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Stroke research at the crossroads – where are we heading?

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Summary

Stroke causes 5.7 million deaths annually. This ranks stroke as the second most common cause of death and, additionally, it is a major cause of disability. Because of an ageing population, stroke incidence and costs will greatly increase in the future. This makes stroke an ongoing social and economic burden, in contrast to the only very limited therapeutic options. In the last decade vast sums were spent on translational research focused on neuroprotective strategies in the acute phase of ischaemic stroke. A plethora of candidate agents were tested in experimental models and preclinical studies, but none was proven effective in clinical trials. This gave rise to discussions about the possible reasons for this failure, ending up mainly with criticism of methodological aspects of the preclinical and clinical studies, or of the relevance of animal studies in drug development. Indeed, the question could rather be whether neuroprotection is the right target for successful stroke treatment. In this context, a paradigm change can currently be observed: the focus of experimental and translational stroke research is shifting from early neuroprotection to delayed mechanisms such as stroke-associated comorbidities, regeneration and plasticity. In this review we highlight a few recently emerging fields in translational stroke research. One such topic is the crosstalk between immunity and the injured brain as key pathomechanism in stroke. On one hand, innate and adaptive immune cells play an important role in the fate of injured brain tissue after stroke; on the other, peripheral immune alterations are critically involved in post-stroke comorbidities. Another emerging research area is the analysis of mechanisms involved in regeneration and neuronal plasticity after stroke. Here, we discuss the current understanding of basic mechanisms involved after brain injury, clinical imaging approaches and therapeutic strategies to promote regeneration in stroke patients.

Key words: *stroke; experimental ischaemia; therapy; neuroprotection; neuroinflammation; brain-immune interactions; plasticity; functional recovery*

Status quo – stroke

Annually, about 16 million first-ever strokes occur worldwide, causing a total of 5.7 million deaths [1]. As a consequence, stroke ranks as the second cause of death in the world population after ischaemic heart disease. Moreover, stroke is a global epidemic: about 85% of all stroke deaths are registered in low- and middle-income countries [2]. In 2002, stroke was the sixth most common reason for reduced disability-adjusted life-years [3]. In the United States, the total direct and indirect cost for stroke was estimated at $ 65.5 billion in 2008 [4]. In all 27 European Union countries the overall costs were estimated € 27 billion in 2008 (European cardiovascular disease statistics 2008). A further increase in stroke incidence and costs can be expected simply as a result of population aging.

Although stroke places such an enormous medical and economic burden on society, thrombolysis with tissue plasminogen activator and mechanical vascular recanalisation are currently the only clinically approved therapies for ischaemic stroke. Moreover, there are well known limitations, including a narrow time window, coagulation abnormalities, intracranial haemorrhage and a list of further contraindications, which make these therapeutic options accessible only to a small percentage of stroke patients [5]. Therefore, prognosis for patients remains poor and the necessity for effective stroke treatment remains an urgent priority. For more than two decades, translational stroke research focused on neuroprotective strategies in the acute phase of ischaemic stroke. More than 1000 neuroprotective compounds have been tested in rodent models with the aim to improve stroke outcome [6]. Early mechanisms of neuronal damage like excitotoxicity, production of reactive oxygen species, cellular energy deficiency and depolarisation were targeted. Indeed, many agents reduced brain damage (in most cases measured as decreased infarct volume) in rodent models of experimental stroke. Out of these candidates approximately 50 neuroprotective agents were tested in more than 100 clinical stroke trials, but none has improved outcome in clinical stroke patients [6].

What are possible reasons for the failure of so many trials? So far, attention in discussions about this failure has been drawn mainly to methodological mistakes. The inappropri-

ate selection of experimental animals in terms of age, sex, comparable physiology and genetic background was discussed, as well as the low replication rate and lack of statistical rigor in preclinical studies [7–9]. Regarding the failure of clinical trials in stroke, other syndromes with strong involvement of the innate immune system, such as sepsis, have been equally resistant to effective drug development. In sepsis, for example, more than 100 randomised phase II and III clinical trials did not result in a single US Food and Drug Administration (FDA) approved drug [10]. Sepsis is a very different disease from stroke, both in preclinical and clinical settings. This poses the question, what are the factors involved in the failure to improve clinical outcome? Is the human immune system really that different from the rodent? Is our focus on suppressing the inflammatory response a dead-end strategy because inflammation is not only harmful but also essential for repair processes and regeneration? These are serious caveats in the varied field of immune-oriented research that will have to be more specifically addressed in future studies. Additionally, after the translation to clinical trials, the time window and dose for administration, as well as patient heterogeneity and inaccurate outcome parameters, were listed as possible sources for failure [11]. This has finally led to the currently widely discussed concept of a "translational roadblock" particularly in stroke research, which becomes obvious through the numerous commentaries, editorials and reviews on this topic [12–14].

However, we have to ask ourselves whether the choice of research tools, protocols and methods are the sole reason for this depressing failure in translational stroke research. Finally, the choice of the therapeutic target itself – acute neuronal death and neuroprotective strategies – also has to be questioned. Consequently, a paradigm shift in translational stroke research can currently be observed: from early neuroprotection to mechanisms involved in subsequent processes such as stroke-associated comorbidities, regeneration and plasticity. In the following text we want to highlight two of these evolving fields.

Brain-immune interactions in stroke

In response to the ischaemic injury, neuroinflammatory responses are relevant pathomechanisms promoting secondary brain injury in the subacute phase after stroke [15]. Brain resident microglia and astrocytes are activated after ischaemic brain injury and release mediators such as free radicals and proinflammatory cytokines that inflict secondary damage on the peri-ischaemic tissue [16]. Activated glial cells play an important role in clearance of cell debris, promoting neuroregenerative processes and controlling the neuroinflammatory reaction, and hence have a beneficial rather than a neurotoxic function after stroke [17–20]. Additionally, the rapid inflammatory response involves infiltration of leukocyte subpopulations (fig. 1; neutrophils, monocytes and lymphocytes). Recruitment seems to occur in a strictly synchronised manner following brain ischaemia; one of the first types of immune cell infiltrating are neutrophils, followed by monocytes and lymphocytes [21, 22].

Our current mechanistic insights about the contribution of immunity to stroke pathophysiology were obtained nearly exclusively in rodent stroke models. Despite the only very limited information about neuroinflammatory mechanisms in human stroke, efforts by academia and pharmaceutical companies have prematurely resulted in testing immunomodulatory drugs in human stroke patients (table 1) [23, 24]. These first clinical trials, which aimed to test immunomodulatory drugs (e.g. fingolimod, natalizumab, table 1) in stroke patients, were particularly hampered by the lack of suitable clinical surrogate markers for post-stroke neuroinflammation. However, such novel parameters, for example blood biomarkers or functional imaging of neuroinflammation, will be indispensable for characterising neuroinflammation in stroke subtypes and analysing the efficacy of immunomodulatory drugs in stroke patients. State-of-the-art imaging modalities such as magnetic resonance imaging (MRI) and positron emission tomography (PET) of microglial activation have already provided valuable information in preclinical studies [25]. Molecular MRI seems to be a promising, relatively noninvasive method to image *in vivo* inflammatory processes in the brain and detect biomarkers (e.g. vascular cell adhesion protein [VCAM] and intercellular adhesion molecule-1 [ICAM-1]) that are not detectable by conventional MRI [26, 27]. Still there are disadvantages, like the low MR sensitivity for cellular neuroinflammation, compared with other molecular imaging modalities such as PET and single-photon-emission-computer-tomography (SPECT). Ultrasmall superparamagnetic iron oxide particles (USPIO) [28], which are used in cancer and cardiac imaging, for example, have high amounts of iron oxide, which can compensate for the low sensitivity. However, repeated usage is limited, as a result of accumulation of the inert particles in liver and kidney. PET imaging of inflammation, using tracers binding the translocator-protein 18kDa (TSPO; formerly known as peripheral benzodiazepine receptor), showed promising results in first clinical approaches in stroke patients [29]. Despite having lower spatial resolution than MRI, the high

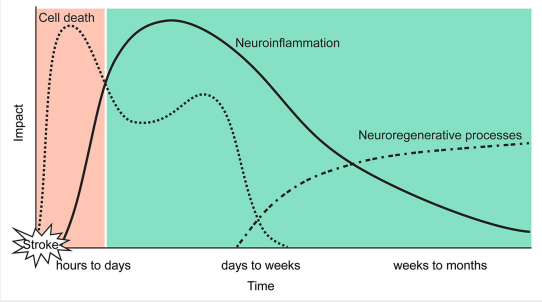

Figure 1

Multiphasic brain interactions after stroke and opportunities for treatment.

Previous neuroprotective strategies targeted pathological mechanisms in a very narrow window of opportunity in the (hyper-) acute phase after stroke (orange). Recently, the focus of translational stroke research has shifted towards understanding pathological processes in the subacute and chronic phase such as neuroinflammation and neuroregeneration (green). These targets have the potential for novel therapeutic approaches which are suitable for a larger population of stroke patients then neuroprotective agents or thrombolysis.

contrast resolution of PET, offering functional and molecular information with high sensitivity in low molar ranges, makes it a potential imaging modality for future clinical trials targeting neuroinflammatory pathways after stroke.

In addition to the neuroinflammatory reaction to the acute brain injury, perturbations of peripheral immune homeostasis have attracted much attention as a relevant complication after stroke. Recent investigations showed that peripheral immune activation has already peaked 4 hours after stroke [30] with highly increased serum concentrations of proinflammatory mediators, both after experimental stroke [31] and in stroke patients [32]. We have recently demonstrated that proinflammatory mediators released from the necrotic brain tissue into the blood circulation after stroke – so-called damage associated molecular patterns (DAMPs) – are critical mediators of a multiphasic peripheral immunomodulation [33]. DAMPs include a plethora of different soluble molecules derived from dying cells and giving rise to new treatment options targeting either DAMPs or their receptors on peripheral immune cells.

Acute immune activation after brain injury is followed by a sudden shift into a subacute immunosuppressive phase caused by exhaustion of innate immune cells and apoptotic lymphocyte death [33]. Subacute immunosuppression results in an increased susceptibility to secondary infections, particularly of the respiratory and urinary tract, which contribute substantially to post-stroke mortality and morbidity [34, 35]. The commonly attributed explanation for this phenomenon is stress-signalling after brain injury, including activation of the hypothalamic–pituitary–adrenal axis and sympathetic innervation of immune organs such as the spleen and bone marrow [36–38]. An alternative explanation proposes exhaustion of innate immune cells upon acute (over)activation and lymphocyte apoptosis due to inadequate costimulatory signalling derived from such exhausted antigen-presenting cells leading to an immunosuppressive phenotype [39]. However, the exact mechanisms of immunodepression following stroke will require further investigation and are a prime example of insufficient reverse translation: while susceptibility to bacterial infections due to functional immunosuppression is a long-standing clinical experience, translation from bedside-to-bench was largely neglected. Future experimental studies will be re-

Table 1: Treatment of post-stroke inflammation.

Target	Preclinical	Model	Clinical	Treatment effect on:			Miscellaneous
	Reference		Reference	Infarct volume	Neurol. deficits	Inflammation	
Downregulation of S1P receptors Fingolimod (FTY720)	Hasegawa Y. et al. 2010, Stroke [62]	tMCAo (rat)		+	+	n.d.	Decreased Casp-3 expression and number of dying neurons
	Wei Y. et al., 2011, Ann Neurol [63]	tMCAo (mouse)		+	+	Reduction of activated microglia and neutrophil infiltration	Decreased dying cells in core and peri-infarct area
	Rolland W. et al., 2013, Exp Neurol [64]	ICH (rat)		n.d.	+	Reduction of circulating leukocytes and ICAM-1+ T cells in brain	Ameliorated brain atrophy and memory performance
	Kraft P. et al. 2013, Stroke [65]	tMCAo (mouse)		+	+	No effect on local inflammatory response	Reduces microvascular thrombosis; no direct neuroprotection or BBB improvement
	Campos F. et al., 2013, Stroke [66]	Thrombo-embolic stroke (mouse)		+	+	n.d.	Combined alteplase and fingolimod administration; BBB improvement
			Fu Y. et al., 2014, PNAS [67]	+	+	Reduction of circulating lymphocytes	Small study: 11 vs 11 patients
			Fu Y. et al., 2014, JAMA Neurol [68]	n.d.	+	Reduction of circulating lymphocytes	Reduction of PHE and relative PHE after administration
			Zhu Z. et al., 2015, Circulation [23]	+	+	Reduction of circulating lymphocytes	Combined alteplase and fingolimod administration
Blockage of VLA-4 Natalizumab (α-CD49d)	Liesz A. et al., 2011, Brain [15]	pMCAo and 30 min tMCAo (mouse)		+	+	Reduced number of infiltrating leukocytes	Anti-CD49d inhibited T cell migration and abrogated their effector mechanisms
	Langhauser F. et al., 2014, Stroke [69]	pMCAo and 30 min tMCAo (mouse)		o	o	Reduced number of infiltrating leukocytes	Anti-CD49d did not influence overall stroke outcome irrespective of model or time
	Llovera G. et al., 2015, Sci Trans Med [70]	pMCAo and 60 min tMCAo (mouse)		+/o	o	Reduced number of infiltrating leukocytes after pMCAo	First preclinical randomised controlled multicentre trial; reduced infarct volume only in small cortical lesions
			Elkins J. et al. "ACTION trial" (ISC 2016)	o	+	n.d.	Improvement in functional independence, cognition and patient-reported stroke impact

BBB = blood-brain barrier; ICH = intracerebral haemorrhage; PHE = perihaematomal oedema; pMCAo = permanent middle cerebral artery occlusion; tMCAo = transient middle cerebral artery occlusion; VCAM-1 = vascular cell adhesion molecule-1; VLA-4 = very late antigen-4

quired for mechanistic understanding and target identification of this clinically important complication after stroke.

Long-term stroke outcome: regeneration and plasticity

Stroke patients experience continuous functional recovery after the stroke for weeks to years [40]. Due to differences in severity and location of the cerebral lesion, large variability between subjects in terms of functional recovery makes it almost impossible to generalise regenerative processes after the acute brain lesion. Although there are standardised scorings for neurological deficits and recovery after stroke in routine clinical use, such as the NIH Stroke Scale or Rankin Scale, current routine clinical tools are still insufficient to assess functional recovery [41]. Despite impressive progresses and convincing preliminary results obtained by use of imaging modalities to investigate neuronal plasticity, functional imaging of brain plasticity and remapping during the spontaneous recovery after stroke is barely used in clinical practice. Rodent studies [42] as well as clinical observations [43] provide important information about the loss and regain of neuronal connectivity after stroke; nevertheless, systematic network analyses and computational mapping remain demanding and require a high amount of methodological expertise. For integration of functional noninvasive imaging into clinical routine further investigations and the development of robust protocols are needed.

Recently, novel *in-vivo* imaging modalities visualising cortical neuronal activity in rodent models have advanced our understanding of reorganisation of cortical functional representation and reorganisation after injury [44]. After experimental stroke, the loss of functional connectivity, represented by breakdown of cortical connectivity maps, is recovered over weeks by establishing new structural and functional circuits (fig. 2a) [42, 45]. *In-vivo* imaging of neuronal activity showed that forelimb-evoked responses re-emerge in peri-infarct areas of the cortex after rodent stroke [26, 46]. The processes of re-establishing neuronal circuits, including axonal sprouting, synapse plasticity and neurogenesis, require a distinct micro-milieu of signalling cues to arise during cortical remodelling processes. Therefore, neuronal "re-wiring" becomes a challenge under the conditions of an adult brain, which is generally inhibitory to axonal sprouting [47]. Instead, for post-stroke recovery neurons must engage a neuronal growth programme. Previous reports have shown that growth-inhibitory molecules are reduced after experimental stroke and neurons themselves activate growth-promoting genes in successive waves after ischaemia [47]. Grefkes and colleagues demonstrated, by using functional magnetic resonance imaging (fMRI), the impact of stroke lesions on cerebral network connectivity [43]. They observed, as in the above mentioned rodent studies, that motor deficits of patients with focal ischaemia are associated with pathological intra- and interhemispheric connections between motor areas (fig. 2b). In the future, combining clinical assessment of disabilities and analyses of connectivity by means of imaging

Table 2: Neuroregenerative approaches after stroke.

Target	(Pre)clinical	Model	Therapeutic effect on:		Miscellaneous
	Reference		Funct. outcome	Regeneration	
Transcranial magnetic or direct current cortical stimulation	Plautz E. et al., 2003, Neurol Res [71]	Bipolar coagulation of vasculature in M1 cortex (squirrel monkey)	+	Large-scale plasticity of movement representation in stimulated cortex	Combination therapy of sub-threshold electrical stimulation and rehabilitative training
	Hummel F. et al., 2005, Brain [72]	Six patients (chronic stroke); double-blind crossover study	+	Functional improvement in paretic hand of all patients, which outlasted stimulation period	Noninvasive cortical stimulation and assessment of functional hand motor skills
	Khedr EM. et al., 2005, Neurology [73]	52 stroke patients in a randomised therapeutic trial	+	rTMS led to improvement of disability scores	10 consecutive days rTMS in addition to best clinical care
	Takeuchi N. et al., 2005, Stroke [74]	20 stroke patients with a first-time cerebral infarct	+	Reduction of transcallosal inhibition by reducing the amplitude of motor-evoked potentials in contralesional M1	Double blind study of real vs sham rTMS
	Grefkes C. et al., 2010, NeuroImage [75]	Eleven patients with unilateral hand weakness after first-ever stroke	+	rTMS over contra-lesional M1 reduced inhibition influence and enables more effective motor processing in lesioned areas	Usage of DCM to assess rTMS influence on effective connectivity within cortical motor system
	Zimerman M. et al., 2012, Stroke [61]	Twelve patients with first-ever subcortical stroke	+	Contra-lesional M1 tDCS improved early online learning period	Association between an intervention-induced SICI within lesional M1 and enhancement of skill acquisition
Motor function therapy & ergorobotics	Wolf S. et al., 2006, JAMA [76]	116 stroke patients in a randomised clinical trial	+	CIMT produced improvements in arm motor function that persist ≥1 year	Measurement of motor function by functional ability and motor activity log
	Staubli P. et al., 2009, J Neuroeng Rehabil [57]	Four patients with chronic stroke and left side hemiparesis	+	Three out of four patients showed improvement in motor functions	Intensive therapy using the robot ARMin II in a functional 3D workspace
	Lo A. et al., 2010, N Engl J Med [77]	127 chronic patients in a multicentre, randomised trial	+	Robot-assisted therapy showed motor function improvement after 12 and 36 weeks	Four-modules robotic system for horizontal, vertical, wrist and grasp movements

CIMT = constraint-induced movement therapy; DCM = dynamic causal modelling; M1= primary motor cortex; SICI = short interval intracortical inhibition; tDCS = transcranial direct current stimulation; rTMS = repetitive transcranial magnetic stimulation

modalities such as fMRI might help to determine the patient's status during the time-course of recovery and to design personalised therapeutic options. Neuronal connectivity analyses will provide insights into how neuromodulatory interventions might target pathological networks that are associated with incomplete recovery. Such novel diagnostic approaches will improve treatment paradigms based on the individual network pathology underlying a particular neurological deficit [48].

Active stimulation of motor function and coordination plays an important role in the recovery and regeneration of cortical circuits. This becomes obvious in experimental stroke studies in which rats housed in an enriched environment, with access to various activities and interaction with other rats, perform significantly better than rats that were housed in a standard environment [49, 50]. Similarly, clinical studies have shown improved cognitive recovery when stroke patients were exposed to music [51], and physical therapy for movement coordination and motor function are well established routine interventions in stroke recovery units [52, 53]. In the last decade, ergo-robotics has become a promising tool in motor-stimulating therapies, although it is not yet used in daily routine. Passively supporting systems like the SwedishHelparm™[54] assist arm movements with counter-weights connected to the arm for fulfilling reach tasks. More advanced systems, like the assisted rehabilitation and measurement (ARM) guide [55], not only support arm movements during therapy, but evaluate the arm impairments to improve further therapy. Recently, state-of-the-art exoskeleton robots provide support, movement guidance and evaluation of movement to individualise rehabilitation therapies, which provided a significantly improved outcome in chronic stroke patients [56, 57]. Additionally, several clinical studies have shown the positive effects of robot-aided neurorehabilitation in comparison with conventional therapy (table 2) [58, 59]. Throughout the last decade, other noninvasive treatment paradigms emerged in the field of chronic stroke regeneration. One paradigm is repetitive transcranial magnetic stimulation (rTMS), relying on the use of an insulated coil placed over the scalp. The coil generates repetitive magnetic pulses, producing changes in brain activity. In several clinical stroke trials, rTMS of the motor cortex led to improved hand function (table 2). A second approach is direct current stimulation (DCS), which uses constant low current delivered via electrodes on the scalp [60]. In a study with patients having a subcortical stroke, contra-lesional M1 area DCS showed an intervention-induced enhancement of skill acquisition [61] (table 2).

Taken together, an integrated view of individual neuronal plasticity after clinical stroke through use of novel imaging modalities and advanced deficit assessment will improve efforts toward personalised and more efficient therapy in stroke recovery.

In summary, after the failure of countless neuroprotective agents in the acute phase, stroke research has to continue transforming from a "neuro-centric" to a multi-disciplinary research field considering the contributions of various brain-resident and invading cell populations to the injured brain, and the complex interplay of the brain and remote organs over a prolonged time course after the stroke. Stroke is more than an acute event, it is a chronic condition and we must not underestimate the potential of therapeutic interventions in the subacute and chronic stages. In the future, promising findings in immune alterations caused by ischaemia and post-stroke brain recovery can provide us with manifold treatment opportunities, which can diminish the burden of stroke.

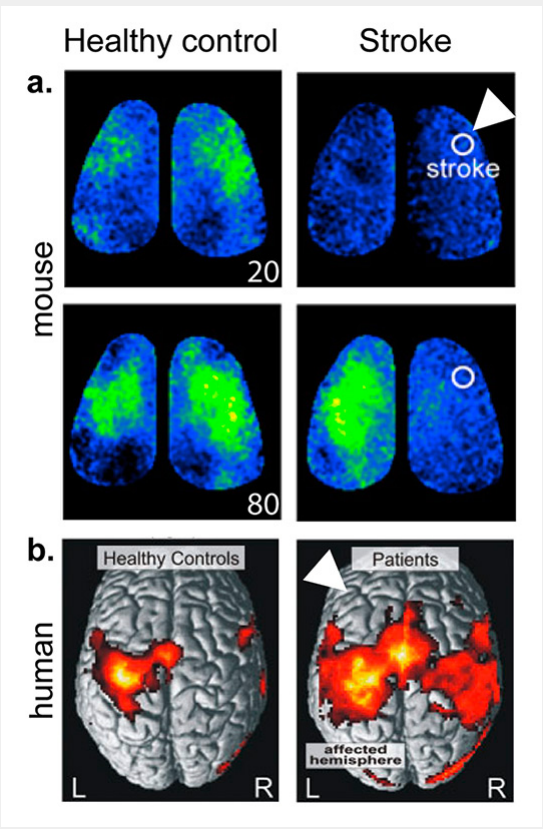

Figure 2

Comparison of neuronal connectivity after stroke in mouse and human.

a. *In-vivo* imaging of mouse cortex during left forelimb stimulation shows sensory-evoked polarisation in control mice emerging immediately (20 ms) in a confined area representing left forelimb function. In contrast, forelimb stimulation after stroke resulted in a more diffuse signal encompassing mainly the contralateral hemisphere. Additionally, there is a significant shift in timing of signal propagation, with a delayed and prolonged response after stroke (compare signal maps at 20 and 80 ms after forelimb stimulation).

b. Connectivity analysis based on significantly activated voxels (BOLD signal, fMRI) during movement of the right hand shows a distinct single-hemispheric cortical pattern in healthy controls. Hand movement in stroke patients were associated with enhanced and more extended neural activity in both hemispheres. White arrowheads mark the affected hemispheres.(Adapted from Mohajerani et al. 2011 [45] and Grefkes et al. 2008 [43], with permission).

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Figures (large format)

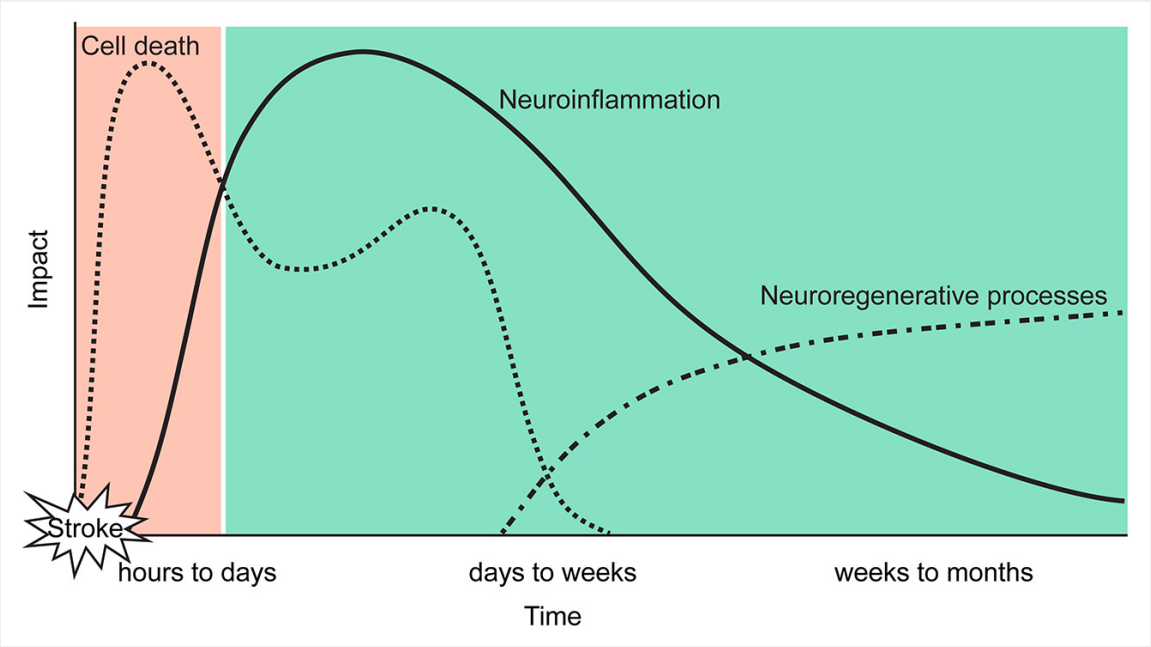

Figure 1

Multiphasic brain interactions after stroke and opportunities for treatment.
Previous neuroprotective strategies targeted pathological mechanisms in a very narrow window of opportunity in the (hyper-) acute phase after stroke (orange). Recently, the focus of translational stroke research has shifted towards understanding pathological processes in the subacute and chronic phase such as neuroinflammation and neuroregeneration (green). These targets have the potential for novel therapeutic approaches which are suitable for a larger population of stroke patients then neuroprotective agents or thrombolysis.

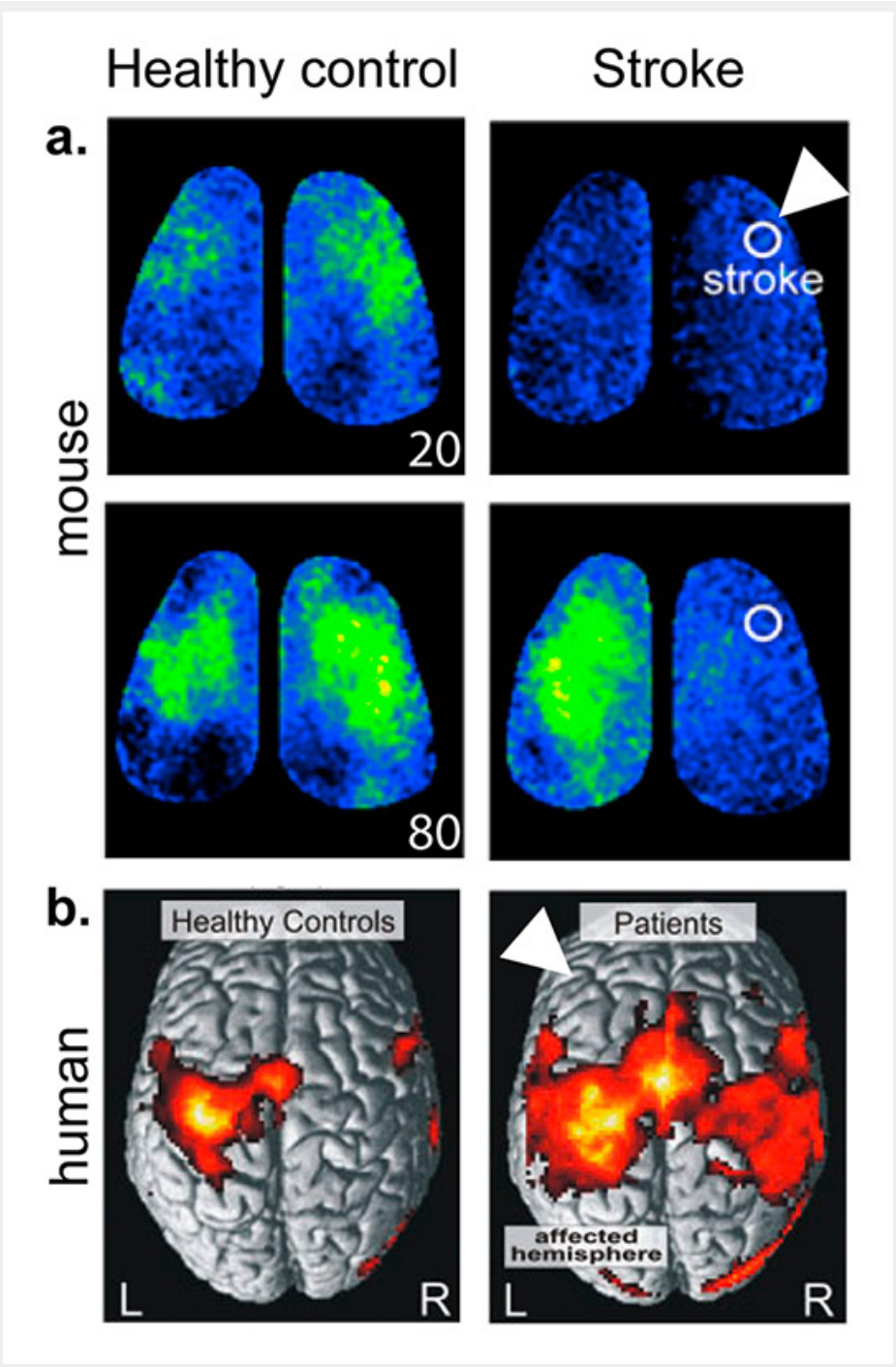

Figure 2

Comparison of neuronal connectivity after stroke in mouse and human.

a. *In-vivo* imaging of mouse cortex during left forelimb stimulation shows sensory-evoked polarisation in control mice emerging immediately (20 ms) in a confined area representing left forelimb function. In contrast, forelimb stimulation after stroke resulted in a more diffuse signal encompassing mainly the contralateral hemisphere. Additionally, there is a significant shift in timing of signal propagation, with a delayed and prolonged response after stroke (compare signal maps at 20 and 80 ms after forelimb stimulation).

b. Connectivity analysis based on significantly activated voxels (BOLD signal, fMRI) during movement of the right hand shows a distinct single-hemispheric cortical pattern in healthy controls. Hand movement in stroke patients were associated with enhanced and more extended neural activity in both hemispheres. White arrowheads mark the affected hemispheres.(Adapted from Mohajerani et al. 2011 [45] and Grefkes et al. 2008 [43], with permission).