abluminal surfaces of blood vessels were accompanied by perivascular edematous changes. Histopathological evaluation suggested milder ischemic edematous changes in MC-deficient rats than in their WT littermates.

BBB disruption (assessed by magnitude of EB albumin extravasation) was highest after treatment with compound 48/80, followed by control figures, and was least in the cromoglycate group (Figure 6A). Furthermore, MC-deficient rats showed significantly less BBB disruption than did their WT littermates (Figure 6B). In the sham-operated animals, no changes occurred in amount of fluorescence between left and right hemispheres (P=0.96).

In accordance with level of BBB disruption, the maximum **brain swelling** was associated with compound 48/80 treatment (an 89% increase compared with control) and, again,

was least in the cromoglycate group (a 39% decrease compared with control) (ANOVA P<0.001). Similarly, MC-deficient rats responded to transient MCAO with 58% less ischemic brain swelling than did their WT littermates (P<0.001). No difference appeared between the volumes of the right and left hemispheres in the sham-operated animals (P=0.71).

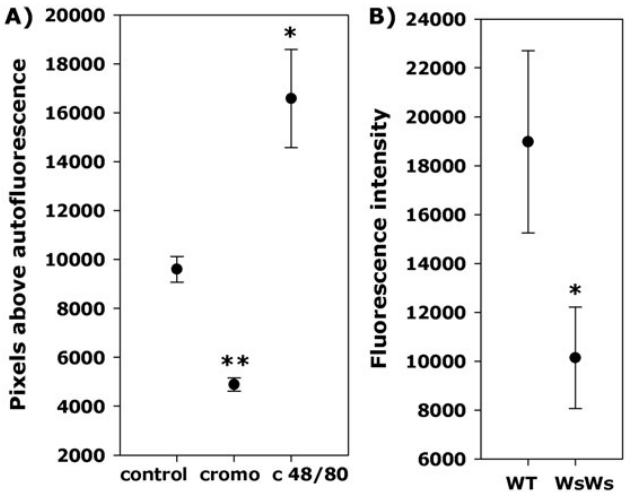

Figure 6. Magnitude of Evans blue fluorescent signal representing intensity of albumin extravasation and BBB leakage. (A) Pharmacologically modulated groups (cromo=cromoglycate, c 48/80=compound 48/80). Kruskal-Wallis ANOVA showed a highly significant effect of pharmacological modulation (P<0.001); post-hoc tests: *P<0.05, **P<0.01. B) MC-deficient rats (WsWs) and their wild-type littermates (WT). *P<0.05 (B). (Figure 2 in I).

Finally, **neutrophil count** was up to 2.5-fold higher in the ischemic hemisphere than in the non-ischemic one or in sham-operated rats (P<0.05) even at this early (4 hours) post-ischemic time point. The lowest neutrophil counts in the ischemic hemisphere were in the MC-deficient rats, being only 47% of their WT littermates (P<0.01). Furthermore, cromoglycate significantly reduced neutrophil density in the ischemic hemisphere (by 37%) compared with controls (P<0.01). This difference was most pronounced in the infarct core and basal ganglia. Cromoglycate reduced not only the number of emigrated neutrophils (P<0.05) but also of those still detectable within the intravascular space (P<0.01).

Treatment with compound 48/80 was associated with a clear trend toward enhanced neutrophil response, with borderline significance in the area of the basal ganglia (35% increase, P=0.06). In sham-operated rats, the pharmacological treatments cause no changes in neutrophil counts that would be comparable to the ipsilaterally elevated levels in the rats that underwent MCA.

5.2 Study II

This study described the involvement of MCs in regulation of BBB damage, formation of edema and hemorrhage, and neutrophil infiltration in experiments with **TPA**. **In vitro** assay of TPA-mediated MC degranulation preceded in vivo MCAO experiments with **pharmacological modulation of MCs** and experiments in **MC-deficient animals**.

In vitro experiments with TPA showed a dose-dependent release of histamine indicating MC degranulation (Figure 7A). TPA-dependent histamine release reached almost 50% of the maximal MC degranulation-associated histamine release achieved by the classic MC-secretagogue compound 48/80. Thus, even small concentrations of TPA, achievable also in vivo during therapeutic thrombolysis, strongly stimulated MCs to degranulate and release histamine in vitro.

No significant differences emerged in **physiological parameters** (MABP, temperature, pH, $PaCO_2$, PaO_2) among study groups.

To confirm successful MCA occlusion and reperfusion, CBF was monitored by **laser-Doppler flowmetry** in Experiments 4 (pharmacological modulation with 3 hours of follow-up), 5 (pharmacological modulation with 24 hours of follow-up), and 6 (gene manipulation with 24 hours of follow-up). No significant differences in CBF appeared among the study groups at monitored time points (unpublished data).

Corrected lesion volumes calculated from TTC-stained brain slices were not influenced by the treatment assignments in any of the experiments (P ranging from 0.44 to 0.96) and ischemic neuronal changes were confirmed by light microscopy of hematoxylin-eosin-stained tissue sections.

Further **histological evaluation** showed that TPA treatment was frequently associated with erythrocyte extravasation (Figure 7D), generally manifested as a perivascular hemorrhagic cuff arising from segmental structural deterioration of the vessel wall (Figure 7B), often accompanied by disseminated intraparenchymal hemorrhages. Similar to Study I, in sections stained with heparin-detecting toluidine blue, metachromatic perivascular cells were found frequently beside cerebral cortical and thalamic penetrating vessels (Figure 7C).

Fluorescent evaluation of EB albumin extravasation revealed significantly diminished **BBB disruption** in cromoglycate+TPA-treated animals compared with saline-treated and TPA-alone-treated ones (Figure 8A). Similarly, MC-deficient rats showed less severe BBB disruption than did their WT littermates (Figure 8B).

Furthermore, pharmacological stabilization of MCs and MC deficiency led to significant reduction in posththrombolytic **brain swelling** at all time-points (II, Figure 3A,B)

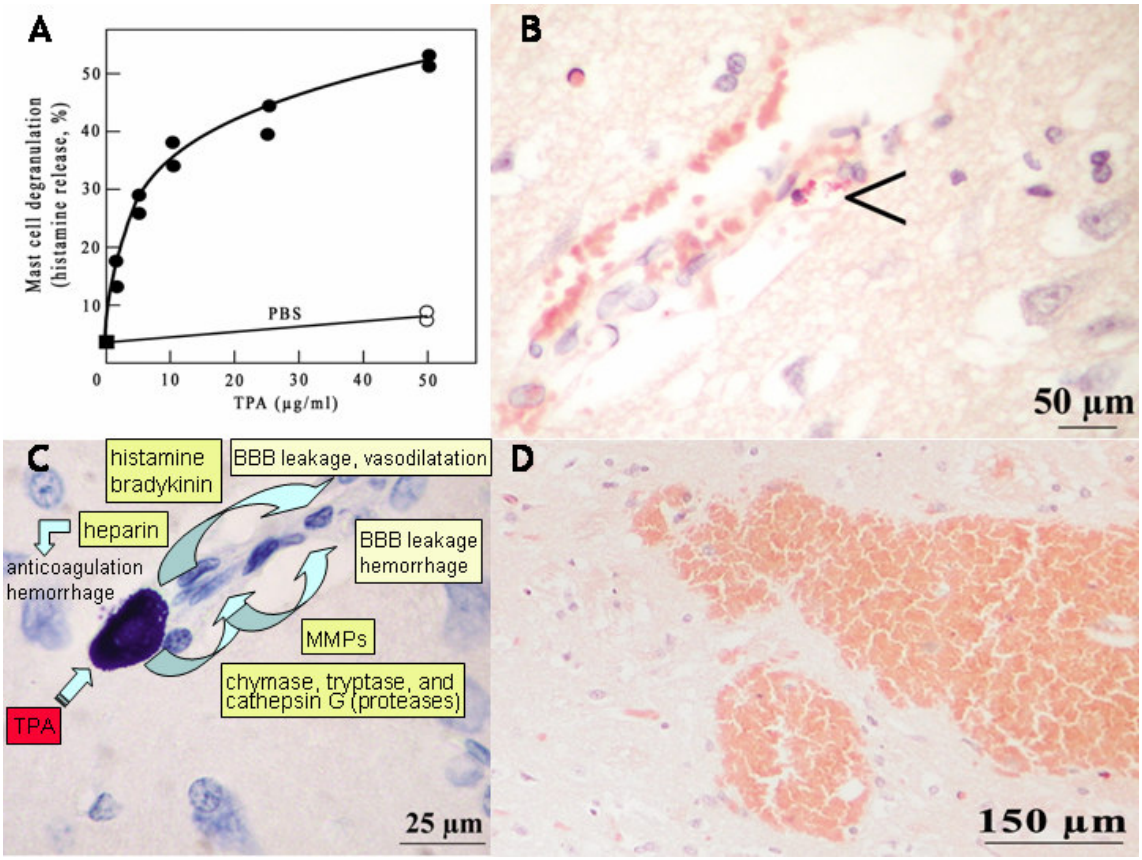

Figure 7. TPA-mediated hemorrhage. A) TPA activates mast cells in vitro. B) Mast cell residing in proximity to a blood vessel (arrow). C) Mediator release from activated mast-cell granules. D) Multifocal areas of hemorrhage formation in a brain section from a TPA-treated rat. (Republished with permission from Duodecim Medical Journal).

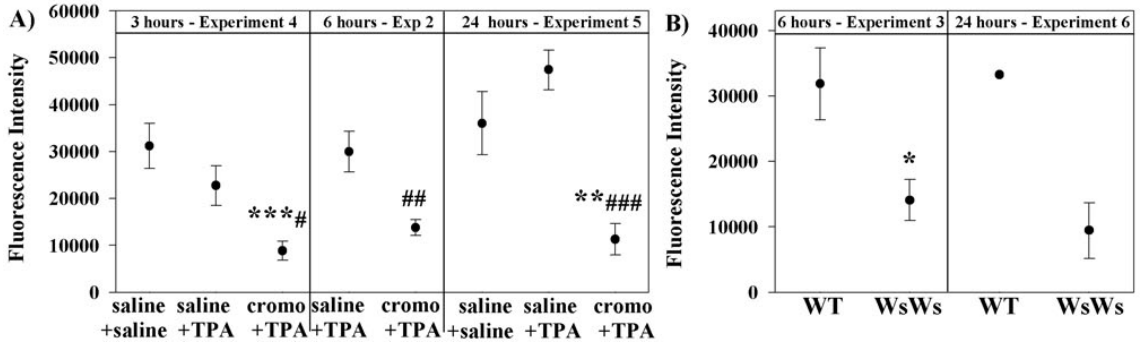

Figure 8. Fluorescent signals representing magnitude of Evans blue albumin extravasation and BBB leakage. A) Pharmacological stabilization of MCs with cromoglycate (cromo) on BBB permeability studied after 3 (ANOVA: P<0.01), 6 (t-test: P<0.01), and 24 hours (ANOVA: P<0.01). B) MC-deficient rats (WsWs) and their wild-type (WT) littermates (only one animal survived until EB albumin administration in the 24-hour WT group). Significance levels of post-hoc (multiple groups) or t-tests (two groups): versus saline+saline-treated group: * P<0.05, ** P<0.01, *** P<0.001; and pharmacological modulation of MCs versus no modulation in TPA-treated rats: ## P<0.01, ### P<0.001. (Additional Figure 2, Supplementary online data in II).

Parenchymal hemorrhages seen on brain sections (Figure 7D) were quantitatively analyzed. TPA caused robust (70- to 100-fold) induction of HF given either before or after reperfusion ($P<0.001$), compared with that of saline-treated controls. Such TPA-mediated hemorrhage was significantly reduced at 3 (95%, $P<0.01$), 6 (75%, $P<0.01$), and 24 hours (95%, $P<0.05$) of follow-up by pharmacological MC stabilization. Importantly, genetic MC deficiency supported the role of MCs, leading to 90% reduction in TPA-mediated hemorrhage at both 6 ($P<0.01$) and 24 hours ($P<0.001$).

Both TPA regimens (either 5 minutes before or 90 minutes after reperfusion) led to a 2- to 3-fold increase ($P<0.05$) in post-ischemic **neutrophil infiltration**, but the earlier infusion had a more substantial effect. Interestingly, a 6-fold increase in neutrophil infiltration took place also in the non-infarcted hemisphere. Notably, MC deficiency led to a 40% reduction in post-ischemic neutrophil counts compared with their WT littermates ($P<0.05$, all regions counted altogether), similarly in emigrated and intravascular neutrophils (data not shown). A comparable reduction was noticed when emigrated neutrophils were analyzed separately by region ($P<0.01$).

Importantly, pharmacological MC stabilization led to significantly ($P<0.01$) better **neurological scores** at 24 hours (median 2) than for saline-treated (median 3) and TPA-alone-treated (median 5) rats. Furthermore, MC-deficient rats had a significantly ($P<0.05$) better neurological outcome (median 1.5) than did their WT littermates (median 5). In addition, in pharmacologically modulated groups, 29% **mortality** occurred in the saline-treated group and 64% in the TPA-alone-treated group. No deaths occurred in the cromoglycate+TPA-treated group ($P<0.05$). I observed 17% mortality (1 dead animal) in the MC-deficient rats, whereas 75% of their WT littermates died ($P<0.05$).

5.3 Study III

Based on the promising results of the previous studies, the possible role of MC modulation (**pharmacological** or **genetic manipulation**) on edema and hematoma growth was examined in an autologous blood injection model of ICH. Further, the influence of such effects on the neurological outcome was addressed.

No significant differences arose in the **physiologic parameters** (MABP, temperature, blood glucose, pH, $PaCO_2$, PaO_2) between study groups.

Importantly, baseline **hematoma volume** did not differ among the pharmacologically modulated groups ($P=0.27$). However, hematoma growth during the next 24 hours was significantly smaller after both iv and icv MC stabilization than in saline-treated controls, as analyzed from MRI ($P<0.001$) and ex vivo obtained digital images ($P=0.02$). Hematoma growth in the compound 48/80 group was not different from that of the

control group (P=0.28), but it was significantly larger than both cromoglycate groups (both P<0.001) in all-pairwise comparison. Furthermore, hematoma volumes in the MC-deficient rats, although being almost significantly larger at baseline than for their WT littermates (P=0.059), showed significantly smaller growth during 24 hours (P=0.04). Similarly, based on the data from ex vivo images, hematoma volumes were larger in the WT littermates than in the MC-deficient rats (P<0.01).

Brain edema volumes calculated from the **hyperintense areas of T2 images** at 24 hours were significantly smaller in both cromoglycate groups than in the saline and compound 48/80 groups (P<0.001), demonstrating a 25% increase in the control and a 46% increase in the compound 48/80 group and approximately a 15% reduction in both cromoglycate groups during 24 hours. Experiments with genetically modified animals revealed that T2-based brain edema calculated at 24 hours was significantly smaller in the MC-deficient rats than in their WT littermates (P=0.036), representing a 10-fold higher increase in the WT littermates compared with the MC-deficient rats during 24 hours.

Furthermore, in the pharmacologically modulated groups, **brain swelling** (**hemispheric expansion seen on T2*-WI**) was, compared with controls, significantly smaller in both cromoglycate groups and significantly larger in the compound 48/80 group even at baseline (P<0.001). This difference was further magnified after the next 24 hours (P<0.001), with brain edema being 75% smaller in the icv cromoglycate, and 48% smaller in the iv cromoglycate group, and 61% larger in the compound 48/80 group than in controls. The difference in brain swelling at 24 hours was even significantly smaller in the icv cromoglycate group than in the iv cromoglycate group (P<0.05). MC-deficient rats developed 42% less brain swelling than did their WT littermates after 24 hours (P=0.035), although it was somewhat larger at baseline. Similarly, the percentage change in brain swelling between baseline- and 24-hour values was significantly smaller in the MC-deficient rats than in the WT ones (P<0.05).

In line with these described MRI-derived observations, planimetric data obtained after 24 hours from the **digital images** of ex vivo tissue slices revealed 83% less **brain swelling** (hemispheric expansion) in the icv cromoglycate, 63% less brain swelling in the iv cromoglycate, and 50% more brain swelling in the compound 48/80 group than in the controls (P<0.001). Similar calculations showed 87% less brain swelling in the MC-deficient rats than in their WT littermates at 24 hours (P <0.001).

Neurological scores obtained at 24 hours were highly significantly (P<0.001) better after MC stabilization (median 1 for both routes of administration) compared with the control (median 4) and compound 48/80 (median 5) groups. The neurological outcome of MC-deficient rats (median 1) was significantly (P<0.001) improved compared to their WT littermates (median 3). **Mortality** at 24 hours was 45% in the control group, 0% in both cromoglycate groups, and 55% in the compound 48/80 group. No deaths occurred in the MC-deficient group, whereas the WT rats experienced 25% mortality.

6 DISCUSSION

6.1 General discussion

Stroke (ischemic and hemorrhagic), a leading cause of death and disability worldwide, consumes a significant portion of the material and immaterial resources available for health care in general. The principal cause of clinical deterioration and premature death following large ischemic hemispheric strokes is **brain swelling**, increased ICP, and distortion of the brain structures, herniation. Several approaches to fight malignant brain swelling clinically are based on empirical support, but scientific evidence for their efficacy is lacking. At present, invasive surgical decompressive craniectomy and hypothermia seems to be promising.

Thus far, **thrombolysis** with intravenously administered TPA is the only proven pharmacological therapy for acute ischemic stroke, although, its application is associated with risk for clinically relevant **parenchymal hemorrhage**. Fear of hemorrhage may be a reason for withholding this otherwise beneficial treatment from a large proportion of patients. A critical step in the formation of edema and hemorrhage is disruption of the **basal lamina** and **the BBB**. These detrimental processes may be aggravated by inflammatory responses, which may be further promoted by therapeutically administered TPA.

In contrast to ischemic stroke, no medical or surgical treatment has thus far been efficacious in **primary intracerebral hemorrhage**, the poor outcome of which is mainly due to the mass effect of the growing hematoma and edema. Furthermore, the hematoma itself induces secondary inflammatory changes. Clearly, novel pharmacological discoveries are needed for the acute stage to improve long-term outcome of the victims.

Studies included in this thesis addressed the possible role of **MCs** in experimental ischemic and hemorrhagic stroke. MCs are resident cellular mediators of immediate hypersensitivity and initiate local phlogistic reactions to mechanical, toxic, and allergic stimuli. They are tissue-based cells and occurred in several end-organs, including the brain. Their metachromatic granules contain potent preformed **vasoactive, proteolytic, anticoagulant,** and **chemotactic** substances. We and others[341,352] have observed MCs positioned abluminally to the basal lamina and BBB (I, Figure 1A,B). In pilot studies,[409] MC density and their state of granulation were altered early following focal transient cerebral ischemia, and MCs were frequently found degranulating in association with edema and hemorrhage formation.

These findings, together with the characteristic **perivascular location of MCs** and the ability of their proteases to degrade the basement membrane proteins,[17,18] led to the hypothesis that following transient focal cerebral ischemia, MCs may be involved in the regulation of BBB permeability, brain swelling, TPA-mediated cerebral hemorrhage, and

inflammatory cell (neutrophil) infiltration. Finally, since the mass effect of a growing hematoma and edema is associated with poor outcome after primary ICH, I studied whether MC blocking would exert a therapeutic effect in an experimental ICH model. Apart from pharmacological modulation of MCs, I induced focal cerebral ischemia and ICH in a genetically manipulated MC-deficient rat strain. In addition, in vitro tests of TPA-mediated MC degranulation were performed.

Briefly, these results demonstrate MC's mediation of BBB permeability, brain swelling, and neutrophil accumulation following focal transient cerebral ischemia (I). Furthermore, in the same model, MCs were found to be involved in formation of hemorrhage associated with the application of TPA, and that hemorrhages and eventual unfavorable outcomes were reduced by cromoglycate treatment (II). Finally, MCs were shown to regulate the growth of hematoma and edema in ICH (III). Importantly, also here the pharmacological and genetic modulations of MC effects described were related to neurological performance and rates of fatal outcome.

6.2 Mast cell mediation of BBB permeability, brain swelling, and neutrophil infiltration after focal transient cerebral ischemia (I)

Studies with the MCAO model demonstrated that cerebral MCs participate in the regulation of early post-ischemic **BBB disruption**, **brain swelling**, and **neutrophil infiltration**. Pharmacological stabilization of MCs reduced these outcome measures, whereas pharmacological augmentation of MC degranulation had the opposite effect. Moreover, the experiments with MC-deficient rats confirmed the specific role of MCs in this scenario.

Although the precise mechanism of **MC activation** in ischemia remains unclear, it may possibly be launched by the blood serum-derived activated complement proteins C3a and C5a, since mixing of serum with CSF in vitro as well as plasma extravasation after BBB damage in clinical stroke and in SAH leads to fast activation of these anaphylatoxins which are potent stimulators of MCs.[432] MC granule constituents (especially histamine, heparin, and bradykinin), in turn, show significant microcirculatory effects, and modulation of their liberation during ischemia may influence the extent of the damage.

What is striking is that extravasation of EB albumin was in direct correlation with degree of expansive cerebral swelling even a few hours after reperfusion (I, Figure 4), suggesting that, even during the very early (ultra-acute) phase of ischemic stroke, **vasogenic volumetric enlargement** is a very dominant mechanism of brain edema in this model. This is important for the future design of anti-edema therapies since it has been widely held that interventions targeting the vascular wall and basal lamina may not be of enormous importance at the ultra-acute stage, when the cytotoxic mechanism of brain edema has been considered to be at its peak.

Accordingly, digestion of the endothelial basal lamina occurs as early as 2 hours after ischemia.[84,85] Such **loss of microvascular integrity** may link blood-protein and blood-cell extravasation to edema and hemorrhage formation.[71,84] A large body of evidence (section 2.1.3) indicates that proteases are involved in this process. Furthermore, MMP-mediated disruption of the tight junction proteins occludin and claudin occurs, and the MMP inhibitor already reduces the BBB damage 3 hours after transient focal cerebral ischemia.[94,96] These results also support the likelihood of rather early BBB disruption after transient MCAO.

Besides vasolytic and anticoagulant properties, I propose five lines of MC involvement in these processes: First, cerebral MC-derived **chymase**[341] is a potent protease which cleaves fibronectin and also activates procollagenases.[18,387] This happens even in the presence of TIMP-1[433]. A key role for MC chymase in the activation of pro-MMP-2 and pro-MMP-9 was demonstrated and confirmed recently.[385] Second, another MC-derived protease, **tryptase**, is also able to activate MMPs.[386] Third, MCs themselves can release the **gelatinases** A (MMP-2) and B (MMP-9),[109] and both MC-derived chymase and tryptase can degrade TIMP-1 protein,[88] suggesting a role for MCs in regulation of this proteolytic system. Fourth, MC granules contain **cathepsin G**, which is able to cleave many components of the extracellular and pericellular matrix, including fibronectin and vitronectin.[390] The role of cathepsins in microvascular matrix degradation has been reported.[86] Finally, apart from proteases, MCs can release various **cytokines** (e.g., TNF-α and IL-1) shown to be involved in upregulation of MMPs[434] and in edema formation.[435,436] Indeed, MCs do possess a potent armamentarium to target the components of the BBB and basal lamina shortly after their activation, whereas de novo production of these and additional mediators reactivates and maintains the process.

In addition to the BBB disruption and brain swelling, RI includes the release of free radicals and **inflammatory** mediators promoting leukocyte infiltration. Neutrophils start to accumulate within hours after reperfusion at the ischemia site,[181,437] a process which also occurs in the human brain.[167] In this inflammatory response, current data suggest MC participation (I, Figure 5). Clinical trials based on inhibition of neutrophils have had no success (section 2.1.3.3) perhaps explained by the fact that leukocytes arrive too late[166] to influence damage-propagation in viable, non-apoptotic neural tissue, or by adverse reactions to heterologous protein.[203]

The extremely potent resident (hence already present at the very outset of ischemia) proinflammatory MCs may offer an alternative and more proximal target for early antichemotactic intervention before the involvement of any circulating inflammatory cells. The fact that MC stabilization was associated with reduction in both the number of transmigrated neutrophils and of those still within the intravascular space (I, Figure 5B, right) suggests a role for MCs not only in reducing passive neutrophil trafficking through BBB breaches, but probably also in attenuating the perivascular chemotactic gradient up which blood-borne neutrophils start to transmigrate. Indeed, tissue-based MCs release potent mediators (PAF, TNF-α, IL-4, IL-5, IL-8 and neutrophil, eosinophil, and

macrophage chemotactic factors) known to attract inflammatory cells. Moreover, MC-derived histamine regulates expression of selectins in ECs with consecutive rolling of leukocytes[376] and plays a role in translocation of P-selectin to the cell surface,[438] further revealing the proinflammatory character of MCs. Traditionally, the main cerebral cellular sources of these chemotactic mediators include ECs, astrocytes, microglia, and neuronal cells, but the present work adds MCs to this list also within the cerebral ischemic cascade.

In this study, **infarct volumes** were not influenced by the MC interventions; this was expected at this early post-ischemic time point and by the fact that we tested a drug, sodium cromoglycate, without any known direct neuroprotective properties. Importantly, experiments performed after sham surgery and pharmacological MC modulation suggest that the changes observed were related to the ischemic response rather than to the pharmacological effects alone. Furthermore, the pharmacological manipulations seemed to cause no significant influence on post-ischemic CBF that could have mediated the effects observed.

Thus far, the MC as a regulator of BBB has not been widely recognized. Current experiments imply the strong involvement of this multi-active and potent cell type (with an armory of bioactive granule mediators able to target vascular basal lamina) in early BBB failure and brain swelling following focal transient cerebral ischemia. Furthermore, stabilization of MCs may provide an early opportunity to prevent infiltration of other inflammatory cells and their consequences such as no-reflow and further release of free radicals, all of which enhances vascular damage. Since the MCs respond rather stereotypically with degranulation of multi-active mediators to a variety of physico-chemical environmental and disease-associated challenges, MC stabilization could be a promising avenue of research also in conditions other than ischemic stroke.

6.3 Mast cell regulation of TPA-mediated hemorrhage formation after focal transient cerebral ischemia (II)

Hemorrhages, together with other components of the vascular RI (the BBB disruption, brain swelling, and neutrophil infiltration), can devastate a good prognosis following successful stroke thrombolysis. Although herniation and edema were once not considered a major problem in TPA-treated patients,[56] the European Cooperative Acute Stroke Study reported herniation- and brain edema-related mortality to be higher than hemorrhage-mediated mortality, and to be more prevalent in the TPA group.[439] Furthermore, cerebral bleedings do occur in patients undergoing thrombolytic treatment for acute myocardial infarction and pulmonary embolism, as well. Fear of post-thrombolytic hemorrhage leads to withholding of this beneficial therapy from number of patients.

The mechanism of **post-thrombolytic hemorrhage** is generally not understood, but it occurs with all fibrinolytic substances such as TPA, streptokinase, prourokinase, and reteplase,[440,441] all of which invariably induce plasminemia.[442] Plasmin, in turn, is a multi-active substance with proinflammatory activity (section 2.1.3.2); it degrades a range of ECM proteins and activates MMPs, which in turn digests matrix proteins[443] and causes brain tissue damage.[102,444]

In addition to proteolytic mechanisms of **BBB disruption**, BBB damage (leading to erythrocyte extravasation) may be mediated by the numerous preformed MC-mediators influencing vascular permeability (e.g., histamine and bradykinin). Perhaps more important in the setting of thrombolytic therapy for cerebral ischemia, hemorrhage development may be further promoted by a strong anticoagulant, heparin, which is produced in mammals by MCs only.[378,379] Heparin released locally from perivascularly positioned MCs may stop the formation of hemostatic plugs to patch BBB breaches to prevent erythrocytes extravasation, thereby contributing to hemorrhagic events. Interestingly, activation of the fibrinolytic system was found in dogs with MC tumors and a similar effect in dogs was achieved after very high levels of MC secretagogue compound 48/80 application,[445] confirming the fibrinolytic potential of MC mediators.

In sum, the present work, as extrapolated from Studies I and II, is in agreement with the recent suggestion that formation of hemorrhage may represent the end stage of a cascade that started as vasogenic edema following the BBB disruption,[446] as suggested even earlier.[71,84] The data in Study II support the fact that TPA aggravates all of these phenomena. In vitro experiments demonstrate that TPA by itself degranulates MCs to a great degree, a finding not reported for any other fibrinolytic drug thus far. Future studies should examine whether this is a class effect for all serine protease thrombolytics. Importantly, based on the present data, these phenomena may not only be modulated by targeted genetic MC manipulation but also be prevented by MC stabilization; this may identify a therapeutic pharmacological target. This study therefore establishes a novel cellular mechanism underlying these catastrophic phenomena.

In the present work, neutrophils were largely intravascular, but the count of neutrophils already having emigrated into the brain parenchyma was also significantly elevated by TPA (data not shown). This occurrence was influenced by MCs. Besides the possible mechanism discussed in the previous section, the **proinflammatory effect of TPA** may be mediated by plasmin, which is able to induce the synthesis of PAF and activates the terminal complement cascade.[447] Furthermore, when injected into the brain, it recruits neutrophils.[444] Interestingly, after TPA treatment, a consistent increase in neutrophil infiltration occurred not only in the ischemic but also in the intact hemisphere (II, Figure 4A). This suggests an independent proinflammatory effect of TPA which has been largely overlooked but may be clinically relevant. The intrinsic proinflammatory effect (increased neutrophil emigration) of TPA in the non-ischemic brain portions appeared also in experiments with pharmacological MC modulation (unpublished data). If the in vitro TPA-

mediated MC degranulation can be reproduced also in vivo, this could in part explain this very phenomenon.

Since we focused on the untoward post-reperfusion effects of TPA rather than therapeutic recanalization, TPA did not reduce **infarct size**. To have better control over the reperfusion per se, we used the suture occlusion and not the blood clot model. The suture model was utilized in experiments studying the effect of TPA on RI and formation of hemorrhage.[153] In that work, reperfusion 6 hours after filament MCA occlusion was not associated with hemorrhage in experiments without TPA (a finding similar to that of Study I), whereas the same experiment with TPA treatment was associated with hemorrhage; this shows the utility of the suture model in studying reperfusion-mediated hemorrhage.

The results show, indeed, the MC as a largely fibrinolytic cell type in the microvascular milieu. This, in the light of current results, clearly potentiates the unwanted side-effects of therapeutic thrombolytics after ischemic stroke. Pharmacological stabilization of MCs is therefore a potential novel adjuvant therapy to prevent the occasional devastating complication from thrombolytics after ischemic stroke as well as after acute myocardial infarction and pulmonary embolism.

6.4 Mast cell regulation of growth of hematoma and brain swelling in experimental intracerebral hemorrhage (III)

This study demonstrated that pharmacologically induced inhibition of MC degranulation led to significant reductions in **hematoma volume** and **brain swelling** at 24 hours after the induction of experimental ICH, whereas pharmacologically stimulated MC degranulation produced the opposite effect. The specific role of MCs in this response was confirmed by experiments in MC-deficient rats and their WT littermates. These results translated into neurological outcome and mortality in favor not only of MC deficiency but also pharmacological MC stabilization, supporting the association between mass effect of hematoma and of swelling and poor outcome after ICH.

Clinically, relentlessly progressing cerebral edema causes neurological deterioration as early as within 24 to 48 hours after ICH,[6] often leading to displacement of brain structures, increased ICP, and fatal outcome[233]—as supported also by the present findings. Extensive experimental studies of the pathophysiology of ICH-associated edema revealed a role for blood degradation products (specifically thrombin) and MMPs (section 2.2.1). Although the precise cellular pathomechanisms of brain edema formation after ICH are still not well established, Study III contributes to the list of potential causes of hazardous expansive tissue displacement and mortality from ICH, specifically, degranulation of MCs and the ensuing liberation of vasoactive, anticoagulant, and proteolytic substances.

However, despite the information gathered in the present studies, the particular pathophysiologic basis of **MC-dependent** brain edema and swelling during ICH remains essentially speculative. It may depend on the release of a host of MC-derived vasoactive substances (histamine, bradykinin) and proteolytic enzymes (tryptase, chymase), which may cause increased vascular permeability. As with ischemia, this may disrupt the basal lamina of the vasculature along with the surrounding extracellular tissue matrix, leading to secondary aggravation of extravasation of blood cells and of plasma proteins.

Moreover, MC-derived chymase and tryptase both activate MMPs, which may further contribute to the disruption. Results from our previous studies in focal transient cerebral ischemia would support the concept of MC-dependent induction of local breaches in BBB that could occur also in perihematomal zones and thereby contribute to the expansive edema formation in ICH. In view of the MC activation by TPA in vitro, it is of interest that PAs potentiate thrombin-induced brain edema,[448] and that—in an experimental setting—TPA used to liquefy the hematoma causes massive edema and inflammation.[449]

Apart from the possible role for the mentioned MC mediators, MC-mediated cytokines (e.g., TNF-α and ILs) may be involved as well. Some human studies show a correlation between plasma concentration of TNF-α and IL-6 (both belonging among MC mediators) and magnitude of perihematomal brain edema and hematoma growth.[254,257] In all likelihood, we are dealing with the mainly multifactorial basis of ICH-related brain expansion.

Hematoma expansion has also been attributed to continuing bleeding from the primary source and to mechanical disruption of the surrounding vessels.[6] In addition to direct tissue-compressing effects and secondary microcirculatory failure, these effects could also trigger local MC degranulation and activation in the perihematomal zone. This view is compatible with recent evidence suggesting that secondary growth of ICH may result from secondary bleeding within the peripheral zone around the ICH.[450]

Although thrombin is considered to be involved in the pathophysiology of ICH-associated edema formation, thrombin inhibitors did not succeed in reducing hematoma size.[273] To add another mechanism to the causes of **secondary bleedings**, MC-derived heparin may, in theory, prolong blood extravasation and also promote secondary hemorrhages, especially in areas where the BBB has been compromised by extravasated blood components and their vasoactive derivatives. This hypothesis is in line with the present data showing significant growth of the hematoma after augmented MC degranulation (with compound 48/80) and in non-treated control animals, and with the absence of hematoma growth after MC stabilization and in MC-deficient rats.

The present results and the mechanisms suggested to explain them support the role of MCs in regulating hematoma and edema growth after experimental ICH. Such regulation translated into neurological outcome and mortality. This makes pharmacological MC modulation interesting for development of clinical strategies to prevent space-occupying

edema and hematoma growth early after the onset of ICH, especially with no medical and surgical treatment for this devastating condition yet proving effective.

6.5 A place for MCs in the neurovascular unit?

The integrity of the microvasculature is provided not only by the BBB and the basal lamina, but by various components of the neurovascular unit (Figure 2, center), as well. Complex interactions between cellular and extracellular components within the unit involve matrix adhesion receptors, the integrins, and dystroglycan (section 2.1.3).

MCs show several types of interactions—some of which still remain speculative—with constituents of the neurovascular unit.

Astrocytes During development, MC association with the vascular bed (preferentially at branching points) is dependent on contact of the blood vessel with astroglial processes. This adhesion to the vascular wall involves MC-expressed α4-integrins.[353] Mature MCs can be grown on astrocytes,[339,451] and astroglial processes elongate in close proximity to MCs.[335] MC cytoplasm, in turn, extends into the neuropil.[351] Adjacent astrocytes can influence the phenotype and the migration of MCs,[346] presumably synthesizing MC growth factors such as IL-3[347,348] and NGF.[349]

Neurons Mast cell products enter neurons at least in three ways (transgranulation) in the dove brain[370]: a) direct fusion of the granule and plasma membranes of both mast cell and neuron; b) engulfment of mast cell processes containing granules or capture of released granule remnants; and c) receptor-mediated endocytosis. Interestingly, the frequency of transgranulation events is related to the activity status of the mast cell.

Endothelial cells Changes in endothelial cell-matrix interactions may be influenced by TNF-α and IL-1β,[452] most likely by down-regulation of integrin receptors of the β1 subfamily.[453] Accordingly, TNF-α reduces ECs' integrin $\alpha_1\beta_1$ expression, leading to decreased adhesion to laminin,[453] whereas IL-1β contributes to early ischemic brain edema, presumably by altering β_1 expression.[436] Both cytokines can be released by different cells, but belong among MC mediators, as well.

Basal lamina and ECM MCs can attach to and migrate on laminin- and fibronectin-coated surfaces.[362] Furthermore, MCs' surface receptors (one of them for laminin) regulate MC trafficking and distribution by engaging ECM components, including the classical integrin receptors.[361]

Considered together, these data suggest some kind of biologically and pathophysiologically relevant interactions between MCs and the neurovascular unit, an idea apparently not previously suggested.

7 SUMMARY AND CONCLUSIONS

The present work on focal transient cerebral ischemia with pharmacological and genetic modulation of **MCs** demonstrates that cerebral MCs participated in regulation of **early BBB disruption, brain swelling,** and **neutrophil infiltration.** That not only genetic MC deficiency but also pharmacological interventions targeting MCs showed similar results suggests a potential novel therapeutic approach to be studied in cerebral insults, where tissue injury is followed by florid extravasation, hazardous brain swelling, and inflammatory cell infiltration. The importance of such an approach is supported by the fact that no effective medical treatment exists for malignant brain swelling after large hemispheric strokes.

The efficacy of the same approaches was reproduced in association with **TPA-mediated hemorrhage**. Furthermore, TPA was found to degranulate MCs in vitro, an observation worth noticing by the manufacturers of thrombolytic compounds. Since the key findings also translated into improved neurological outcome and reduced mortality, they are relevant to the understanding and prevention of the occasional devastating complications following administration of thrombolytics (acute ischemic stroke, acute myocardial infarction, pulmonary embolism). If found useful also in humans, the safety of thrombolytic therapy could improve, so that more stroke patients could receive this beneficial treatment.

Since neither medical nor surgical treatment has been beneficial in **primary ICH**, findings of reduced hematoma and edema growth and of improved neurological outcome as well as of reduced mortality associated with MC stabilization suggest that it is an interesting, novel therapeutic avenue also in this life-threatening condition. All these antihemostatic effects, superimposed on the vasculopathic proteolytic capacity, emphasize the fibrinolytic properties of MCs in both ischemic and hemorrhagic brain insults, properties that could well be utilized in future research.

Future studies on this subject could also include more detailed dissection of the mechanism underlying MC-mediated BBB disruption and its consequences, as well as elucidation of how, exactly, MCs influence hemorrhage and edema growth in ICH. Of further interest would be the nature and behavior of particular MC mediators during ischemia, since some of these may also serve in tissue protection. For instance, histamine release may promote vasodilatation in areas of secondary microcirculatory ischemia. Further work might involve application of MMP antagonists, antihistamines; more detailed morphological studies of the early BBB opening after ischemia could reveal the interplay of MCs with other components of the neurovascular unit at different points during evolving brain damage.

Since brain edema is a key contributor to increased mortality and morbidity in numerous brain conditions apart from stroke, further study would be useful in the area of brain trauma, brain tumors, and infections. However, the most important task is to examine

whether any of these promising data can be translated into human medicine, since differences arise in the biological tasks of MCs among species. It is possible that the cerebral MCs are less abundant in humans and may possess a different operative repertoire than in rodents.

Cromoglycate, the model compound, is a well-known and safe drug used topically for decades for allergic clinical conditions. However, for purposes of the present work, the fact that it has presumably too low penetration through the BBB presents a problem that needs expertise from those in, for example, the pharmaceutical industry. However, the observation that intravenously administered cromoglycate in an ICH model showed the same positive effects as did intracerebroventricular application might speed its translation into human trials.

Another limitation of this study is that only one model compound was used to substantiate the utility of MCs as a pharmacological target for limiting deleterious effects. Moreover, the issue of appropriate dosages must be further addressed.

Novel therapeutic strategies are necessary to prevent loss of microvascular integrity. Interactions within the **neurovascular unit** are complex. This calls for interventions at multiple levels (e.g., those between individual mediators and their target receptors or structures, and the level of cellular activation and interactions) rather than a single-impact molecular target approach. Such a single-pathway approach (glutamate release, calcium antagonism, free radical release) prevailed in recent failed clinical trials of neuroprotective agents in ischemic stroke.

MCs reside in the pivotal perivascular position and contain an abundance of vasoactive, proinflammatory, anticoagulant, and proteolytic mediators. Hence, **MC stabilization may represent a multiple-level strategy, blocking simultaneously several molecular cascades that participate in progressive tissue damage instead of blocking just one single pathway.** The present results propose the MC to be considered an essential player in the neurovascular unit, where it contributes to cell-cell and cell-matrix interactions as well as controls vascular permeability and participates in recruitment of blood-derived inflammatory cells.

To reproduce the findings of these studies in human disease—to reduce suffering after large hemispheric strokes and intracerebral hemorrhages as well as to improve the safety of thrombolysis—is my earnest intention.

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