

Pathophysiological basis of translational stroke research

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Abstract

The high incidence and the devastating consequences of stroke call for efficient therapies but despite extensive experimental evidence of neuroprotective improvements, most clinical treatments have failed. The poor translational success is attributed to the inappropriate selection of clinically irrelevant animal models, the inappropriate focus on clinically irrelevant injury pathways and the inappropriate estimation of the length of therapeutic windows. To substantiate this conclusion, the pathophysiology of experimental stroke is reviewed. Particular emphasis is placed on the importance of collateral pathways, the penumbra concept and the viability thresholds of ischaemia, the haemodynamic and molecular mechanisms of injury evolution and the effect of secondary complications, notably inflammation and brain oedema. The comparison of permanent and transient focal ischaemia, on the one hand, and between mechanical and thrombolytic reperfusion, on the other, reveal basic differences in the mechanisms and dynamics of injury evolution which are of paramount importance for the proper targeting and time window of therapeutic interventions. These differences must be considered for adequate modelling of preclinical stroke studies to avoid unsuccessful translation of experimental data to the clinical setting.

Key words: Stroke, pathophysiology, penumbra, viability thresholds, injury pathways, secondary complications, treatment, therapeutic window, animal models, translation.

General remarks on experimental stroke research

Experimental stroke research aims at understanding stroke pathophysiology as the basis for the development of new clinical preventive and therapeutic interventions. Such developments are urgently needed because the prevalence and the costs of stroke are steadily increasing due to the growth of the elderly population and the continuous improvement of long-term stroke survival.

To deal with this problem, stroke research priorities have been established in the United States [16] and the European Community [48] but concerns have been raised that progress in experimental research does not successfully translate to the clinical setting. In fact, despite the publication of over 1000 experimental treatments, evidence-based clinical improvements are sparse [57]. So far, the only Food and Drug Administration (FDA)-approved stroke treatment is recombinant tissue plasminogen activator (rtPA)-induced thrombolysis within 3 hours of stroke onset,

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but none of the other neuroprotective interventions exhibited significant improvement of outcome in randomized double-blind class III trials. This failure has been attributed partly to deficiencies in selecting the experimentally most efficacious drugs for stroke trials, but concerns have also been raised that a substantial part of experimental stroke research cannot be translated because of limited clinical relevance.

This concern is based mainly on three arguments: the inappropriate selection of clinically irrelevant animal models; the inappropriate focus on clinically irrelevant injury pathways; and the inappropriate estimation of the duration of the therapeutic window.

The reason for these misconceptions can be traced back to a paradigm shift in the methodology of experimental stroke research during the past decades. Up to the late eighties the preferred experimental stroke model was permanent transorbital middle cerebral artery (MCA) occlusion in cats or primates. This model replicates clinical stroke fairly well but for a number of reasons it has been widely replaced by intraluminal thread or similar vascular occlusion techniques in small rodents. The main reasons are that these models are surgically less demanding, less expensive and ethically less debatable, and that complex injury pathways can be investigated in genetically modified mutants. However, as permanent middle cerebral artery occlusion in small rodents produces large infarcts with high mortality, perfusion is often restored to reduce lesion size. Both permanent and transient MCA occlusion produce focal necrosis but the pathways of injury and the time windows for therapeutic interventions are basically different. If these differences are ignored, experimental data are inappropriately translated to the clinical setting, which may lead to erroneous predictions of clinical outcome and could explain some of the poor results of clinical trials.

The following chapter is based on the Mossakowski lecture held in December 2008 at the Mossakowski Medical Research Centre in Warsaw, and attempts to analyze the pathophysiology of stroke in this particular aspect to provide appropriate guidelines for future research.

Topography of brain infarcts

Cerebral infarction has been defined as brain cell death due to prolonged ischaemia. This definition is based on the tissue state and categorizes as infarction both pan-necrosis and neural drop-outs, or – in the old terminology – complete and incomplete infarcts [65].

The size and localisation of brain infarcts depends on the site of vascular occlusion and the efficacy of collateral blood flow (Fig. 1). The six main supplying arteries of the brain, i.e. the right and left anterior, middle and posterior cerebral arteries, are interconnected by two major collateral systems: the *circle of Willis* at the base of the brain, which provides low resistance connections between the origins of these arteries, and *Heubner's leptomeningeal anastomoses*, which connect the distal cortical branches (for review see [43]).

Extracranial vascular occlusion of a carotid or vertebral artery is largely compensated by the circle of Willis, which distributes the residual blood supply evenly to all six brain arteries. Only in species with an incomplete circle of Willis, such as the gerbil, or if more than two vessels are occluded, may brain ischaemia evolve. In this situation blood flow is most severely reduced

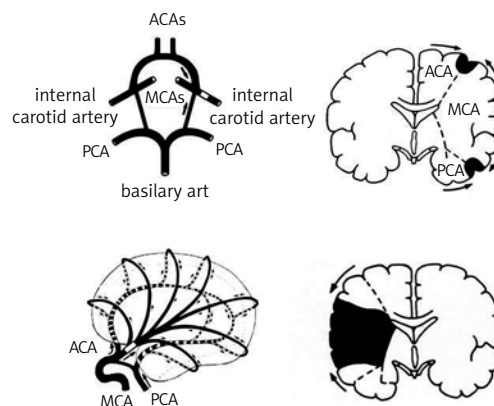


Fig. 1. Topography of brain infarcts. Above: Extracranial vascular occlusion. The circle of Willis provides low resistance connections between the origins of the anterior (ACA), middle (MCA) and posterior cerebral arteries (PCA). Reduction of blood supply by extracranial vascular obstruction lowers blood perfusion pressure mostly in the peripheral branches of the brain arteries, leading to focal ischaemia in the border zones between the supplying territories ('last field' phenomenon). Below: Intracranial vascular occlusion. Heubner's leptomeningeal anastomoses connect the peripheral branches of the brain arteries and provide collateral blood flow to the peripheral parts of the adjacent vascular territories. Reduction of blood supply by intracranial occlusion of a brain artery lowers perfusion pressure mostly in the proximal branches, leading to focal ischaemia in the centre of the vascular territory (modified from [76]).

in the distal branches of the brain arteries (the “last meadow” phenomenon), and infarcts are manifested in the peripheral zones of the supplying territories (borderline or boundary zone infarcts).

Intracranial vascular occlusion distal to the circle of Willis interrupts blood supply to the territory of the occluded vessel with partial retrograde compensation by the collateral system of Heubner’s pial anastomoses. As collateral flow is supplied to the peripheral branches of the artery, ischaemia is most severe in the central part of the vascular territory (territorial infarcts).

Depending on the haemodynamic capacity, i.e. the number and diameter of collateral vessels, infarct size may vary largely even under constant experimental conditions. This is a known problem of surgical vascular occlusion, notably the transorbital middle cerebral artery occlusion model. As the manipulation of the vessel may induce peripheral vasoconstriction resulting in the reduction of collateral blood supply, the size of infarction depends on the microsurgical skills of the investigator and tends to decline with improving experience.

A particular type of cerebral infarction is that induced by *microembolism*. Depending on the size and number of emboli, such interventions induce more or less widespread necrosis throughout the territory of the affected vessel. However, in contrast to ischaemic lesions induced by large vessel occlusion, microembolism produces a heterogeneous pattern of flow disturbances with complete flow arrest in the embolized and highly increased flow rate in the unobstructed microvessels. The immediate consequence of the flow redistribution is a massive breakdown of the blood-brain barrier and the formation of severe vasogenic brain oedema which, in fact, may be the leading cause of the evolution of tissue injury [74]. The relevance of this model for ischaemic infarcts is, therefore, limited.

Evolution of ischaemic injury

The evolution of focal ischaemic injury is multi-form and varies with the kind and density of ischaemia. Under conditions of transient ischaemia it also depends on the duration of ischaemia and the latency and dynamics of post-ischaemic reperfusion. Accordingly, focal ischaemia has to be differentiated into sudden or gradual, transient or permanent, and complete or incomplete, and the resulting tissue da-

mage into reversible or irreversible, acute or delayed, and primary or secondary. Below these aspects will be discussed in the context of general stroke pathophysiology.

The penumbra concept of ischaemia

The intact mammalian brain covers its energy needs almost exclusively by oxidation of glucose. Opitz and Schneider [59] were the first to draw attention to the fact that impairment of energy production induced by a reduction of oxygen supply affects the energy-consuming processes in a sequential way: first the functional activity of the brain is impaired, followed, at a more severe degree of hypoxia, by the suppression of the metabolic activity required to maintain structural integrity. The concept of two different thresholds of hypoxia for the preservation of functional and structural integrity was later refined by Symon et al. [72], who investigated the effect of graded ischaemia in a model of permanent focal ischaemia. These studies revealed that EEG and evoked potentials are disturbed at substantially higher flow rates than the potassium gradient across the plasma membranes. Since the preservation of this gradient is a sign of cell viability, Symon and his colleagues speculated that neurons located in the flow range between “electrical” and “membrane” failure are functionally silent but structurally intact. In focal ischaemia, this flow range corresponds to a crescent-shaped region intercalated between the necrotic core and the normal brain; it has been termed the “penumbra” in analogy to the partly illuminated area around the complete shadow of the moon in full eclipse [2].

The penumbra is viable only for a limited time but as long as energy metabolism and membrane function are preserved, injury is fundamentally reversible. Changes in the viability status of the penumbra are, therefore, of critical importance for the progression of tissue damage and determine the window of opportunity for therapeutic interventions.

Viability thresholds of focal ischaemia

In the classical description of the penumbra, only two flow thresholds were differentiated, but with more elaborated methods, evidence has been provided that different brain functions break down over a wide range of flow levels (Fig. 2). Progressing from the periphery to the core of the infarct, the most sen-

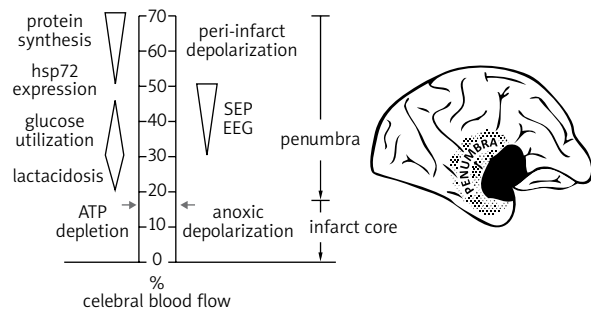


Fig. 2. Thresholds of metabolic (left) and electrophysiological (right) disturbances during graded reduction of cortical blood flow. The infarct core is the region in which blood flow decreases below the threshold of energy failure, and the penumbra is the region of reduced blood supply in which energy state is preserved. SEP: somatically evoked potentials; EEG: electroencephalogram (modified from [72] and [31]).

sitive parameter is protein synthesis, which is inhibited by 50% at about 0.55 ml/g/min, and is completely suppressed below 0.35 ml/g/min [51]. These values are clearly above the thresholds for disturbances of mRNA synthesis at 0.25-0.30 ml/g/min [37] or those for energy metabolism, which begins to deteriorate at even lower flow values [61]. Glucose utilisation transiently increases at a flow rate below 0.35 ml/g/min before it sharply declines below 0.25 ml/g/min [61]. This range corresponds to the beginning acidosis and the beginning accumulation of lactate. At flow rates below 0.26 ml/g/min tissue acidosis becomes very pronounced and both PCr and ATP begin to decline [53].

Anoxic depolarisation occurs at even lower flow values. The sodium/potassium ratio of brain tissue increases at flow values below 0.10-0.15 ml/g/min [32] and extracellular ion changes occur between 0.06 and 0.15 ml/g/min [24]. At the same threshold extracellular calcium declines due to the opening of calcium channels. The metabolic and ionic disturbances in the periphery of focal ischaemia thus proceed in the following order: initially protein synthesis is inhibited (at a threshold of about 0.55 ml/g/min), followed by stimulation of anaerobic glycolysis (below 0.35 ml/g/min), breakdown of energy state (at about 0.20 ml/g/min) and anoxic depolarisation of the cell membranes (below 0.15 ml/g/min).

As far as functional disturbances are concerned, the first change is the suppression of EEG activity,

which occurs between 0.15 and 0.23 ml/g/min [53]. Evoked potentials disappear between 0.15 and 0.25 ml/g/min [52], and spontaneous unit activity at a mean value of 0.18 ml/100g/min [28]. Neurological studies suggest that reversible hemiparalysis appears at about 0.23 ml/g/min, followed by irreversible paralysis below 0.17-0.18 ml/g/min [35]. All these values are distinctly below the threshold of the suppression of protein synthesis and even below that of the beginning activation of anaerobic glycolysis, but they fall into the range of the beginning energy crisis.

This is also true for the release of neurotransmitters into the extracellular compartment, as measured by interstitial dialysis techniques. According to these investigations, both inhibitory and excitatory neurotransmitters are released at about 0.20 ml/g/min with a possibly slightly higher threshold for glycine, adenosine and GABA than for glutamate [46]. The release of neurotransmitters is probably unspecific because other intracellular metabolites are co-released.

A direct consequence of the metabolic disturbances associated with focal ischaemia is the rise of cell osmolality, which causes an osmotically driven shift of water from the extra- into the intracellular compartment. The resulting decline in the fluid volume of the extracellular compartment can be detected by measurements of electrical impedance or by diffusion-weighted NMR imaging (DWI), both of which are sensitive to cell volume changes. Two hours after vascular occlusion the threshold of the beginning rise of electrical impedance is about 0.30 ml/g/min [47] and that of the rise of signal intensity in diffusion-weighted imaging is 0.41 ml/g/min [41]. These thresholds are distinctly higher than the thresholds of energy failure and extracellular ion changes, indicating that the area of increased DWI is larger than the infarct core and includes parts of the peri-infarct penumbra.

In contrast to the biochemical and functional changes which appear shortly after vascular occlusion, histological lesions require some time before they become visible. The threshold of histological changes, therefore, depends on both the density and the duration of flow reduction. Under conditions of permanent ischaemia, the threshold of pan-necrosis is between 0.17 and 0.24 ml/g/min [35]. When ischaemia is reversed within 1 to 2 hours, the tissue is able to survive a reduction of flow to 0.12 ml/g/min. At flow values below 0.80 ml/g/min, i.e. far above the threshold of pan-necrosis, selective neuronal loss may occur [49]. Interestingly, this loss is not threshold-dependent; the

flow rate correlates linearly with the number of surviving neurons, which suggests a coupled decrease in parallel to the reduced metabolic requirements of the tissue. This interpretation is in line with the hypothesis that the peri-infarct brain tissue suffers pathological changes which are not directly related to the reduction of blood flow (see below).

Most of these thresholds have been determined at only one time point after a few hours following the vascular occlusion. However, studies dealing with the dynamics of infarct development clearly indicate that the thresholds may change with time. The threshold of ATP depletion increases from 0.13 ml/g/min after 30 min to 0.19 ml/g/min after 2 h vascular occlusion and further to 0.23 and 0.32 ml/g/min after 6 and 12 h, respectively [51]. Similarly, the threshold of glutamate release rises from 0.20 ml/g/min after 1 h to 0.30 ml/g/min after 6-15 h ischaemia [46]. The threshold of the irreversible suppression of spontaneous neuronal unit activity rises from 0.05 to 0.12 ml/g/min during the initial 2 h of vascular occlusion [27], and that of the signal intensity in diffusion weighted imaging – which reflects alterations in the intra/extracellular water compartmentation – from 0.41 to 0.47 ml/g/min between 30 min and 2 h of vascular occlusion [41]. In contrast to these gradually progressing threshold values, the threshold for the suppression of protein synthesis remains remarkably stable at about 0.55 ml/g/min during the initial 12 h of ischaemia [51]. These data are in line with the concept that the infarct core grows into the penumbra,

but the border between penumbra and normal tissue does not shift.

Reversal of focal ischaemia

In most instances of ischaemic stroke, blood flow improves over time. Under clinical conditions recanalisation of vascular occlusion is observed in up to 50% of patients within 4 days after stroke [38] but in most instances reperfusion is initiated too late to reverse primary ischaemic injury. Experimental models of transient vascular occlusion, therefore, replicate clinical stroke only if reperfusion is similarly delayed.

Haemodynamics of transient ischaemia

Experimentally, different patterns of flow restoration can be distinguished, depending on the model of vascular occlusion (Fig. 3). Sudden mechanical occlusion of a major brain artery causes instantaneous circulatory arrest in the supplying territory of the occluded vessel, followed within a few minutes by partial restoration of flow via unobstructed collateral blood vessels [32]. This interval is caused by the delay in collateral “opening”, which is a vasodilatory response initiated by the fall in intraluminal pressure and enhanced by the metabolic acidosis developing in the ischaemic territory. The haemodynamic capacity of the collateral system determines the severity and extension of focal ischaemic injury and is a crucial modulator of the infarct volume [4].

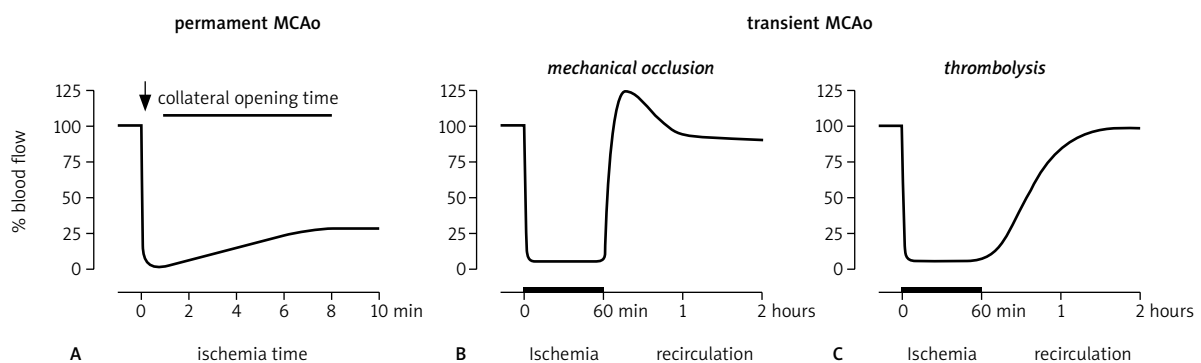


Fig. 3A-C. Schematic presentation of flow profiles after different types of focal brain ischaemia. **A.** Permanent occlusion of middle cerebral artery results in instantaneous flow arrest in cerebral cortex, followed by partial restoration of flow due to “opening” of Heubner’s pial anastomoses. **B.** Transient mechanical occlusion of middle cerebral artery. Release of vascular occlusion results in instantaneous reperfusion and transient hyperaemia. **C.** Clot embolism of middle cerebral artery reversed by intra-arterial thrombolysis. Blood flow is not instantaneously restored but slowly increases over protracted times, depending on the speed of clot lysis.

The onset of vascular occlusion induced by *clot embolism* is similar to sudden mechanical obstruction but after some time blood flow further improves due to clot lysis [8]. This process can be accelerated by thrombolytic drugs but even under optimal experimental conditions, i.e. high dose and topical application of thrombolytic agents, up to 60 minutes elapse until blood flow returns to normal [39].

Finally, flow can be restored by *release of mechanical occlusion*, e.g. by clip removal or withdrawal of an intraluminal suture. In this situation blood flow is instantaneously restored and may even temporarily overshoot (reactive hyperaemia) but after an interval which roughly corresponds to the duration of the preceding ischaemia, it declines below normal (post-ischaemic hypoperfusion) and may remain at this reduced level for extended periods [42].

The different haemodynamics of post-ischaemic recirculation are reflected by differences in the evolution of brain infarcts and the therapeutic window for the alleviation of the ischemic lesions (Fig. 4). Early reperfusion due to collateral opening reverses initial energy failure in accordance with the viability thresholds of ischaemia and, therefore, directly determines the size of the ischaemic infarct. The slow recanalisation during spontaneous or drug-induced

thrombolysis produces initially only a “trickle” of flow which in the core is not able to restore normal oxidative metabolism. Quite contrary, the beginning resupply of glucose, oxygen and water may promote anaerobic glycolysis, reoxygenation injury and oedema formation, which tend to aggravate the severity of injury. Only in the penumbra, where blood flow does not decline below the threshold of energy failure, even minor improvement of flow is of benefit because it enhances the haemodynamic reserve capacity and reduces the mismatch between oxygen supply and oxygen requirements of the tissue (see below).

After transient mechanical vascular occlusion full restoration of blood flow promotes almost instantaneous recovery of energy metabolism provided the duration of ischaemia does not exceed 1-2 hours [26]. However, after some time the combination of post-ischaemic hypoperfusion with molecular disturbances may result in secondary deterioration of metabolism and delayed necrosis. The magnitude of delayed necrosis and the interval between primary recovery and secondary deterioration depend on the duration of ischaemia. After 30 min middle cerebral artery occlusion secondary necrosis affects only the central parts of the vascular territory and may be delayed for as long as 3 weeks [15]. After one hour of ischaemia,

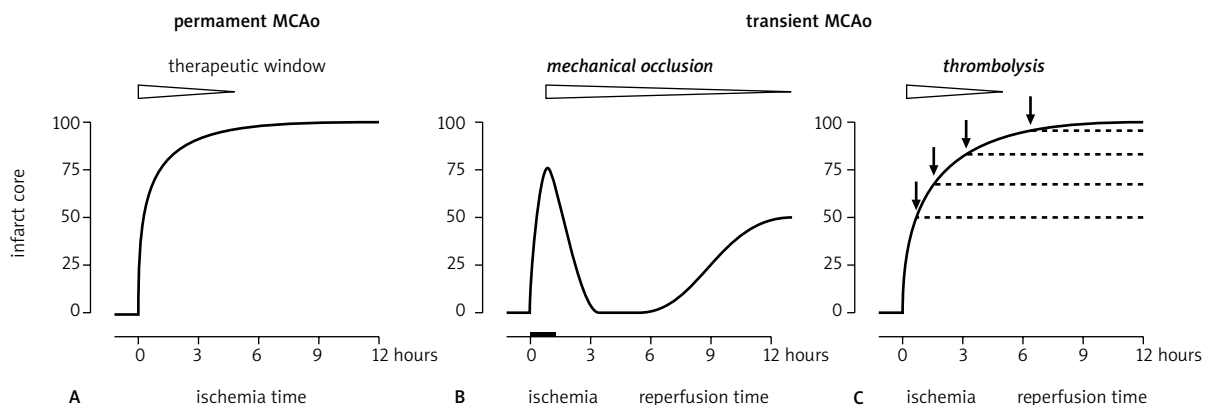


Fig. 4A-C. Evolution of brain infarcts and therapeutic window for the alleviation of ischaemic lesions after various types of focal ischaemia. **A.** In permanent ischaemia the brain infarct expands to near maximum size within 3-6 hours due to growth of the infarct core into the penumbra. Chances for therapeutic intervention vanish in unison with the disappearance of the penumbra. **B.** Sudden reversal of focal ischaemia by release of mechanical occlusion results in primary restitution of energy metabolism followed, after an interval of several hours, by secondary energy depletion. Chances for therapeutic intervention exist throughout this interval until final infarction evolves. **C.** Gradual improvement of blood flow induced by thrombolysis does not reverse primary energy failure but prevents infarct expansion into the penumbra. With increasing delay between clot embolism and thrombolysis, the chance for tissue protection vanishes in unison with the disappearance of the penumbra, as during permanent ischaemia. Infarct core: relative brain tissue volume in which energy metabolism has ceased [31].

secondary necrosis develops between 12 and 24 hours, and it expands more peripherally into the area of primary energy failure. After even longer ischaemia times, primary recovery occurs only in the most peripheral parts of the middle cerebral artery, and after longer than 3 hours ischaemia infarcts do not differ from those induced by permanent occlusion [25].

Viability markers of transient ischaemia

When blood flow is restored after transient ischaemia, the outcome of injury cannot be longer predicted on the basis of the classical viability thresholds. This is of particular importance for the ischaemic penumbra, which by definition is reversed when blood flow is resumed. A more appropriate characterization of tissue injury under these conditions is the description of the metabolic alterations. Comparisons of various models and durations of transient ischaemia revealed that tissue fate can be robustly predicted by imaging energy and protein metabolism [30]. Absence of primary recovery of energy state is equivalent to primary necrosis, whereas recovery of energy state in unison with that of protein synthesis predicts tissue survival. As long as recovery of energy metabolism is dissociated from that of protein synthesis, the tissue is at risk of delayed post-ischaemic cell death: either protein synthesis recovers after some delay and the tissue survives, or it does not and energy metabolism secondarily fails, resulting in delayed tissue necrosis. The dissociation between energy metabolism and protein synthesis is reminiscent of the metabolic disturbances in the penumbra during permanent vascular occlusion, but to avoid possible confusion, the term “metabolic” penumbra should be used.

Mechanisms and mediators of ischaemic injury

According to the classical threshold concept of brain ischaemia, fatal tissue injury evolves when blood flow declines below the minimal rate required for the supply of adequate amounts of oxygen and nutrients to support energy metabolism. In normothermic animals, this rate is about 15% of control flow and corresponds to the core of brain infarction. In the penumbra flow rate is above this threshold, and

energy metabolism is preserved but only as long as the oxygen requirements of tissue do not increase and/or secondary molecular alterations do not activate energy-independent injury pathways. Similarly, post-ischaemic recovery after transient ischaemia depends not only on the restoration of an adequate supply of oxygen and nutrients but also on the prevention of increased oxygen demands and/or of potentially fatal molecular injury cascades that evolve after blood flow has been restored [14]. Below, the most important mechanisms of injury evolution will be described.

Haemodynamic mechanisms

The primary cause of ischaemic stroke is the fall of blood flow below the threshold of functional integrity. Flow disturbances may be aggravated by cardiovascular insufficiency, rheological alterations (caused by aggregation of platelets at low shear or by post-ischaemic disseminated coagulopathy), and by oedema formation. Following transient focal brain ischaemia, post-ischaemic hypoperfusion may develop due to microvascular compression or post-ischaemic vasospasm. Although post-ischaemic hypoperfusion rarely reaches the threshold of primary energy failure, it carries the risk of oxygen mismatch, particularly under conditions of increased metabolic activity. Post-ischaemic injury due to haemodynamic disturbances, therefore, cannot be predicted by fixed thresholds of blood flow but depends on the individual mismatch between oxygen supply and oxygen consumption.

Molecular mechanisms

The term “molecular mechanisms” has been introduced to describe injury pathways which contribute to ischaemic cell death beyond primary energy failure (Fig. 5). From a conceptual point of view such mechanisms are only of pathophysiological relevance when the residual blood flow is high enough to cover the oxygen demands of the tissue. In permanent focal ischaemia this occurs only in the penumbra but in transient ischaemia molecular mechanisms may also contribute to injury in areas in which energy metabolism has recovered after ischaemia. Under experimental conditions, this applies only to transient mechanical vascular occlusion because restoration of blood flow by spontaneous or thrombolytic

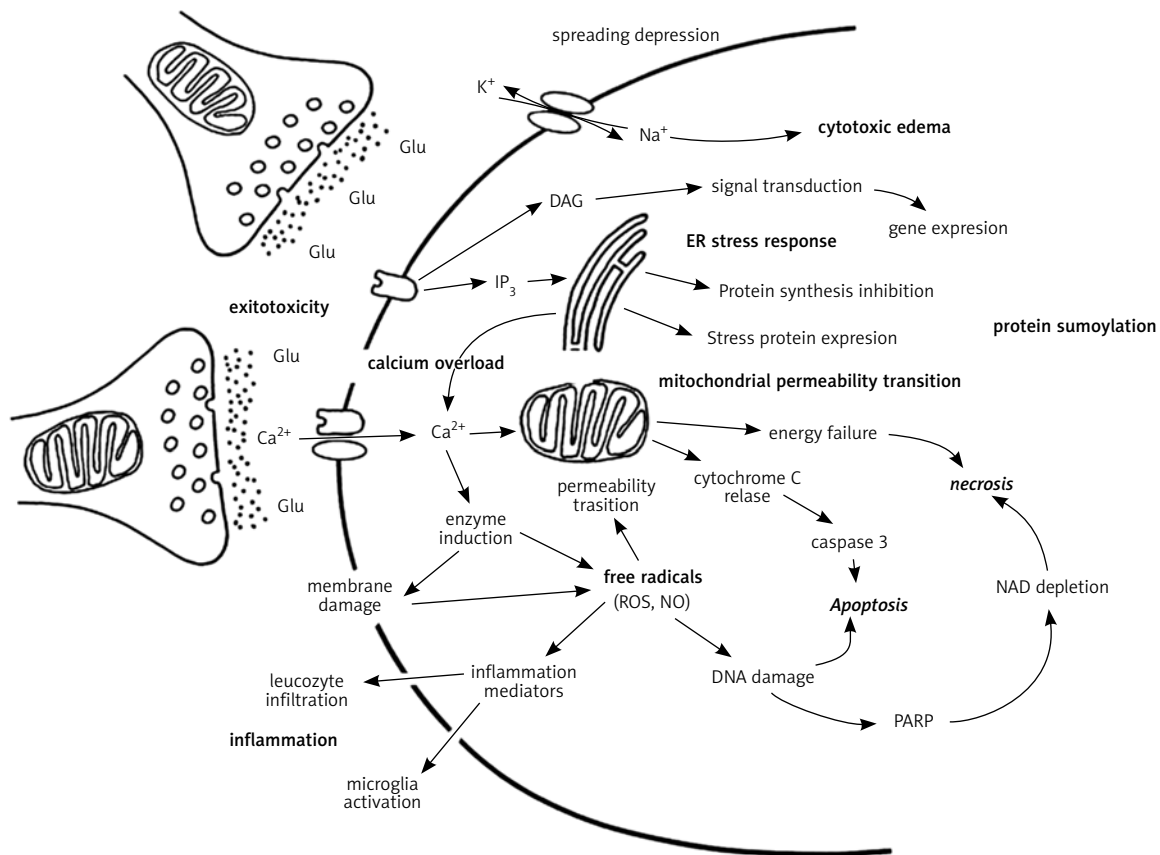


Fig. 5. Schematic representation of ischaemia-induced molecular injury pathways leading to mitochondrial failure, the endoplasmic reticulum stress response and the evolution of necrotic and apoptotic injury. Injury pathways can be blocked at numerous sites, providing multiple approaches for the amelioration of both necrotic and apoptotic lesions.

recanalisation is too slow to reverse primary energy failure (see above). Given that transient mechanical vascular occlusion is not a model of clinical stroke, the practical relevance of molecular mechanisms is restricted to peri-infarct injury, i.e. the growth of brain infarcts into the penumbra.

Acidotoxicity: During ischaemia oxygen depletion and the associated activation of anaerobic glycolysis cause accumulation of lactic acid, which depending on the severity of ischaemia, blood glucose levels and the degree of ATP hydrolysis results in a decline of intracellular pH to values between 6.5 and below 6.0. As the severity of acidosis correlates with the severity of ischaemic injury, it has been postulated that acidosis is neurotoxic. Evidence has been provided that ASICs (acid-sensing ion channels) are glutamate-independent vehicles of calcium flux, and

that blockade of ASICs attenuates stroke injury. This suggests that acidosis may induce calcium overload, and that this effect is one of the mechanisms of acidotoxicity [69].

Another acidosis-induced effect is endoplasmic reticulum (ER) stress [1], which is known to result in prolonged inhibition of protein synthesis and delayed cell death (see below). Acidosis, therefore, may be involved in both primary and secondary evolution of ischaemic injury.

Excitotoxicity: Shortly after the onset of ischaemia, excitatory and inhibitory neurotransmitters are released, resulting in the activation of their specific receptors. Among these neurotransmitters, particular attention has been attributed to glutamate, which under certain experimental conditions may produce excitotoxicity [9]. The activation of iono-

tropic glutamate receptors results in the inflow of calcium from the extracellular into the intracellular compartment, leading to mitochondrial calcium overload and the activation of calcium-dependent catabolic enzymes. The activation of metabotropic glutamate receptors induces the IP_3 -dependent signal transduction pathway, leading among others to the stress response of endoplasmic reticulum, and by induction of immediate-early genes (IEG) to adaptive genomic expressions. At very high concentration, glutamate results in primary neuronal necrosis. However, following pharmacological inhibition of ionotropic glutamate receptors, an apoptotic injury mechanism evolves that may prevail under certain pathophysiological conditions. The importance of excitotoxicity for ischaemic cell injury has been debated [73], but this does not invalidate the beneficial effect of glutamate antagonists for the treatment of focal ischaemia. An explanation for this incongruity is the pathogenic role of peri-infarct depolarisations in infarct expansion (see below). As glutamate antagonists inhibit the spread of these depolarisations, the resulting injury is also reduced.

Calcium toxicity: In the intact cell, highly efficient calcium transport systems assure the maintenance of a steep calcium concentration gradient of approximately 1:10.00 between the extra- and the intracellular compartment on the one hand, and between the cytosol and the endoplasmic reticulum (ER) on the other. During ischaemia anoxic depolarization in combination with the activation of ionotropic glutamate and acid-sensing ion channels causes a sharp rise of cytosolic calcium [68]. At the onset of ischaemia this rise is further enhanced by activation of metabotropic glutamate receptors which mediate the release of calcium from the endoplasmic reticulum (ER), and after recovery from ischaemia by activation of transient receptor potential (TRP) channels which perpetuate intracellular calcium overload despite the restoration of ion gradients (Ca^{2+} paradox) [44]. Calcium dysregulation is also sustained by calpain-mediated inhibition of calcium efflux [5]. The changes in intracellular calcium activity are highly pathogenic. Prolonged elevation of cytosolic calcium causes mitochondrial dysfunction and induces catabolic changes, notably by activation of Ca^{2+} -dependent effector proteins and enzymes such as endonucleases, phospholipases, protein kinases and proteases that damage DNA, lipids and proteins. The release of calcium from the ER evokes an ER stress response, which media-

tes a great number of ER-dependent secondary disturbances, notably inhibition of protein synthesis. Calcium-dependent pathological events are therefore complex and contribute to a multitude of secondary molecular injury pathways.

Free radicals: In brain regions with low or intermittent blood perfusion, reactive oxygen species (ROS) are formed which produce peroxidative injury of plasma membranes and intracellular organelles [7]. The reaction with nitric oxide leads to the formation of peroxynitrate, which causes violent biochemical reactions. Secondary consequences of free radical reactions are the release of biologically active free fatty acids such as arachidonic acid, the induction of endoplasmic reticulum stress, the induction of mitochondrial disturbances and fragmentation of DNA. The latter may induce apoptosis and thus enhance molecular injury pathways related to mitochondrial dysfunction. The therapeutic benefit of free radical scavengers, however, is limited, as recently documented by the therapeutic failure of the free radical-trapping agent NXY-059 [67].

Nitric oxide toxicity: Nitric oxide (NO) is a product of NO synthase (NOS) acting on arginine. There are at least three isoforms of NOS: eNOS is constitutively expressed in endothelial cells, nNOS in neurons and the inducible isoform iNOS mainly in macrophages. Pathophysiologically, NO has two opposing effects [11]. In endothelial cells the generation of NO leads to vascular dilation, an improvement of blood flow and the alleviation of hypoxic injury, whereas in neurons it contributes to glutamate excitotoxicity and – by formation of peroxynitrate – to free radical-induced injury. The net effect of NO thus depends on the individual pathophysiological situation and is difficult to predict.

Zinc toxicity: Zinc is an essential catalytic and structural element of numerous proteins and a secondary messenger which is released from excitatory synapses during neuronal activation. Cytosolic zinc overload may promote mitochondrial dysfunction and generation of reactive oxygen species (ROS), activate signal transduction pathways such as protein kinase C or enhance glutamate toxicity by inhibiting GABAA channels and blocking excitatory amino acid transporters. However, zinc may also exhibit neuroprotective properties, indicating that cells may possess a specific zinc set-point by which too little or too much zinc can promote ischaemic injury [66].

ER stress and inhibition of protein synthesis: A robust molecular marker for the progression of ischaemic injury is inhibition of protein synthesis, which evolves shortly after the onset of ischaemia and persists until the manifestation of cell death [29]. It is initiated by the ischaemia-induced release of calcium stores from the endoplasmic reticulum (ER), which results in ER stress and various cell biological abnormalities such as accumulation of unfolded proteins, expression of stress proteins and global inhibition of the protein synthesizing machinery [60]. The latter is due to the activation of protein kinase R (PKR), which causes phosphorylation and inactivation of the alpha subunit of eukaryotic initiation factor eIF2. This in turn causes selective inhibition of polypeptide chain initiation, disaggregation of ribosomes and inhibition of protein synthesis at the level of translation.

Other consequences of ER stress in focal ischaemia are *ubiquitination* and trapping of proteins which are crucial for cellular function, and *SUMOylation* (i.e. conjugation with the small ubiquitin-like modifier SUMO), which causes suppression of most transcription factors [10,75]. The former is presumably the reason for the irreversibility of translation arrest because protein aggregates include components of the translation complex [12]. Obviously, persistent inhibition of protein synthesis is incompatible with cell survival but as the maturation interval between onset of ischaemia and the manifestation of cell death greatly varies, other factors are also involved.

Mitochondrial disturbances: The concurrence of increased cytosolic calcium activity with the generation of reactive oxygen species leads to an increase in permeability of the inner mitochondrial membrane (mitochondrial permeability transition, MPT), which has been associated with the formation of a permeability transition pore (PTP). The PTP is thought to consist of a voltage-dependent anion channel (VADC), the adenine nucleotide translocator (ANT), cyclophilin D and other molecules. The increase in permeability of the inner mitochondrial membrane has two pathophysiologically important consequences. The breakdown of the electrochemical gradient interferes with mitochondrial respiration and, in consequence, with aerobic energy production. Furthermore, the equilibration of mitochondrial ion gradients causes swelling of the mitochondrial matrix, which eventually will cause disruption of the outer mitochondrial membrane and the release of pro-apoptotic mitochondrial proteins (see below).

Ischaemia-induced mitochondrial disturbances thus contribute to delayed cell death both by impairment of the energy state and the activation of apoptotic injury pathways [56].

Apoptosis: Apoptosis is an evolutionary conserved form of programmed cell death that in multicellular organisms matches cell proliferation to preserve tissue homeostasis. It is an active process that requires intact energy metabolism and protein synthesis, and it is initiated essentially by two pathways: an extrinsic death receptor-dependent route, and an intrinsic pathway which depends on the mitochondrial release of pro-apoptotic molecules such as apoptosis-inducing factor (AIF) and cytochrome C. Both pathways involve a series of enzymatic reactions and converge in the activation of caspase-3, a cysteine protease which contributes to the execution of cell death. An end stage of this process is the ordered disassembly of the genome, resulting in a ladder pattern of oligonucleosomal fragments as detected by electrophoresis or terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labelling (TUNEL).

Although apoptosis is mainly involved in physiological cell death, it is widely assumed to contribute to the pathogenesis of diseases, including cerebral ischaemia [34]. In the context of stroke this is difficult to understand because in areas with primary cell death the obvious cause is energy failure, and in regions with delayed injury protein synthesis – an essential requirement for apoptosis – is irreversibly suppressed. However, ischaemia induces a multitude of biochemical reactions that are reminiscent of apoptosis, such as the expression of p53, JNK, c-jun, p38, cyclin-dependent kinase 5 or caspase 3, all of which correlate to some degree with the severity of injury. It has also been suggested that the consumption of NAD(+) by poly(ADP-ribosylation), an enzymatic reaction involved in DNA repair, could result in energy failure [6]. Ischaemic cell death, therefore, may be a hybrid of necrosis and apoptosis, appearing on a continuum with the two forms of cell death at its poles [45].

Functional mechanisms/spreading depression

The anoxic depolarisation of cell membranes in the infarct core is associated with the release of large amounts of potassium and excitatory amino

acids, which by diffusion or astrocyte interconnections may evoke spreading depression-like depolarisations in the surrounding tissue [70]. These depolarisations are generated at irregular intervals and spread centripetally at a speed of 2-3 mm/min over the entire ipsilateral – but not the contralateral – hemisphere. The energy required for the repolarisation of cell membranes increases the energy turnover by about 50%. In the non-ischaemic tissue the associated increase in oxygen demands is covered by the coupled increase in blood flow but in the penumbra the haemodynamic capacity of the collateral system is exhausted, leading to relative hypoxia and stimulation of anaerobic glycolysis [19]. Depending on the residual flow rate the resulting mismatch between energy production and energy requirements causes a gradual prolongation of the time required for repolarisation until, after some time, depolarisation becomes irreversible. Another aggravating effect of peri-infarct depolarisation is spreading ischaemia in the surrounding of the infarct core [71], possibly due to blockade of nitric oxide synthase [62] or post-depolarisation alkalosis [3].

Secondary complications of ischaemic injury

Primary tissue necrosis induced by focal ischaemia may be aggravated by secondary events. Other than primary haemodynamic or molecular injury they contribute only marginally to the early manifestation of brain infarcts, but they inflict additional injury which enhances the severity and – in the case of large “malignant” brain infarcts – even determines the final outcome of stroke.

Brain oedema

Focal ischaemia induces two types of brain oedema: an early cytotoxic and a delayed vasogenic type of oedema. The early *cytotoxic oedema* is initiated within minutes after the onset of ischaemia. It is an osmotically driven increase in intracellular water content in response to the massive ion shifts following cell depolarisation, and the accumulation of lactic acid and other osmotically active particles under conditions of anaerobic glycolysis. Cell swelling induced by cytotoxic oedema is initially compensated by a reduction of the extracellular fluid volume, but with ongoing ischaemia time the osmotic pres-

sure gradient between the increased osmolality of the brain and the unchanged osmolality of blood and cerebrospinal fluid results in the gradual increase of net water content of the brain [40].

Intracellular water accumulation is most pronounced in astroglia, where the swollen pericapillary astrocytic endfeet may compress the microcirculation. This mechanism contributes to the pathogenesis of the no-reflow phenomenon, which is an important limiting factor for the restoration of blood flow – and hence tissue recovery – after prolonged circulatory arrest.

Cytotoxic brain oedema develops in the infarct core and, to a lesser degree, in those parts of the penumbra where the accumulation of lactate increases intracellular osmolality and hence water accumulation. It is strictly confined to the injured tissue and does not spread into the surrounding non-ischaemic tissue. Its contribution to ischaemic brain swelling, therefore, depends on the size of the ischaemic lesion and is most pronounced in large territorial infarcts (“malignant” brain infarcts).

The late *vasogenic type of oedema* is caused by the breakdown of the blood-brain barrier in the infarct core. During permanent middle cerebral artery occlusion, the blood-brain barrier becomes permeable to circulating serum proteins after 4-6 hours, i.e. at a time when the first histological signs of tissue necrosis become visible. Putative mediators of the ischaemic barrier lesion are enhanced expression of matrix metalloproteinases (MMPs) [17], activation of cytosolic phospholipase A(2) [55] and Rho kinase-mediated endothelial cell contraction [36]. The breakdown of the blood-brain barrier is accompanied by the extravasation of a protein-rich filtrate of blood that accumulates in the extracellular compartment. It spreads along hydrostatic pressure gradients from the ischaemic into the surrounding non-ischaemic tissue, preferentially along the long fibre tracts of the white matter. The peak of oedema is reached after 1-2 days and may cause tissue swelling by as much as 100%. As a result, intracranial hypertension may develop and supratentorial parts of the brain may herniate, causing fatal compression of the brain stem.

A particular type of vasogenic brain oedema is associated with *microembolism*. In this condition, the blood-brain barrier breaks down almost instantaneously after embolism, probably due to the sudden multifocal pressure changes within the embolized

microcirculation. The massive extravasation of blood serum causes an increase in tissue pressure and, thereby, aggravates the primary reduction in blood flow.

Inflammation

Brain infarcts evoke a strong inflammatory response which is thought to contribute to the progression of ischaemic brain injury. Gene expressions related to this response have, therefore, been extensively investigated to search for possible pharmacological targets. The inflammatory response of the ischaemic tissue has been associated, among others, with the massive activation of the peripheral immune system [58], and the generation of free radicals in reperfused or critically hypoperfused brain tissue (for review see [64]). The prostaglandin synthesizing enzyme cyclooxygenase-2 (COX-2) and NF-kappa B, a transcription factor that responds to oxidative stress, are strongly upregulated and may be neurotoxic, as suggested by the beneficial effect of COX-2 inhibitors. Infarct reduction was also observed after genetic or pharmacological inhibition of matrix metalloproteinase (MMP)-9, but this effect has been disputed.

A key player in the intracellular response to cytokines is the JAK (janus kinase)/STAT (signal transducer and activator of transcription) pathway, which induces alterations in the pattern of gene transcription. These changes are associated with either cell death or survival and suggest that inflammation may be both neurotoxic and neuroprotective [63]. Inflammatory reactions and the associated free radical-mediated processes are, therefore, important modulators of ischaemic injury, but the influence on the final outcome is difficult to predict.

Progression of ischaemic injury

With the advent of non-invasive imaging techniques, evidence has been provided that infarcts grow. After acute occlusion of the middle cerebral artery in small rodents, infarct expansion is most pronounced during the initial 3 hours, followed by a more gradual increase to the maximum volume within 6-12 hours. The main reason for infarct growth is the expansion of the infarct core into the penumbra. This is reflected by the time-dependant increase of the threshold of energy metabolism, which steadily rises until it merges with the thresholds of penumbral altera-

tions, notably the inhibition of protein synthesis. An additional factor for infarct expansion is spreading ischaemia which develops in the peri-infarct penumbra and presumably is related to peri-infarct depolarisation [71].

Regarding the mechanisms of infarct growth, two concepts have been proposed. The *mismatch concept* postulates that blood flow in the penumbra does not match the tissue requirements of oxygen and nutrients under conditions of increased metabolic activity [30]. The main reason for metabolic mismatch is peri-infarct spreading depolarisation, but transient haemodynamic insufficiencies or increases in metabolic activity due to molecular injury pathways may also contribute. Experimental evidence of infarct growth due to metabolic mismatch has been provided by polygraphic co-registration of spontaneous or induced depolarisations with diffusion-weighted images or measurements of penumbral lactate concentration. These experiments revealed that each depolarisation results in an increase of the tissue content of lactate and the volume of DWI-visible tissue injury [19]. After passage of the depolarisation these changes do not or only partly reverse, supporting their mechanistic contribution to infarct growth.

The *sandwich model* postulates that cell death occurs when the tissue's self-protecting capacity is overloaded [13]. As ischaemia induces a great number of apparently unrelated injury pathways, each of which is corrected by the tissue in a different way, irreversible cell damage will occur only when the combined "sandwich" of stress loads exceeds the inherent capacity of self-repair. This model could explain that with ongoing ischaemia time the penumbra is gradually overloaded by the combined effect of all stressors, and that the prevention or therapeutic alleviation of any of these stressors may reduce the total load below the threshold of tissue damage.

The common denominator of both models is the abrupt transition from reversible to irreversible brain injury once the reserve capacity of the system has been exhausted. In the sandwich model this transition is implicit in the hypothetical threshold of cellular self-repair. The mismatch model predicts that with the decrease of oxygen supply below the metabolic requirements of the tissue, energy production has to be covered by anaerobic glycolysis. As the ATP yield (after subtraction of transport energy) of anaerobic glycolysis is less than 10% of oxidative glucose metabolism, glycolysis is sharply up-regulated

to compensate for this difference. The resulting accumulation of lactic acid induces acidotoxicity, mitochondrial calcium overload and cytotoxic brain oedema, which in turn aggravate the mismatch between blood flow and metabolism. This explains that the increased energy demand resulting from just one spreading depolarisation increases the volume of the infarct core by more than 20% [50], and that this expansion of injury can be equally well prevented by suppressing the spread of depolarisation or by improving blood flow.

Therapeutic window of focal ischaemia

In permanent focal ischaemia primary necrotic injury in the infarct core is irreversible but the expansion of the core into the penumbra can be alleviated by appropriate therapeutic interventions. The dynamics of this expansion determine the duration of the therapeutic window (Fig. 4). In small rodents submitted to permanent middle cerebral artery occlusion, infarcts expand within 3 hours to about 80% of their final size [26]. The volume of the salvageable penumbra declines accordingly, and after more than 3 hours becomes negligible. The therapeutic window of this model, therefore, has a maximum duration of 3 hours and the efficacy declines the later the intervention is started.

After transient ischaemia the situation is different. Depending on the kind and duration of ischaemia, primary tissue injury may recover, followed after a variable interval by secondary deterioration (see above). The therapeutic window for the prevention of this delayed type of injury is much longer than that for the treatment of infarct growth during permanent ischaemia and covers the whole length of the free interval between the primary recovery and the secondary failure of energy metabolism.

For the clinical translations of experimental therapies the two windows should not be mixed up. This is of particular importance for the translation of results obtained in transient mechanical occlusion models, which exhibit a much longer therapeutic window than the clinically more relevant models of permanent or gradually reversed vascular occlusion.

The inappropriate translation of therapeutic windows may be one of the reasons why experimentally highly efficient drugs failed to improve stroke under clinical conditions. In fact, the maximum time to treatment of all larger randomized clinical trials of

neuroprotection was 6 hours or even longer, i.e. an interval after which infarcts resulting from permanent vascular occlusion have already grown to their maximal size (see Table 4 in [18]). The same also applies to the published class III stroke trials. These trials include haemodynamic and molecular approaches with therapeutic windows mostly of 6 hours. The only exception is the rt-PA trial of the NINDS Stroke Study Group in which thrombolysis was initiated within 3 hours, and which was positive [54]. A thrombolysis trial with alteplase 3 to 4.5 hours after ischaemic stroke was also positive but only after exclusion of patients with brain haemorrhages and large infarcts [21]. All the other trials, including both ECASS and the recent DIAS thrombolysis trials, had therapeutic windows of longer duration and were negative [20,22,23].

Obviously, it cannot be excluded that in some of the negative trials clinically irrelevant injury pathways have been targeted, but here again the appropriate selection of clinically relevant animal models would have improved prediction of outcome. The detailed analysis of stroke pathophysiology is, therefore, of considerable importance for the proper design of preclinical studies.

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