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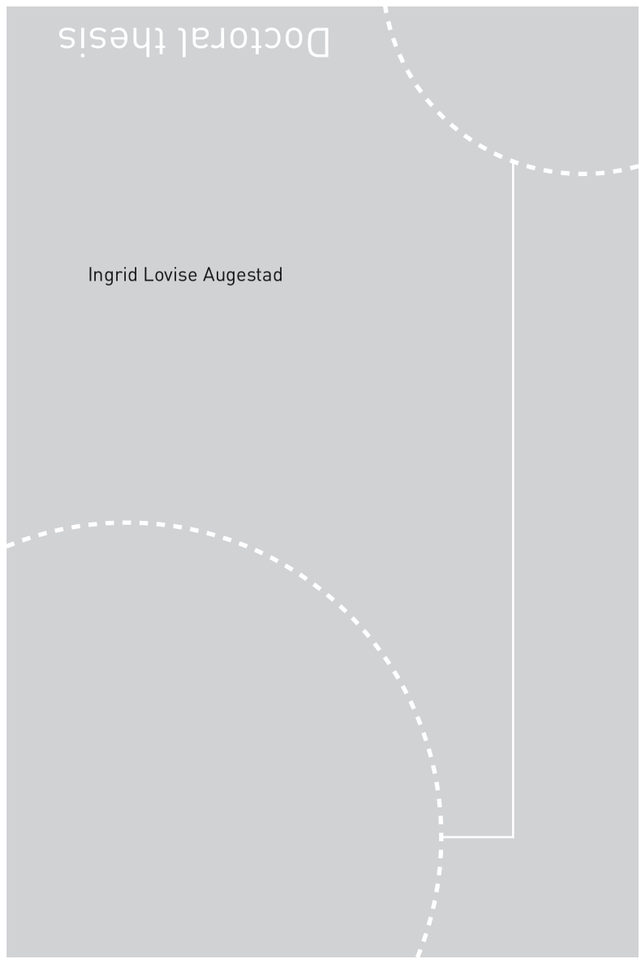
Ingrid Lovise Augestad

Combinatory strategies for promoting tissue remodelling and endogenous plasticity after experimental ischemic stroke



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Thesis for the Degree of Philosophiae Doctor

Trondheim, May 2018

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Preface

This thesis is submitted to the Norwegian University of Science and Technology (NTNU) in partial fulfilment of the requirements for the academic title of PhD in Neuroscience. The research work presented in this thesis was carried out at the Sandvig Lab at the Department of Neuromedicine and Movement Science, NTNU, the Comparative Medicine Core facility (CoMed), the MR Core facility, the Histology lab at the Cellular and Molecular Imaging Core Facility (CMIC), as well as specific equipment at the Kavli Institute/Centre for Neural Computation, NTNU. The work has been supervised by Dr. Ioanna Sandvig PhD, Professor Asta Kristine Håberg MD, PhD, and Associate Professor Axel Sandvig MD, PhD. Funding for this project was provided by The Liaison Committee for education, research and innovation in Central Norway (Samarbeidsorganet Helse Midt-Norge RHF).

Ulike strategier for å fremme regenerering og endogen plastisitet i hjernen etter eksperimentelt iskemisk slag

Hjerneslag er på verdensbasis en av sykdommene som rammer flest mennesker i tillegg til å ha høy dødelighet. Vi kan kategorisere hjerneslag i to hovedtyper; slag forårsaket av en blødning i hjernevevet, eller slag forårsaket av en blokkering av blodtilførselen i hjernen, også kalt iskemiske slag. De fleste pasienter som rammes av slag, rammes av iskemiske slag, enten ved trombose eller en embolisme som begge innebærer at plakk eller en blodpropp føres med blodstrømmen opp i hjernen og blir sittende fast der.

Per i dag er det kun trombolyse, oppløsning av blokkeringen i arterien med tissue plasminogen activator (tPA), som er godkjent som klinisk behandling av slagpasienter. Dessverre er tPA kun effektivt i et kort tidsrom på ca. 3-4,5 timer etter blodtilførselen opphører, noe som fører til at mange pasienter ikke kan motta denne typen behandlingen grunnet forsinket ankomst til sykehuset. Med økende levealder og en økende eldre befolkning forventes det også at antallet nye slagtilfeller vil bli flere i årene som kommer.

Sykdomsutviklingen og patologien til iskemiske slag er en sammensatt prosess som utvikler seg over tid, i tillegg til store forskjeller mellom pasienter, noe som gjør behandling svært utfordrende. En stor del av behandlingen dreier seg derfor i mange tilfeller om rehabilitering etter skaden har oppstått. Dette understreker hvorfor tilnærming til behandling bør fokusere på å kombinere ulike strategier, enten når det gjelder å bremse skadeutviklingen, eller reparere skaden som allerede har oppstått. Humane nevronale stamceller (hNSCs) har vist seg i dyremodeller å ha positiv effekt på mikromiljøet i skadeområdet og på inflammasjon. Det er også en mulighet for at hNSCs kan erstatte noe av det tapte vevet, men også stimulere hjernens eget potensiale for plastisitet og regenerasjon.

I arbeidet som er presentert i denne avhandlingen beskrives det ulike tilnærminger til behandling av hjerneslag i eksperimentelle slagmodeller. Hovedmålet med arbeidet har vært a) å kombinere stamcelleterapi enten med andre celletyper eller med farmakologisk behandling for å se om dette øker effekten i forhold til stamcellebehandling alene; b) studere effekten av å modulere inflammatoriske prosesser på utvikling av skaden; c) ta i bruk histologiske metoder for å se på celleimplantatenes overlevelse og integrering i vevet; d) studere hjernens egen (endogene) kapasitet for regenerasjon og effekten av de ovennevnte behandlingene på denne responsen til skade; e) sammenligne to forskjellige dyremodeller og diskutere potensialet for klinisk translasjon ved bruk av disse i slagforskning.

I avhandlingen presenteres og diskuteres resultatene av arbeidet, potensialet av å integrere forskjellige behandlinger, samt bruk av dyremodeller med tanke på klinisk translasjon.

Navn kandidat: Ingrid Lovise Augestad
Institutt: Institutt for nevromedisin og bevegelsesvitenskap
Veileder(e): Ioanna Sandvig, Asta Kristine Håberg, Axel Sandvig
Finansieringskilde: Samarbeidsorganet Helse Midt-Norge RHF

Ovennevnte avhandling er funnet verdig til å forsvares offentlig
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First, I would like to thank my supervisors, Ioanna Sandvig, Axel Sandvig and Asta Kristine Håberg for taking a chance on me despite my little experience with neuroscience at the time of starting my PhD. I am grateful for all your support and encouragement, helpful advice and for teaching me most of what I now know. I hope we will continue to work together in the years to come. A special thanks to Ioanna for the many hours spent together in the animal facility, at the cell lab and in the office, for pushing me to do better and for always making an effort to help me when I have needed it. I would not have made it through my PhD without you.

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Thanks to Prof. Mike Modo at the McGowan Institute for Regenerative Medicine at the University of Pittsburgh for welcoming me as a visiting researcher during my stay abroad. Thanks to Francesca Nicholls, Jessie Liu, Harman Ghuman, Madeline Gerwig and Julia Donnelly for being my lab group away from home.

Thanks to Marius Widerøe and Deborah Katherine Hill at the MR Core Facility at the Department of Circulation and Medical Imaging for teaching me how to perform MRI. Thanks to the fMRI group and MR Cancer group at the MR Center, for creating such a nice and including research environment despite working on very different topics.

Thanks to Susan Carol Barnett, Axel Karl Gottfrid Nyman and Alex Ignatius Costa for the collaborations on our publication.

Thanks to Professor Pavla Jendelova and Lucia Urdzíková-Machová at the Institute of Experimental Medicine of CAS, Department of Tissue Culture, University of Prague for teaching me photothrombotic lesion model.

Thanks to Professor Menno Witter at the Norwegian Brain Centre at the Kavli Institute, for allowing access to equipment and the scanning microscope in his lab, and to staff engineer Bruno Monterotti and senior engineer Grethe Mari Olsen for technical assistance.

I want to thank the core facilities at NTNU for enabling my work; especially the Comparative Medicine Core facility (CoMed) and the staff for teaching me how to work with laboratory animals and helping me take care of my animals; and EM Lab and CMIC for teaching me how to use the vibratome and cryostat.

Thanks to my family and friends for supporting, encouraging and believing in me. And finally, to my wonderful parents for always loving me, believing in me and supporting me in whatever I set out to do. Without you I would not be where I am today, and I am forever grateful. I dedicate this work to you.

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Augestad, Ingrid Lovise; Nyman, Axel Karl Gottfrid; Costa, Alex Ignatius; Barnett, Susan Carol; Sandvig, Axel; Håberg, Asta Kristine; Sandvig, Ioanna. *Effects of neural stem cell and olfactory ensheathing cell co-transplants on tissue remodelling after transient cerebral ischemia in the adult rat*. *Neurochemical Research*: 2017:1-11.

Paper II
Augestad, Ingrid Lovise; Valderhaug, Vibeke Devold; Sandvig, Axel; Håberg, Asta Kristine; Sandvig, Ioanna. *Minocycline treatment combined with neural stem cell transplantation attenuates inflammation and promotes endogenous neurogenesis after transient focal cerebral ischemia in rats*. Submitted to Translational Stroke Research.

Paper III
Augestad, Ingrid Lovise; Valderhaug, Vibeke Devold; Sandvig, Axel; Håberg, Asta Kristine; Sandvig, Ioanna. *Pharmacological immunomodulation as a strategy for improving stem cell graft survival after cortical ischemia*. Manuscript in preparation.

Paper IV
Ioanna, Sandvig; **Augestad, Ingrid Lovise**; Håberg, Asta Kristine; Sandvig, Axel. *Neuroplasticity in stroke recovery. The role of microglia in engaging and modifying synapses and networks*. Submitted to the European Journal of Neuroscience.

Additional publications

Article

McDonagh, Birgitte Hjelmeland; Singh, Gurvinder; Hak, Sjoerd; Bandyopadhyay, Sulalit; **Augestad, Ingrid Lovise**; Peddis, Davide; Sandvig, Ioanna; Sandvig, Axel; Glomm, Wilhelm. *L-DOPA-Coated Manganese Oxide Nanoparticles as Dual MRI Contrast Agents and Drug-Delivery Vehicles*. *Small* 2015; Volum 12. s. 301-306.

Posters and conference contributions

Augestad, Ingrid Lovise; Valderhaug, Vibeke Devold; Håberg, Asta Kristine; Sandvig, Axel; Sandvig, Ioanna. *Modulation of the inflammatory response after transient cerebral ischemia in rats through minocycline treatment combined with human neural stem cell transplantation*. 2nd Nordic Neuroscience Conference, Karolinska Institute, Stockholm, Sweden – 2017: 07.06-09.06. **(Poster)**

Augestad, Ingrid Lovise; Valderhaug, Vibeke Devold; Håberg, Asta Kristine; Sandvig, Axel; Sandvig, Ioanna. *Effects of Minocycline treatment on human neural stem cell survival and microglia response after transient cerebral ischemia in adult rats.* 4th National PhD Conference in Neuroscience, Hurdalssjøen, Hurdal, Norway – 2016: 21.09-23.09. **(Poster and blitz presentation)**

Augestad, Ingrid Lovise. *Co-transplantation of olfactory ensheathing cells and rat neural stem cells in a rat model of focal cerebral ischemia*. 22nd Annual Conference of the American Society of Neural Therapy and Repair, Clearwater Beach, Florida, US – 2015; 30.04-02.05. **(Oral presentation)**

McDonagh, Birgitte Hjelmeland; Singh, Gurvinder; Hak, Sjoerd; Bandyopadhyay, Sulalit; **Augestad, Ingrid Lovise;** Sandvig, Ioanna; Sandvig, Axel; Glomm, Wilhelm. *L-DOPA-coated manganese oxide nanoparticles as dual MRI contrast agents and potential drug delivery vehicles.* 1st Nordic Neuroscience Conference – 2015: 10.06-12.06. **(Poster)**

Nyman, Axel Karl Gottfrid; **Augestad, Ingrid Lovise**; Widerøe, Marius; Sandvig, Axel; Håberg, Asta; Sandvig, Ioanna. *Cellular, anatomical, and functional effects of neural stem cell and olfactory ensheathing cell co-grafts in a rat model of transient.* 1st Nordic Neuroscience Conference – 2015: 10.06-12.06. **(Poster)**

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Augestad, Ingrid Lovise; Willner, Nadine; Hill, Deborah Katherine; Sandvig, Axel; Håberg, Asta Kristine; Sandvig, Ioanna: *The effects of Minocycline on survival and functional outcome of neural stem cell grafts in experimental stroke.* ERC Meeting: New trends in CNS regeneration and treatment, Prague, Czech Republic – 2014: 15.09-17.09. Travel grant award. **(Poster)**

Augestad, Ingrid Lovise. *Stroke repair. Minocycline treatment combined with hNSC transplantation after transient cerebral ischemia in rats*. Workshop: CNS function damage and repair, NTNU, Trondheim, Norway – 2016: 16.12. **(Oral presentation)**

Augestad, Ingrid Lovise. *Stroke repair: What can we learn from animal models?* Workshop: Stroke and spinal cord injury and repair, NTNU, Trondheim, Norway – 2016: 04.11. **(Oral presentation)**

Augestad, Ingrid Lovise. *Internationalization*. Presentation for all new PhD candidates at the Faculty of Medicine and Health Sciences, NTNU, Trondheim, Norway – 2016: 15.09.

Abbreviations

AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARRIVE	Animal Research: Reporting *In Vivo* Experiments
BBB	Blood-brain-barrier
BDNF	Brain-derived neurotrophic factor
CBF	Cerebral blood flow
CCA	Common carotid artery
CNS	Central nervous system
CSPGs	Chondroitin sulphate proteoglycans
CT	Computed tomography
DALYs	Disability-adjusted life-years
DAMPs	Damage-associated molecular patterns
DNA	Deoxyribonucleic acid
DWI	Diffusion weighted imaging
ECA	External carotid artery
ECs	Endothelial cells
ESCs	Embryonic stem cells
ET-1	Endothelin-1
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
GABA	Gamma (γ)-Aminobutyric acid
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary associated protein
HT	Hemorrhagic transformation
ICA	Internal carotid artery
IL	Interleukin
iNOS	inducible nitric oxide synthase
iPSCs	Induced pluripotent stem cells
MCA	Middle cerebral artery
MCAo	Middle cerebral artery occlusion
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
NG2	Also known as oligodendrocyte progenitor cells

NGF	Nerve growth factor
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
NPCs	Neural progenitor cells
NSCs	Neural stem cells
OECs	Olfactory ensheathing cells
PSD	Post-stroke dementia
PWI	Perfusion weighted imaging
ROS	Reactive oxygen species
SD	Spreading depolarization
SGZ	Subgranular zone
STAIR	Stroke Therapy Academic Industry Roundtable
STEPS	Stem cell Therapies as an Emerging Paradigm in Stroke
SVZ	Subventricular zone
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
tPA	Tissue-plasminogen activator
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Ethical aspects

All procedures involving laboratory animals were approved by the Norwegian Animal Research Authority and conducted in accordance with the laws and regulations controlling experimental procedures in live animals in Norway and with the EU Directive 86/609/EEC.

1. Introduction to stroke

Stroke is one of the leading causes of mortality and disability-adjusted life-years (DALYs), worldwide (1). With approximately 10.3 million new incidents and 6.5 million deaths caused by stroke every year (2), few neurological diseases are as complex and devastating as stroke (3, 4). On a global basis more than 90% of the stroke burden can be attributed to modifiable risk factors related to lifestyle (*e.g.* smoking, poor diet, and low physical activity), and the physical environment (*e.g.* air pollution and lead exposure) (5). While there is little difference globally between genders, European women have a higher stroke mortality rate than men (1, 6). Despite an overall decrease in mortality over the last years, the number of stroke incidents is expected to increase due to the steadily growing population of elderly people, placing more and more impact on socio-economic costs (7, 8). Given the fact that stroke is largely considered a preventable disease, which creates a major burden, especially in low- and middle-income countries, the World Health Organization (WHO) has made it one of their goals to globally reduce the number of stroke incidents by 2025 (9).

There are two main types of stroke; hemorrhagic stroke, which accounts for 13%, and ischemic stroke, which accounts for 87% of all stroke incidents (Figure 1) (1). While hemorrhagic stroke is the result of bleeding in the brain or in the subarachnoid space by the rupture of a vessel, ischemic stroke is caused by an obstruction of blood supply to the brain caused by a thrombosis or an embolus (10). The standard clinical treatment for ischemic stroke is thrombolysis, aimed to restore blood flow by dissolving the blood clot (11). However, few patients are eligible for thrombolytic treatment as it is limited by a short therapeutic time window of only 3-4.5 hours (12). Although efforts are being made to shorten the onset-to-treatment times, many patients arrive at the hospital too late to receive treatment.

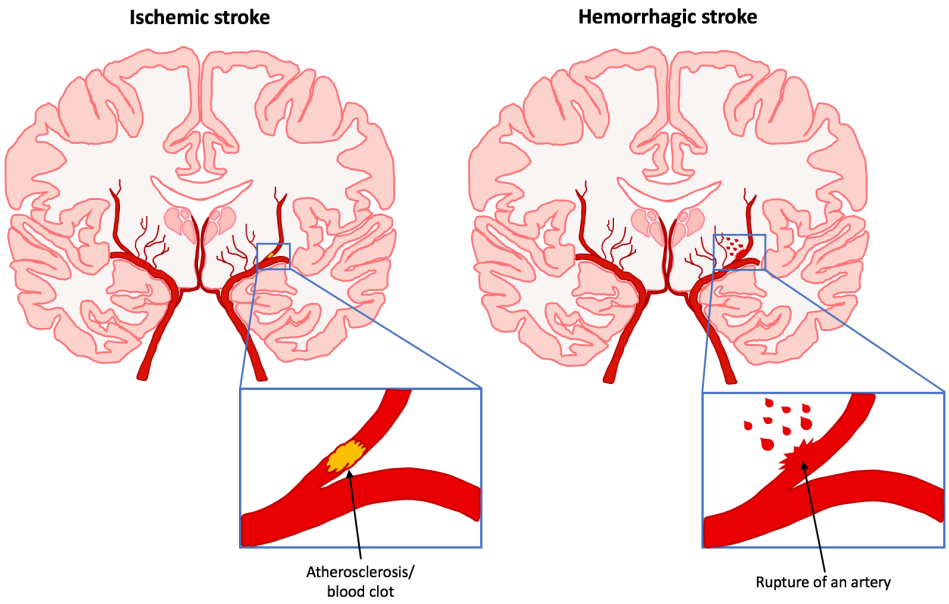

Figure 1: The two main classifications of cerebral stroke. Ischemic strokes are caused by an obstruction of a vessel by a thrombus or an embolism, leading to reduced blood supply to the brain. Hemorrhagic strokes arises when an artery in the brain ruptures and causes bleeding into the tissue or in the subarachnoid space. Augestad, IL 2017

People suffering from stroke can experience short- as well as long-term functional deficits. At the onset of a stroke, partial paralysis, loss of vision, and impaired speech are well-known physical signs. More long-term deficits (~80% of stroke survivors) involve persistent motoric deficits and cognitive dysfunctions impairing memory and executive functions (13-15). In fact, one of the main causes of dependency (*i.e.* daily assistance, home attendant or admission to nursing home) is the development of post stroke dementia (PSD) in about 30% of stroke survivors, which is related to the brain atrophy caused by the ischemic attack (16, 17).

The lack of treatment options for stroke patients suggests that there is an urgent need to further understand underlying pathophysiological mechanisms of ischemic lesions, both at the cellular and molecular level (18). Although the CNS has some inherent capacity for regeneration and remodelling, these endogenous responses are not sufficient to repair the brain after an ischemic event. The field of tissue engineering may represent a promising approach to promote repair and tissue remodelling after ischemic injury (19). By combining (stem) cells, biomaterials, and biological cues (*e.g.* growth factors) in preclinical *in vitro* and *in vivo* models, different aspects of stroke pathology and brain responses can be targeted or enhanced, especially in the context of neuroprotection, replenishing and/or restoring damaged tissue, and enhancing endogenous neurogenesis (19, 20).

1.1 Pathophysiology of ischemic stroke

Brain ischemia is most commonly caused by transient or permanent occlusion of the middle cerebral artery by an embolus or thrombus, which causes a reduction in blood flow to the brain to the extent that normal cellular function and homeostasis are significantly compromised (21). The sudden lack of blood flow triggers a cascade of cellular and molecular events, such as excitotoxicity (22, 23), peri-infarct neuronal depolarizations (24), production of free radicals such as reactive oxygen species (ROS) and nitric oxide (NO) (25-28), and inflammation leading to cell death (involving both necrosis and apoptosis). This process is specifically known as the ischemic cascade (*e.g.* Figure 2).

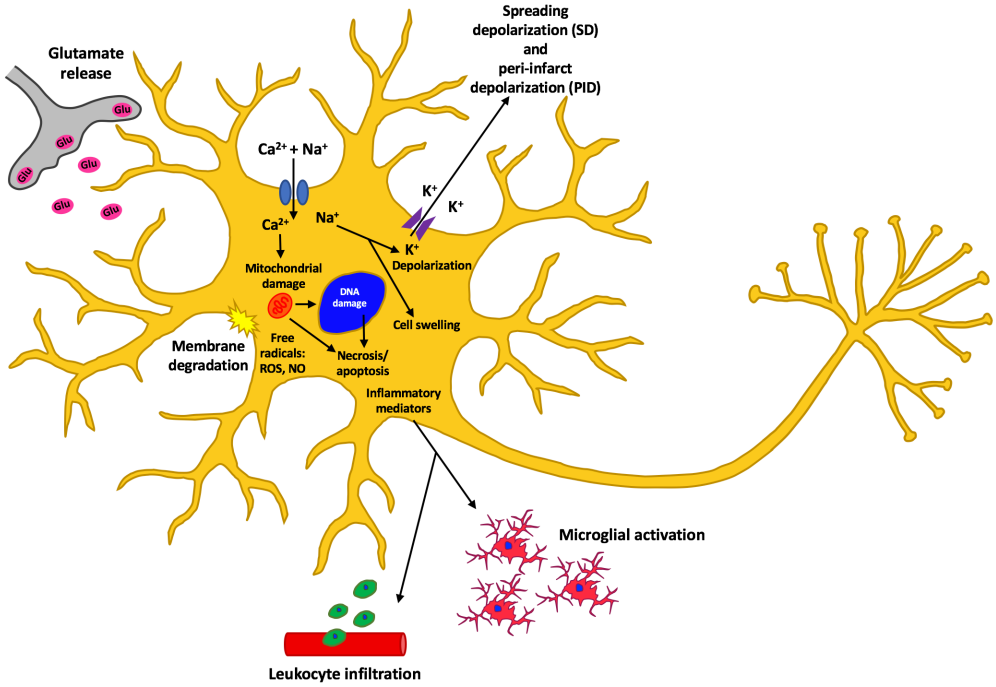

Figure 2: Stroke triggers an ischemic cascade resulting in neuronal death. Ischemia triggers a cascade of complex cellular and molecular events. Extracellular accumulation of glutamate leads to excitotoxicity causing an influx of calcium (Ca^{2+}) and sodium (Na^+), which will eventually lead to mitochondrial damage and cell swelling, respectively. The cell membrane of the affected neuron will degrade, and inflammatory mediators and free radicals will be released from the neuron triggering activation of microglia. Damage to mitochondria will furthermore trigger DNA damage and necrotic and apoptotic cell death. When the blood-brain-barrier breaks down, peripheral inflammatory cells infiltrate the brain contributing to injury. Adapted from Hossmann[30].

Reduction in the amount of available nutrients, particularly oxygen and glucose, leads to failure in maintaining ionic gradients thereby causing a breakdown in normal cellular function and homeostasis (29). Excessive accumulation of excitatory neurotransmitters in the extracellular space due to

increased release and impeded uptake of these, leads to excitotoxicity (29, 30). Excitotoxicity overactivates several downstream signaling pathways leading to increased intracellular calcium levels (31). The failure of cellular energy metabolism furthermore leads to swelling of brain cells, also known as cytotoxic edema (32).

The disruption of ionic gradients triggers neuronal depolarization, which in turn leads to release of excessive glutamate causing an influx of calcium into the intracellular compartment, the latter being termed excitotoxicity (33, 34). Overload of intracellular calcium causes neuronal oxidative stress, calpain activation, and exacerbates mitochondrial dysfunction, and DNA fragmentation (35, 36). In addition, ischemia leads to spreading depolarization (SD), which induces a wave of strong excitation followed by a wave of suppression of all spontaneous or evoked electrical activity in the affected region (37-40). Consequences of SD include propagated loss of ion homeostasis, altered vascular response, changes in synaptic architecture and subsequent depression in electrical activity across the cortex (41).

In the ischemic core, where blood flow is most severely disrupted, necrotic and excitotoxic cell death are likely to occur as early as 5-10 minutes after the onset of stroke (4, 42). Tissue damage in the infarct core is pan-necrotic, *i.e.* death of all cellular constituents including neurons, glia, and vascular cells (43). The tissue in the periphery of the ischemic core is electrically inactive and metabolically compromised but still buffered by collateral blood flow and therefore potentially salvageable (4, 44, 45). This area of hypoperfused yet transiently viable tissue is called the penumbra, and is viable only for as long as energy metabolism and membrane function are preserved (30). Unless blood flow is restored as early as possible after stroke onset, this tissue will eventually form part of an extended infarct core due to excitoxicity. At later stages, cell death in the extended infarct area occurs *via* mechanisms such as apoptosis and inflammation. The subsequent progression of the injury depends on different factors such as type of ischemia, duration of occlusion, and degree of reperfusion (30).

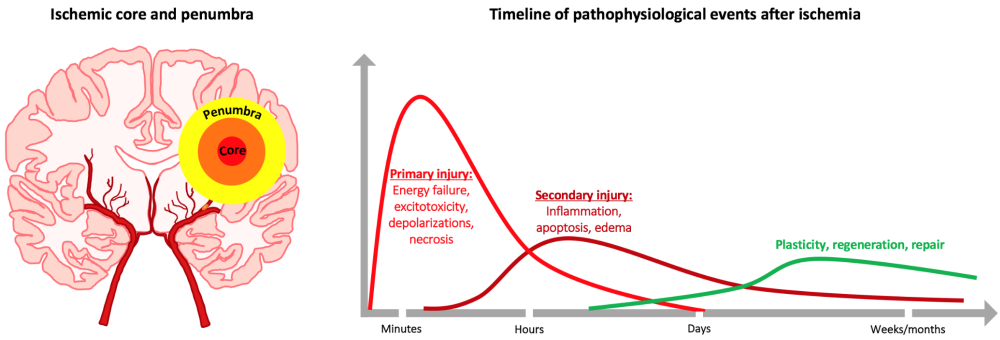

Figure 3: Development of pathophysiological processes after onset of stroke. The area of the brain where blood flow is most severely disrupted is called the ischemic core, and necrotic and excitotoxic cell death is likely to occur within 5-10 min after onset of ischemia. The surrounding area, which is still buffered by blood from collateral arterial branches and therefore still represents salvageable tissue, is called the penumbra. Unless blood flow is restored as early as possible after onset of stroke, the cells within the penumbra will die as part of an extended infarct core. During the time course after an ischemic event, different mechanisms are involved in primary and secondary injury in the acute (minutes-hours) and subacute (hours-days) phase, and plasticity and repair in the chronic (weeks-months) phase. Adapted from Dirnagl, Iadecola and Moskowitz[29].

An important brain structure affected by ischemia is the blood-brain-barrier (BBB), which is composed of a tightly sealed monolayer of endothelial cells (ECs) that form the walls of the capillaries, together with pericytes, and the end-feet of astrocytes (46). The BBB creates specific physiological barriers consisting of enzymes and transporters that prevent free exchange of components of the circulating blood system to come into contact with neurons (47, 48). ECs are therefore crucial in the response to ischemic events because they create a diffusion barrier against potentially harmful blood-borne solutes (49). Normal brain function and the communication between neurons are highly reliant on the integrity of the neuronal-vascular relationship/coupling as the precise regulation of the ionic microenvironment around synapses and axons is critical for neural signaling (46, 48). However, when the homeostatic mechanisms of the BBB fail due to cellular death/injury (matrix and intercellular signal exchange damage), solutes, water and peripheral immune cells can move across the BBB and into the extracellular space of the brain parenchyma further exacerbating ischemic injury (50, 51). Matrix metalloproteinases (MMPs), a family of zinc-dependent proteolytic enzymes, are involved in BBB breakdown, especially MMP 2 and MMP 9, which are upregulated 1-3h after onset of ischemia (51, 52).

Breakdown of the BBB leads to influx of water into the brain parenchyma, *i.e.* vasogenic edema, causing an increase in the volume of the extravascular space in the brain and increased tissue pressure (32). This increased tissue pressure may in turn compromise capillary perfusion and further exacerbate

the damage caused by ischemia (53). Cytotoxic edema is observed in the early phase of ischemia and mostly affects gray matter, whereas vasogenic edema is a common complication of stroke and spreads preferentially through white matter (54). This is thought to be related to the difference in the cellular organization of the two structures (54).

The temporal progression of ischemic stroke injury can be differentiated into three phases; during the acute phase energy failure and depolarization of cell membranes leads to tissue injury within minutes after onset of ischemia (55). If blood flow is not restored at an early stage, the infarct core expands into the penumbra in the subacute phase (from 4-6 h after onset). The largest increment of infarct volume occurs during this phase. Finally, in the chronic phase (days-weeks), secondary phenomena such as vasogenic edema, inflammation and apoptosis may cause further injury (Figure 3) (55).

1.1.1 Role of inflammation and microglia

In addition to the ischemic cascade, stroke also triggers an inflammatory cascade (56). Ischemic tissue, *i.e.* dying cells in the brain parenchyma, releases pro-inflammatory factors such as cytokines, neurotransmitters and damage-associated molecular patterns (DAMPs) which leads to activation of microglia and astrocytes (57). Microglia are the tissue-resident macrophages in the CNS and respond to ischemia through activation and proliferation followed by migration to the site of injury (58, 59). The microglial response occurs along a spatial gradient with the highest level of proliferation closest to the infarct edge.

In their resting state, microglia have very motile processes while their somata more or less remain in the same position (Figure 4) (60). Constant surveillance of the microenvironment for accumulating metabolic products and deteriorated tissue components enables microglia to respond quickly to ischemic or other injuries in the brain (60-62). Once ischemia occurs, microglia enter a hypertrophic state and start secreting pro-inflammatory mediators such as cytokines, chemokines, cell adhesion molecules and MMPs, exacerbating neuronal injury (Figure 4) (31, 63). Some of the key pro-inflammatory cytokines which are secreted by microglia and contribute to ischemic brain injury include tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-1β and IL-6 (52). In fact, microglia together with macrophages are the major sources of TNF-α secretion (64).

Microglia can be broadly defined based on their dual function in response to ischemia (Figure 4), the classic M1 and the alternative M2 activation, and the balance between these states may determine the fate of injured neurons (65). M1 microglia produce pro-inflammatory cytokines and reactive oxygen and nitrogen species, while M2 microglia promote anti-inflammatory responses and are

involved in wound repair and debris clearance (63, 65, 66). However, the specific phenotype microglia display is not a static shift from pro-inflammatory to anti-inflammatory, rather, microglia can switch between these two states depending on inflammatory signals from the microenvironment (63). Interestingly, microglia activation after ischemic insult may influence proliferation and migration of neural progenitors from neurogenic niches in the brain (67-69). Indeed, increasing evidence suggests that microglia are involved in neurogenesis after stroke (70). The relationship between inflammation and neurogenesis is not well understood, however, elucidation of the underlying mechanisms might add to the development of new therapies that can promote reparative processes after brain ischemia (70).

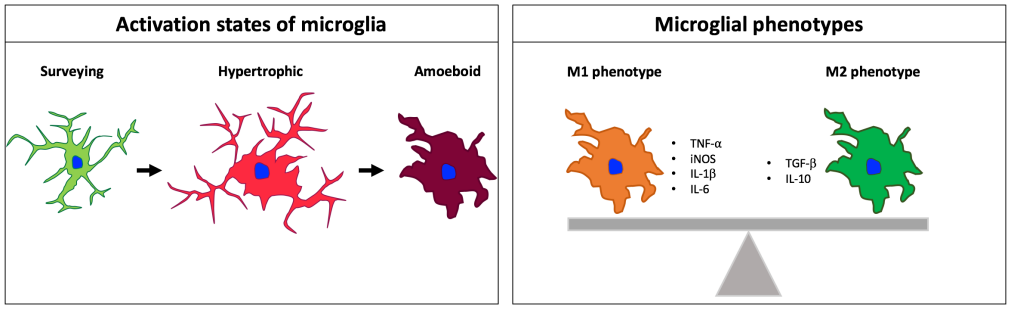

Figure 4: Ischemic stroke triggers activation of microglia. In a normal state, microglia are constantly surveying the environment for signs of injury; when they detect inflammatory cues from *e.g.* injured neurons they go into a hypertrophic state. Upon detecting dead or dying neurons, microglia take on an amoeboid shape and assume a phagocytic role. Amoeboid microglia can be further classified into two different phenotypes, the classic M1 and the alternative M2 type. M1 microglia mediate pro-inflammatory processes by secreting cytokines such as TNF-α and IL-6, while M2 microglia mediate anti-inflammatory processes thereby contributing to tissue clearance. Adapted from Hu et al. [54].

1.1.2 Reactive astrocytes and the glial scar

Astrocytes are the most abundant type of cells in the human brain and play a critical role in maintaining normal brain function, physiology and homeostasis. In addition, astrocytes are important for brain architecture due to their involvement in the neurovascular unit and close relationship with the BBB (71, 72). Some of the roles of astrocytes include ion buffering, neurotransmission, controlling cerebral blood flow, transport of water, release of antioxidant substances, and immunomodulation (73). In response to ischemia, however, the excessive accumulation of ions in the extracellular space overloads the buffering capacity of the astrocytes (33, 73). This causes changes in the molecular expression and morphology of the astrocytes, a process commonly referred to as reactive astrogliosis, where astrocytic processes become hypertrophic (72, 74).

The activation of astrocytes can be observed as a progressive change in gene expression with the upregulation of glial fibrillary associated protein (GFAP) and nestin (75). Following ischemia, astrocytes undergo several alterations over time, resulting in reactive astrocytes, which together with activated microglia, form the glial scar (also known as the glia limitans). The glial scar provides a functional and structural barrier at the interface between intact tissue and the ischemic lesion (76). Furthermore, there is an upregulation of inhibitory molecules secreted by the glial cells/astrocytes in the border area surrounding the lesion, which has an inhibitory effect on axon outgrowth (77). Inhibitory molecules include extracellular matrix components, such as chondroitin sulphate proteoglycans (CSPGs) produced by reactive astrocytes, and NG2-positive oligodendrocyte precursor cells (78).

In the same manner as microglia, the role of astrocytes in ischemia can be detrimental but also beneficial in ischemic conditions (52). Reactive astrocytes are involved in the immune response by mediating pro-inflammatory signals in the brain, thereby influencing physiological and behavioural responses (73). Specifically, several reports have shown that reactive astrocytes may help promote neuronal survival through various processes supporting tissue remodelling, including release of neurotrophic factors and promotion of synaptic plasticity (74, 79, 80). Furthermore, increasing evidence suggests that reactive astrocytes can contribute to remodelling of damaged tissue and penumbra networks after stroke through active phagocytosis (81).

1.2. Clinical assessment and management

The main aim of acute stroke treatment is to restore blood flow to the affected part of the brain, and rescue penumbral tissue (82). There are two main approaches for clinical treatment of ischemic stroke. The first approach, thrombolysis, utilizes tissue plasminogen activator (tPA), a serine protease, to catalyze the conversion of plasminogen to plasmin, thus dissolving the blood clot (83). However, the therapeutic time window of tPA is very short, only up to 4.5 hours after onset (12). Permanent tissue loss rapidly develops after onset of ischemia, within as early as a few hours up to 3-4 days after stroke, emphasizing the need for early intervention (84). As a result, fewer than 7% of stroke patients are eligible for treatment with tPA, primarily because patients reach the hospital too late for the initiation of therapy (85). The second approach, thrombectomy, involves different methods to surgically remove the clot (31, 86). The time window of thrombectomy, however, can be up to 6-8 hrs and in some cases even up to 24 hrs after onset of ischemia (87, 88).

Tissue PA was the first approved drug for intra-arterial thrombolytic treatment of stroke patients by the American Food and Drug Administration (FDA) (89). However, the short therapeutic time-window

of tPA as well as the risk of bleedings after reperfusion, also known as hemorrhagic transformation (HT), suggests that the current available treatments are not adequate (89). The risk of hemorrhage also increases with delayed onset of tPA treatment (90). Especially patients diagnosed with hemorrhagic strokes should not be administered tPA as this can cause additional bleeding and further damage to the brain. The sooner the patient receives thrombolytic treatment, the greater probability for a better outcome (91). Thus, reperfusion within the "golden hour" (*i.e.* 60 min) after onset of stroke, can make a major difference in salvaging 'at-risk' brain tissue (92).

It has been estimated that an average stroke patient loses 1.2 billion neurons, 8.3 trillion synapses, and 7140 km myelinated fibers, leading to an accelerated aging of 36 years on average per stroke patient (Table 1) (93). Indeed, for every minute the stroke remains untreated, about 2 million neurons are lost. This not only emphasizes the importance of starting treatment as soon as possible, but also the fact that the rapid development of the ischemic lesion has major implications for the patient.

	Neurons lost	Synapses lost	Myelinated fibers lost	Accelerated aging
Per stroke	1.2 billion	8.3 trillion	7140 km	36 years
Per hour	120 million	830 billion	714 km	3.6 years
Per minute	1.9 million	14 billion	12 km	3.1 weeks

Table 1: Estimated pace of neural circuitry loss in acute ischemic stroke. Ischemic lesions develop rapidly after onset and often have severe implications for the patient. It has been estimated that about 2 million neurons, 14 billion synapses and 12 km of myelinated fibers are lost per minute during an ischemic event, which highlights the urgent need for early intervention. Adapted from Saver[93].

1.2.1 Imaging of the ischemic brain

Diagnostic imaging of the brain is an invaluable tool in clinical stroke medicine because it provides information about the nature, location and volume of the stroke lesion (94). The standard imaging technique used to acquire anatomical information is computed tomography (CT), however, owing to its superior soft-tissue contrast, magnetic resonance imaging (MRI) is also more frequently being used to acquire details about the stroke physiology beyond lesion structure, as seen with T2- and T1-weighted MRI (94). Diffusion-weighted imaging (DWI) is the most sensitive and specific MRI technique for detection of acute infarction because it uncovers cytotoxic edema which is the initial pathophysiological event (see above) (43). Also perfusion-weighted imaging (PWI) gives important clinical information about the cerebral blood flow. Together, DWI and PWI constitute the MRI scans that can delineate the penumbra.

Apart from assessing the severity of stroke, diagnostic imaging can also help determine which patients are eligible for thrombolytic treatment, *i.e.* by confirming that the stroke is ischemic, not haemorrhagic (95). Verification of a haemorrhagic stroke is an absolute contraindication to thrombolysis, whereby administration of tPA to patients under these circumstances may cause further bleeding in the brain and a worse outcome.

1.2.2 Stroke rehabilitation

It is widely accepted that early rehabilitation is an important feature of effective stroke care (96). This is associated with reduced costs of care, and can significantly speed up the recovery process. Ischemic stroke results in damage to neuronal networks and the impairment of sensation, movement or cognition (97). Despite the severe disabilities with which many stroke patients live, many patients also experience spontaneous recovery, which can be improved further by rehabilitative therapy.

Evidence from animal studies suggests that there is a time-limited window of neuroplasticity following a stroke, and it is during this period that the greatest gains in recovery can occur. Plasticity mechanisms include activity-dependent rewiring and synapse strengthening, and the challenge for improving stroke recovery is to understand how to optimally engage and modify surviving neural networks, to provide new response strategies that compensate for tissue lost to injury (97). Recovery is further complicated by the heterogeneity of lesion location and degree of cell loss within a given group of stroke patients. Functional recovery thus depends on restoration of connectivity in multiple neuronal circuits, as functional connectivity of adjacent regions can be affected by stroke through retrograde degeneration between existing neuronal networks (98).

1.3 Targets of therapeutic strategies

1.3.1 Neuroprotection

Neurons are particularly sensitive to ischemia, suggesting that neuroprotective strategies should be applied in the acute phase of stroke (99). Neuroprotection can be defined as “any strategy which directly targets the brain parenchyma with the aims of antagonizing the harmful molecular and cellular events responsible for the ischemic damage, allowing brain cells to survive the reduced cerebral blood flow (CBF) and to stabilize the penumbra” (100). Given the number of neurons lost within a short time period after onset of stroke, research on neuroprotection has been focused on the different factors that influence neuronal death (101, 102). Numerous molecular processes involved in the ischemic cascade and pathological mechanisms have been investigated in order to achieve neuroprotection including mechanisms underlying excitotoxicity, calcium influx, ROS scavenging, NO production,

inflammatory reactions and apoptosis (Figure 2) (100). Furthermore, some of the different neuroprotective agents that have been investigated include: glutamate receptor blockers such as NMDA (*N*-methyl-D-aspartate) agonists, calcium channel blockers, GABA (γ-Aminobutyric acid) and AMPA (Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) agonists, small cation channel modulators, administration of growth factors, inflammatory inhibitors and free radical scavengers (102, 103). Despite the fact that many of these approaches have shown positive results in preclinical models, they have generally failed to produce equivalent results in clinical trials (102, 104-106).

Considering the multiple pathways involved in the pathophysiology of ischemia, effective neuroprotection might require a combination or addition/sequence of drugs that target distinct pathways during the protracted development of ischemic injury (107). Excitotoxicity is important during the initial 1-2 hrs after onset of stroke (Figure 3) and the differences in timing of neuroprotective treatments between animal models and in clinical trials may be part of the explanation for the lack of translational success of neuroprotective agents (107). Furthermore, the majority of *in vivo* experiments on stroke are performed on healthy young animals where comorbidities such as hypertension and diabetes are not factored in. Finally, animal models do not fully represent the heterogeneity in stroke lesions as seen in human patients (102-104), neither the complexity of the human brain.

1.3.2 Modulation of inflammation

The neuroinflammatory response to ischemic stroke starts with the activation of microglia followed by infiltration by circulating inflammatory cells and reactive astrocytosis (108). Neuroinflammation is a fundamental response that protects the CNS, however, uncontrolled or prolonged inflammation is potentially harmful and may result in increased cellular damage (65). Ischemic stroke leads to the release of cytokines, superoxide NO and proteases, and production of ROS, which can further exacerbate ischemic brain injury (Figure 2) (109). Different approaches to modulate these responses include agents inhibiting activation and proliferation of microglia (*e.g.* minocycline), as well as agents reducing inducible nitric oxide synthase (iNOS), IL-1β and cyclooxygenase-2 (110).

Several preclinical studies have used anti-inflammatory approaches and shown promising results in modulating the inflammatory response after stroke (111). However, attempts at clinical translation have been unsuccessful, likely because of the heterogeneity in the underlying mechanisms of post-ischemic inflammation and the uncertain time window at which inflammation should be targeted (111).

There are many theoretical advantages of anti-inflammatory treatment such as increasing the therapeutic time-window and limiting inflammation associated with reperfusion (108). However, inflammatory cells play complex and multiphasic roles after ischemic stroke, displaying both adverse and beneficial effects. Based on experimental findings, inflammatory cell infiltration is predominantly deleterious in the early phase after ischemic stroke and targeting a single cell type/only inflammation will most likely not be a feasible way to treat human stroke (70). Furthermore, inhibiting the immune response at the wrong time could be more damaging than beneficial, and immune modulating interventions should therefore be tailored to specific phases of stroke (112).

Minocycline

Of particular interest in the context of pharmacological therapy aimed to modulate stroke lesion pathology is minocycline. Minocycline is a tetracycline derivative, known for its anti-apoptotic, neuroprotective, as well as anti-inflammatory properties and reduction of microglial activation (112-115). Several preclinical and clinical studies suggest a positive, safe effect of minocycline on modulating inflammation and reducing tissue loss. Indeed, minocycline is considered suitable for widening the therapeutic window beyond 4-5 hrs of thrombolysis due to its alleged stabilizing effect on the BBB (11, 116-119). Importantly, minocycline may facilitate an alternative activation of microglia/macrophages into a neuroprotective phenotype (120). Furthermore, minocycline has been shown to reduce the expression of TNF-α and IL-1β, while at the same time increasing the expression of anti-inflammatory cytokines transforming growth factor beta (TGF-β) and IL-10 four weeks after middle cerebral artery occlusion (MCAo) in rats (120), without affecting the differentiation potential of transplanted neural stem cells (NSCs) (121).

1.3.3 Reperfusion/angiogenesis

There are two processes that lead to vessel formation, vasculogenesis and angiogenesis (122). Whereas vasculogenesis is the formation of new vessels by *de novo* production of endothelial cells, angiogenesis is the formation of new vessels from pre-existing ones (123). One of the key factors in the post stroke regeneration process is angiogenesis, and proangiogenic therapies may thus help to decrease infarct size and restore vascular networks. New vessel growth is most pronounced in the ischemic penumbra where small changes in perfusion might make the difference between cell death and survival (124).

Although angiogenesis may act as a positive and necessary regenerative process after stroke, the endothelial barrier function is severely compromised during the vascular remodelling process (105).

Vascular endothelial growth factor (VEGF) is the most potent trigger for inducing angiogenesis showing a high upregulation within 1 hour after stroke onset (105). However, while VEGF can have a positive effect on brain perfusion by promoting angiogenesis, pathological levels induce BBB breakdown and vessel leakage (125). In animal models of stroke, VEGF administration within 1 h after onset of ischemia leads to increased brain edema and worse neurological outcome (126). Later administration however, enhances angiogenesis in the ischemic penumbra and significantly improves neurological recovery (126).

1.4 Transplant-mediated repair

Transplant-mediated repair, where exogenous cells are transplanted into the brain as part of a cell replacement strategy, has emerged as one of the main approaches in pre-clinical studies in the last years. Cell loss after ischemic stroke is not limited to a single population of neurons, but involves different neuronal populations, as well as glia and endothelial cells (127), suggesting a promising role for stem cells in transplant-mediated stroke repair. However, the main consensus regarding grafted cells is that they are not so much integrated into residing neural networks but rather promote neurological recovery through paracrine mechanisms (128).

The development of ischemic injury over time suggests that stem cell therapy will have different effects depending on the time point of transplantation (99). In the acute phase of stroke, stem cell therapy may exert neuroprotective effects, while sub-acute delivery of stem cells is thought to prevent early cell death, inhibiting inflammation, mitochondrial impairment, oxidative stress and apoptosis. Finally, in the chronic phase, administration of stem cells is intended to replace lost tissue and influence tissue remodelling by stimulating endogenous stem cell proliferation and initiating reparative processes such as angiogenesis, vasculogenesis, neurogenesis and synaptogenesis (99).

Challenges associated with successful clinical translation of stem cell therapy include choice of cell type, autologous vs. allogeneic transplantation, the age of the patient, stroke subtype and location, treatment strategy, including selection of time-point, delivery route and cell dose, and finally, validation of the treatment with functional assessment and neuroimaging (129). Depending on the cell type chosen, there may also be ethical issues related to origin, as in the case of embryonic stem cells (ESCs). However, cells of other origins, such as mesenchymal stem cells (MSCs), NSCs, and induced pluripotent stem cells (iPSCs) do not pose the same ethical issues as ESCs as these cell types can be derived from non-embryonic sources. Finally, stroke patients as well as experimental animals may

often experience additional complications, such as immunodepression, which may also affect functional outcome (130).

1.4.1 Mesenchymal stem cells

MSCs are the most widely used cell source in clinical stroke studies because of the broad experience with these cells in other clinical contexts (128). MSCs are multipotent cells derived from bone marrow, umbilical cord blood cells, placenta, muscle, and skin, and can secrete different cytokines and growth factors, including VEGF and angiopoietin, directly or through endogenously induced mechanisms (131, 132). MSCs can differentiate into cell phenotypes that are relevant for repair (including neuronal and glial phenotypes), and may influence multiple mechanisms that may aid in post-stroke outcome, such as induction of angiogenesis, promotion of neurogenesis, prevention of apoptosis and immunomodulation (133). Transplantation of MSCs in animal models of stroke has been reported to result in improvement in sensory-motor function, enhanced synaptogenesis, promotion of nerve regeneration, amelioration of tPA-induced brain damage, and anti-inflammatory and immunomodulatory effects (134).

1.4.2 Human embryonic stem cells

Human embryonic stem cells (hESCs) are pluripotent cells that can differentiate into cells from all three germ layers (135). Unlike other sources of stem cells there is almost an unlimited self-renewal capacity of these cells, and they have therefore been an ideal source of cells for regenerative medicine (136). However, just because of this pluripotent ability there are risks and concerns associated with hESCs when it comes to their application in transplantation studies, such as the risk of teratoma formation, in addition to ethical considerations regarding their sourcing/origin (137).

1.4.3 Neural stem cells

NSCs are self-renewing multipotent progenitors that can differentiate into neurons as well as astrocytes and oligodendrocytes, a property that renders them highly relevant for stroke repair (138, 139). Furthermore, NSCs are known to secrete a host of different neurotrophic and immunomodulatory factors, which have been associated with improved functional outcomes in stroke repair (140-142). NSCs can be derived from ESCs or iPSCs, but also directly from other sources of adult stem cells from different tissues. Although there are many positive effects of NSC, survival of these cells in the brain parenchyma is very poor, and differentiation and migration of the engrafted cells are very much dependent on time point, location, and mode of delivery (120). The above pose major challenges in the translational success of this approach. As a result, NSCs are under intense

investigation in terms of developing strategies that may enhance their post-transplantation survival and function in stroke.

1.4.4 Olfactory ensheathing cells

Although few studies have used olfactory ensheathing cells (OECs) in stroke models, their properties make them an interesting approach to cell-based therapy. OECs, the glia surrounding the primary olfactory neurons throughout their trajectory from the olfactory mucosa to the olfactory bulb glomeruli, possess several attributes that make them particularly interesting in the repair of CNS lesions, including cerebral ischemia (143). OECs secrete a host of growth factors, including VEGF, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and nerve growth factor (NGF), all of which are important for neural, glial and endothelial cell function as demonstrated in several experimental studies (144-149). Furthermore, OECs share similar functional features with both Schwann cells and astrocytes, but compared to Schwann cells, OECs induce less astrocytic response in both *in vitro* confrontation assays (150) and following transplantation into adult CNS white matter (151).

1.5 Harnessing endogenous plasticity

We have learned from animal models that mammals retain some capacity for regeneration after stroke injury. This involves predominantly two mechanisms; endogenous neurogenesis and neural network remodelling. Neurogenesis after stroke in rodents occurs mainly in two different niches where neural progenitor cells (NPCs) reside in the brain: the subventricular zone (SVZ) lining the lateral ventricle, and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (Figure 5) (139). In the former zone, there is continuous neuronal replacement, in which newly formed NPCs from the SVZ migrate to the olfactory bulb (142). Interestingly, in response to stroke, both the SVZ and the SGZ are stimulated (152-157). However, the most dramatic response is observed in NPCs from the ipsilesional SVZ, which proliferate and start migrating on ectopic pathways towards the ischemic lesion, where surviving progenitors may contribute to tissue remodelling through partial cell replacement and/or bystander effects such as modulation of inflammation and secretion of growth factors (84, 142, 158, 159). However, relatively little is known about the exact mechanisms regulating neurogenic responses to ischemic injury and how to improve NPC survival, differentiation, migration and functional integration to promote brain repair after stroke (69, 70, 160, 161).

Several other factors pose significant challenges in understanding and harnessing endogenous neurogenic responses in stroke. For example, most studies are done on young animals, while stroke

tends to affect older individuals, where neurogenesis may already be relatively limited. Furthermore, there are large differences in the neurogenic potential between different species (162, 163). Survival of newly formed neurons after insults such as stroke has been estimated to be about 0.2% in rodents, and it is still unknown to what extent these cells may contribute to reconstruction of neural circuitry and functional recovery (164).

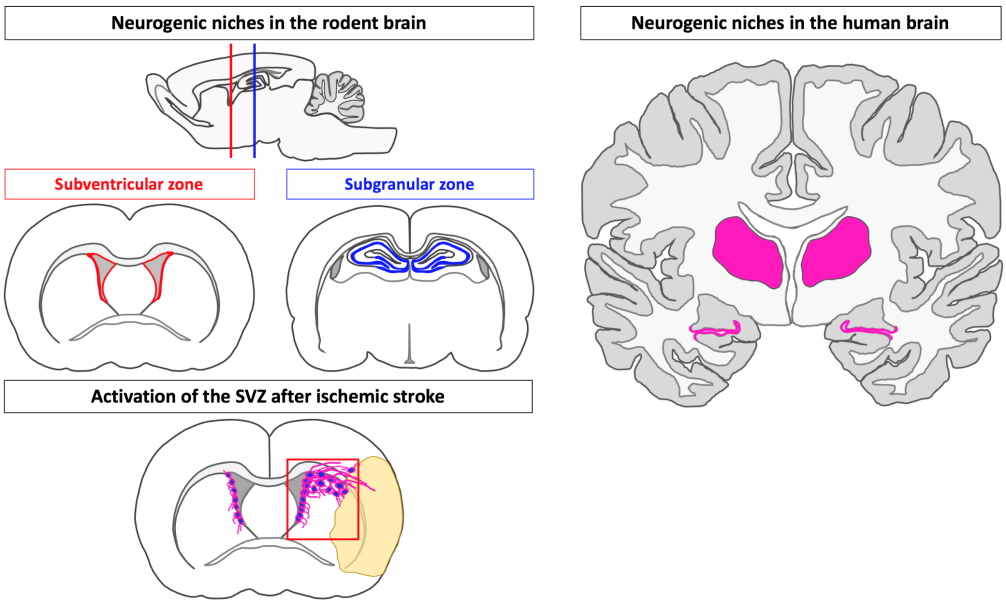

Figure 5: Illustration of neurogenic niches in the rodent and human brain. In the rodent brain, there are two neurogenic niches; the subventricular zone (SVZ) in the lateral ventricles (red), and the subgranular zone (SGZ) in the dentate gyrus in the hippocampus (blue). After an ischemic stroke, these neurogenic niches, especially the SVZ, are activated and neural progenitors proliferate and migrate out of the niche and towards the lesion area. Most of the evidence we have of neurogenic niches in the adult brain comes from animal studies. However, evidence also suggest adult neurogenesis in the striatum and dentate gyrus in the human brain (magenta). Adapted from Magnusson and Frisén[159].

Animals with ischemic stroke, but also stroke patients, exhibit a form of adaptive plasticity post-injury manifested as partial spontaneous recovery of sensorimotor and/or cognitive functions (165). This involves perilesional, cortical and sub-cortical remapping, as well as alterations in intrahemispheric balance, either of which may lead to adaptive, but also maladaptive reorganization of neural networks (166). By combining different approaches such as pharmacological intervention, rehabilitation, or other adjuvant strategies, neuroplasticity can be promoted (167). Despite current available interventions, the limited improvement of lesion induced deficits as well as the potential for

maladaptations strongly suggest that we need to improve our understanding of the underlying mechanisms of plasticity and spontaneous recovery to design more efficient and targeted treatment strategies to translate into the clinic (165, 168).

1.6 Experimental stroke models

As a result of experimental stroke research, we have gained an understanding of pathophysiological processes and the molecular, cellular and systemic mechanisms involved in ischemic stroke (169). Animal models of stroke have been developed to induce ischemia within the middle cerebral artery (MCA) territory to reflect the most common clinical cases of ischemic stroke (Figure 6) (170). Such models may involve either transient or permanent ischemia. The most commonly used animal in stroke studies is the rat because its cerebrovascular system and physiology resembles that of humans, as well as due to the ease of conducting reproducible studies due to its smaller size compared to larger animals (170). Rat models of ischemic stroke can produce long-term neurological disability, sensorimotor and cognitive impairments replicating what is observed in human patients. Some of the clinical features modelled in rats include hemiplegia, spatial neglect, tactile extinction, and learning and memory deficits (171).

Although several experimental stroke models exist, the following paragraphs present in more detail the two main stroke models on which the experimental work in this thesis is based, namely MCAo and photothrombotic lesion models, followed by a brief description of other relevant stroke models.

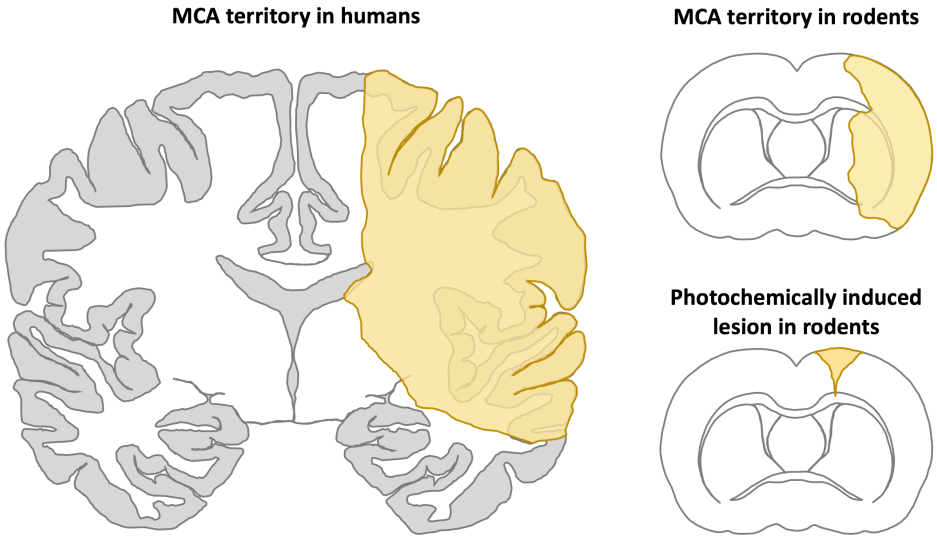

Figure 6: Brain areas affected by obstruction of the MCA or lesioned by photothrombosis. Illustration of the area of the human and rodent brain supplied by the middle cerebral artery (MCA) in yellow; regions shown in a coronal plane. Areas typically affected by ischemic strokes include striatal and cortical areas such as the primary motor cortex. Photochemically induced injury typically leads to smaller lesions, mainly in cortical areas, compared to MCAo. Augestad, IL 2017.

1.6.1 Middle cerebral artery occlusion

The MCA is the most commonly affected vessel in stroke (43). This artery supplies most of the outer convex brain surface, most of the basal ganglia, and the anterior and posterior internal capsules. In the rat, the MCA supplies the frontal, sensorimotor, auditory and occipital cortices, and the striatum (172). Therefore, experimental models investigating occlusions of the MCA make them highly relevant for ischemic stroke in the context of clinical translation. The main model of MCA occlusion (MCAo) is the intraluminal filament technique originally developed by Longa et al. (173). Here a filament is introduced into the common carotid artery and advanced up past the MCA to obstruct blood flow in the MCA vascular territory (Figure 7). The filament can subsequently be withdrawn to enable reperfusion or left permanently in place, thus the MCAo model can be used to induce either transient or permanent focal cerebral ischemia. Furthermore, depending on the length of occlusion, the technique can effectively induce lesions of various degrees of severity (173-175).

1.6.2 Photothrombotic lesion

Photothrombotic lesion is a minimally invasive method of inducing ischemic damage to a specific cortical area (Figure 7) (176, 177). Photoactivation of an injected light-sensitive dye leads to singlet

oxygen that damages components of endothelial cell membranes. Blood flow is interrupted by the subsequent platelet aggregation and thrombi formation in the vessels reached by the cold light source. Furthermore, photothrombosis causes progressive reduction in blood flow, blood-brain-barrier (BBB) dysfunction and breakdown, and cell damage within few hours after onset of damage (178).

1.6.3 Other models of stroke

In addition to MCAo and photothrombotic lesion there are several other experimental models of stroke (130). These include, embolic, thromboembolic and electrocoagulation models of MCAo, as well as stereotactic injection of Endothelin (ET)-1, Endothelin-1 a potent vasoconstrictor into the brain (175, 179).

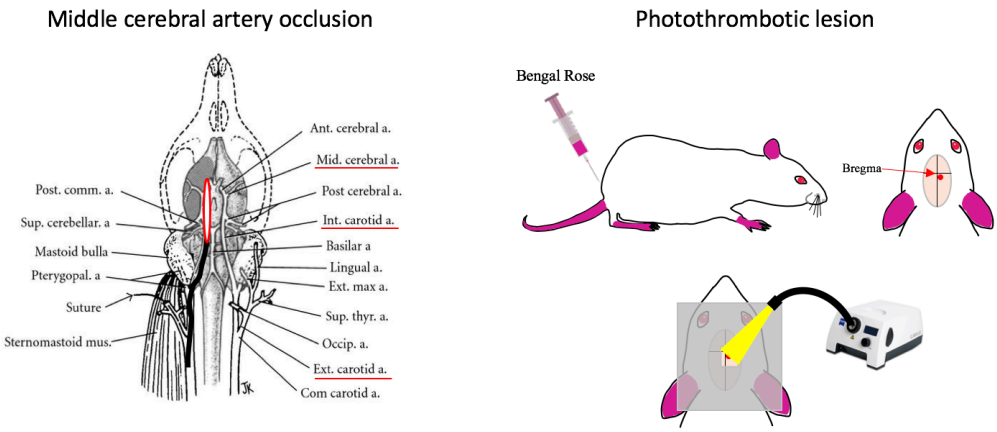

Figure 7: Main models of experimental ischemic stroke. MCAo is induced by inserting a silicone coated filament into the common carotid artery and advancing it up into the MCA. The filament is left in place for 30-120 min before allowing reperfusion by drawing the filament out (Adapted from Longa et al. [172]). Photothrombotic lesion is induced by intravenous injection of the photosensitive dye Bengal Rose. Following Bengal Rose injection an incision is made on the scalp to locate bregma and the area above the cortex (red dot) to be illuminated by white light. A piece of aluminium foil with a cut out of about 0.5x0.5 cm is placed on top of the animal´s head to prevent more than a small area of the cortex to be lesioned (Augestad, IL 2017).

1.6.4 Neurological and functional assessment tests in experimental stroke research

Characterization of functional recovery is critical for evaluating the efficacy of potential therapeutic agents and treatment strategies for ischemic stroke lesions (180). We can divide functional and behavioural assessments into two main categories; sensorimotor tests, including neurological assessment, that can determine the extent of sensory and motoric deficits, and cognitive tests to investigate *e.g.* memory deficits. Choosing the appropriate test may be crucial for the success of translational research because the test must be sensitive to the area of brain damage but also to the

interventions being applied, as well as the experimental model in question (180). Stroke patients, and rodents with induced stroke lesions, can display a large degree of spontaneous recovery after stroke, and the neurological impairments assessed by different neurological scales, (*e.g.* the Bederson scale assessing rodents) usually disappear during the first weeks after onset of ischemia (181). Furthermore, patients suffering from stroke often experience cognitive deficits in learning, memory and inhibitory control, which underlines the importance of including a battery of tests (cognitive as well as sensorimotor tests) in animal studies in order to improve clinical translatability (182, 183). Cognitive tests can include different mazes (*e.g.* the Morris Water Maze, Barnes Maze and radial-arm maze), open field test and object-recognition tasks (184-186). While motor deficits are relatively objective endpoints in ischemic stroke models, assessment of cognitive deficits in rodents is more challenging because the sensorimotor deficits may act as confounding factors in complex behavioural tests (170, 187). Following is a short description of some of the main sensorimotor tests used in the field.

The Cylinder test (lateral asymmetry test)

This test assesses asymmetries in spontaneous forelimb use by placing the animals in a transparent cylindrical enclosure which allows vertical exploration of the environment (182). Placing mirrors behind the cylinder and video recording the animals, gives the experimenter the opportunity to track movements from all angles. Placements of the good, *i.e.* unaffected, forelimb, affected forelimb, or both on the cylinder wall is then counted. This test is sensitive to long-term deficits in forelimb use that might otherwise be masked by motor relearning (182).

The staircase test

This test is used to assess independent use of the forelimbs after unilateral lesions of *e.g.* the sensorimotor cortex (188). The animal is placed on top of a platform inside a smaller box, with a removable double staircase inserted at the end of the box so that a row of steps is on either side of the platform. On each step of the staircase, there is a shallow well that can hold a small food pellet. At the end of the test, the number of remaining pellets on either side is counted (188).

Adhesive removal test (sticky tape test)

Focal cortical or striatal lesions often cause contralateral sensory neglect (182). This asymmetric tactile response can be tested using the adhesive removal test by placing a small piece of tape on each front paw of the animal, and compare the time it takes for the animal to remove the piece of tape from each paw. If there is unilateral lesion to the striatum or forelimb region of the sensorimotor cortex, the animals will first respond to the piece of sticky tape on the good forelimb and remove it with their

mouth, before responding to the piece of tape on the bad forelimb. Depending on the extent of injury, this sensorimotor asymmetry slowly diminishes with time or can be stable and chronic (182).

1.6.5 Challenges with animal models of stroke related to clinical translation

One of the current challenges with the use of animal models is the fact that they tend to be poor predictors for the success of different therapies in the clinic (171). This may be related to the young age of animals included in studies, lack of co-morbidity with other diseases such as diabetes, and the fact that most preclinical stroke models include only male animals (189). In addition to the difficulty of representing human brain complexity in animals (29, 34), there is also a large discrepancy in the manner in which animal studies are designed and conducted across different laboratories and countries, which makes comparison of results challenging. Valuable opinions on how to advance the field of stroke research can be found in the following guidelines and recommendations: The Animal Research: Reporting *In Vivo* experiments (ARRIVE) guidelines (190), the Stroke Therapy Academic Industry Roundtable (STAIR) recommendations (191, 192) and the stem cell therapies as an emerging paradigm in stroke (STEPS) (193). The STAIR recommendations call for better standardization across studies using animal models to increase validity and reproducibility of results *e.g.* blinded studies, defined time windows and dose-response curves of pharmacological compounds, and histological and functional assessment of acute and long-term outcomes. The STEPS guidelines focus on how to develop and improve cell-based restorative therapies for stroke (194).

1.7 Combinatory approaches to stroke therapy

As previously discussed, multiple cellular and molecular pathways contribute to the evolution of the complex stroke pathology. It is therefore reasonable to aim to develop combination therapies targeting several of these mechanisms simultaneously and/or consecutively (51). A better understanding of the complex ischemic cascade, including endogenous neuroplasticity mechanisms, and the development of more targeted therapeutic strategies should remain the basis for clinical translation. Thus, successful intervention paradigms in preclinical models should also aim to combine different, complementary therapeutic approaches in a timely manner. Within the above context, this thesis employs different, clinically relevant combinatory paradigms, and studies their efficacy in promoting reparative mechanisms after ischemic lesion in the adult rat.

2. Overview of methods

This section describes the key experimental methods used in the papers that comprise this thesis. A detailed description of the rest of the methods used is provided in the materials and methods section of each paper.

	Method	**Paper**
	Surgical procedures	
2.1	Middle cerebral artery occlusion	I, II
2.2	Photothrombotic lesion	III
2.3	Intracerebral cell engraftment	I, II, III
	Cell culture	
2.4	Olfactory ensheathing cells	I
2.5	Human neural stem cells	I, II, III
	In vivo MRI and data analysis	
2.6	T2-weighted imaging	I, II
2.7	Behavioural tests and analysis	I, II
2.8	Immunohistochemistry	I, II, III

Surgical procedures

2.1 Middle cerebral artery occlusion

MCAo (60 min occlusion time) was performed on Sprague Dawley rats using the intraluminal filament technique under 1.5%-2% isoflurane anaesthesia. Briefly, the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed following a right midline incision to the neck. A silicone rubber-coated monofilament was then advanced to the ostium of the MCA and ligated before temporarily suturing the wound. The animals were assessed for neurological signs of stroke during the occlusion period. Prior to reperfusion the animals were re-anaesthetized, the surgical wound reopened and the filament withdrawn.

2.2 Photothrombotic lesion

Unilateral cortical lesions were induced photochemically in Sprague Dawley rats, under 1.5%-2% isofluorane anaesthesia, by intravenous injection of the photosensitizing dye Rose Bengal. A scalp incision was made to expose the area of the skull above the right somatosensory cortex to a cold white light source for 10 min. A sheet of aluminum foil with a cut out window of about 0.5 cm x 0.5 cm was placed over the rats' heads to expose a minimal area to the light.

2.3 Intracerebral cell engraftment

The animals were placed in a rat head stereotactic frame (Kopf Instruments) under 1.5%-2% isofluorane anaesthesia. The skull was exposed by a midline incision and the Bregma located before making a burr hole at predetermined coordinates targeting the globus pallidus/striatum (Paper 1 and 2) or the somatosensory cortex (Paper 3). A total volume of 3 μl of a single cell suspension ($1x10^5$ cells/μL) was injected at a rate of 1 μL/min using a Hamilton syringe attached to an UltraMicroPump (UMP3) with a SYS-Micro4 Controller (World Precision Instruments Ltd, Hitchin, Herdfordshire, UK).

Cell preparation and culture

2.4 Olfactory ensheathing cells

OECs were dissected and harvested from the olfactory bulbs of 4-5 P7 Sprague Dawley rats and purified by fluorescence activated cell sorting (FACS) by selecting for galactocerebroside-negative and O4-positive cells. OECs were then cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1.25% gentamicin, 5% FBS, 500 ng/mL FGF2, 50 ng/mL heregulin, and 10–6 M forskolin. Finally, OECs were passaged at confluence. Purity of the OECs populations was assessed by p75NTR specific labelling and was always at 98–100%.

2.5 Human embryonic neural stem cells

hNSCs were cultured and fed with Complete StemPro® serum-free NSC medium (Invitrogen, life Technologies), kept in a standard humidified air incubator with 5% CO2, at 37°C, and passaged twice at about 90% confluence before transplantation.

Data analysis

2.6 MRI analysis of lesion volumes

Animals were imaged in a Bruker Biospin 7T magnet. A T_2- weighted RARE multi-gradient echo (MGE) sequence was acquired to confirm the presence of lesion as shown by hyperintense areas on the MR images. Furthermore, the lesion volumes for each animal were calculated based on the hyperintense

areas in the ipsilesional hemisphere compared to the contralesional hemisphere. Hyperintense areas were outlined in each image slice using an Intuos Pen and Creative Tablet (Wacom Company, Ltd), before computing ROI volume in OsiriX (Pixmeo, Geneva, Switzerland).

2.7 Behavioural testing and analysis

The animals' forelimb locomotor asymmetry was evaluated using the cylinder test, by placing them in a glass cylinder while being video recorded for five minutes. The number of contacts between the forepaw and the cylinder wall were counted and registered as left (NL) or right (NR). Only contacts involving all digital pads and the palmar pads were registered. A normalized forepaw preference ratio (FP) for each animal was calculated for the left and right forepaw.

2.8 Immunohistochemical staining

Tissue was collected at 5 weeks post lesion and sectioned 40 μm in thickness. Free-floating sections were permeabilized in blocking solution for 1 h before applying primary antibodies and incubated at 4°C overnight. Primary antibodies were rinsed off with PBS before adding the appropriate secondary antibodies and incubating the tissue for 3 h at room temperature. To visualize all cell nuclei Hoechst was added 5-10 min before rinsing off the secondary antibodies. Sections were then mounted on gelatin-coated slides and imaged using a Zeiss AxioVert A1 fluorescence microscope (Carl Zeiss, Germany).

3. Aims and objectives of thesis

The overarching aim of this thesis was to combine therapeutic approaches to attenuate lesion sequelae and promote tissue remodelling after experimental ischemic lesion in the adult rat.

Key objectives included:

a) Investigate the efficacy of different therapeutic approaches in ischemic models of stroke including:
   - Co-transplantation of human neural stem cells (hNSCs) with olfactory ensheathing cells (OECs) as part of a novel *in situ* tissue engineering strategy;
   - hNSC transplantation in combination with clinically relevant pharmacological treatment.

b) Evaluate the extent to which the different therapeutic approaches influence:
   - Cell transplant survival and integration with host-tissue;
   - inflammatory processes; and
   - endogenous neurogenesis/neuroplasticity

Secondary objectives included:

i) Comparing the efficacy of the same combinatory approach, applying stem cell and pharmacological treatment, in two different experimental models of cerebral ischemia;

ii) Reviewing the complex role of microglia in tissue and synaptic remodelling in response to brain ischemia, focusing on possible implications for stroke repair.

4. Summary of papers

4. 1. Paper I – Effects of neural stem cell and olfactory ensheathing cell co-transplants on tissue remodelling after transient focal cerebral ischemia in the adult rat

Augestad IL, Nyman AKG, Costa AI, Barnett SC, Sandvig A, Håberg AK, Sandvig I. *Neurochemical Research* (2017) 42(6): 1599-1609. https://doi.org/10.1007/s11064-016-2098-3
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BACKGROUND
Human neural stem cells (hNSCs) are promising reparative candidates for stroke induced lesions, however, their survival and integration with the host-tissue post-transplantation is poor. The aim of this study was to address the challenges associated with survival and integration of hNSCs with host-tissue in ischemic lesions by co-transplantation with olfactory ensheathing cells (OECs) as part of a novel *in situ* tissue engineering paradigm.

METHODS
Transient focal cerebral ischemia was induced in adult rats by a 60-min middle cerebral artery occlusion (MCAo) followed by reperfusion. Two weeks after MCAo surgery, animals with confirmed ischemic injury were randomly assigned into three groups for intrastriatal cell transplantation; i) control + saline group (*n* = 4), ii) NSCs group (*n* = 6) and iii) NSCs + OECs group (*n* = 7). The effect of treatment on functional outcome was investigated with the cylinder test.

RESULTS
We found evidence of an extensive vascular network in the lesion core of severely lesioned animals in the co-transplant group as well as increased survival of hNSCs compared to the hNSCs only group.

CONCLUSIONS
Our results suggest a neuroprotective effect of OECs on the transplanted NSCs, which could be attributed to secretion of neurotrophic factors and cytokines. These findings support a possible role of OECs as part of an *in situ* tissue engineering paradigm for transplant mediated repair of ischemic brain lesions. More importantly, this study provides the first evidence of a synergistic effect of co-transplantation of OECs and NSCs in promoting vascular and/or tissue remodelling in the ischemic brain.

4.2. Paper II – Minocycline treatment combined with human neural stem cell transplantation attenuates inflammation and promotes endogenous plasticity after ischemic brain injury

Augestad IL, Valderhaug VD, Håberg AK, Sandvig A, Sandvig I
Submitted to *Translational Stroke Research* (Springer).

BACKGROUND
Post-ischemic inflammation is mediated by microglia, and previous studies suggest that minocycline, a neuroprotective and anti-inflammatory compound, facilitates activation of microglia into a neuroprotective/anti-inflammatory phenotype. In this study, we investigated the potential synergistic effects of minocycline and transplanted hNSCs as a strategy for modulating the inflammatory response and promoting endogenous plasticity after ischemic brain injury.

METHODS
Transient focal cerebral ischemia was induced by 60-min MCAo using the intraluminal filament technique on Sprague Dawley rats (270-315 g). Immediately following reperfusion, daily intraperitoneal injection of 50 µl minocycline (3mg/kg) treatment for 14 days was started. To confirm injury, T2-weighted MR imaging was performed 10 days post-lesion followed by intracerebral cell engraftment at 2 weeks post-lesion. Animals were randomly assigned into three groups for intrastriatal cell transplantation; i) control + hNSCs (*n* = 3), ii) hNSCs-only group (*n* = 5) and iii) hNSCs + minocycline group (*n* = 5). Brain tissue was collected at 3 weeks post-lesioning (wpl) and assessed with immunohistochemistry.

RESULTS
The main finding of the study was a significant regulation of the inflammatory response combined with evidence of extensive vascular remodelling and robust graft survival and integration with the host tissue in the minocycline treated group. Additionally, we observed increased hNSC survival, less severe neurological deficits and improved functional outcomes in the hNSCs-minocycline group. Finally, we observed prominent expression of doublecortin (DCX), a marker for neurogenesis, in the dorsal SVZ of the ipsilesional hemisphere in both treatment groups and the presence of DCX positive cells in the perigraft area in the hNSCs-minocycline group.

CONCLUSIONS
Taken together, our findings provided evidence of positive synergistic effects of a clinically relevant therapeutic approach combining minocycline treatment and stem cell transplantation to modulate the inflammatory response and promote tissue remodelling and endogenous plasticity after experimental brain ischemia.

4.3. Paper III – Pharmacological immunomodulation as a strategy for improving stem cell graft survival after cortical ischemia

Augestad IL, Valderhaug VD, Håberg AK, Sandvig A, Sandvig I
Manuscript

BACKGROUND
The complex lesion pathology that follows ischemic stroke suggests the need for combinatory treatment strategies that aim to address, key aspects of related pathology, such as neuronal loss and inflammation. In this study, we investigated the effect of the neuroprotective and anti-inflammatory drug, minocycline, as a pre-treatment before engraftment of hNSCs in a photothrombotic model of cortical ischemic damage.

METHODS
Adult Sprague Dawley rats (185-223g) (n=21) were randomly assigned into three groups; i) control (sham PCL) (*n* = 5); ii) PCL + hNSCs (*n* = 8) and iii) PCL + minocycline + hNSCs (*n* = 8). Focal cortical ischemia was induced by injection of 0.5 mL in saline (8mg/kg, Sigma) of the photosensitizing agent Rose Bengal into the right femoral vein, followed by exposure of the skull and photoactivation of the dye by applying a light source to designated coordinates over the right somatosensory cortex. Immediately after induction of photochemical thrombosis, animals in the minocycline group started receiving a daily intraperitoneal injection of 50 µl minocycline (3mg/kg) for 14 days prior to cell engraftment. For intracranial injections, a total volume of 3 µl of cell suspension (*i.e.* 300 000 cells) was injected at the same coordinates used for lesioning and at DV -0.5-0.7 at a rate of 1 µl/min using an UltraMicroPump with SYS-Micro4 Controller. Brain tissue was collected at 3 weeks post-lesioning (wpl) and assessed with immunohistochemistry.

RESULTS
Our results suggest that minocycline modulated the inflammatory response, as shown by reduced expression of microglia/macrophage marker CD68 and hypoxia-inducible factor 1 alpha (HIF-1α). However, we found no conclusive evidence of improved cell survival or tissue remodelling as a result of minocycline administration, nor any observable differences in activation of neurogenic niches.

CONCLUSIONS
The lack of convincing evidence of cell survival suggests that the chosen treatment regime with minocycline failed to modulate the inflammatory response to the extent of supporting cell survival. Based on our observations, it is difficult to conclude whether or not minocycline modulated inflammation to the extent of significantly promoting neurogenesis in the ipsilesional hemisphere.

4.4. Paper IV – Neuroplasticity in stroke recovery. The role of microglia in engaging and modifying synapses and networks

Sandvig I, Augestad IL, Sandvig A, Håberg AK,
Submitted to the European Journal of Neuroscience

BACKGROUND
Numerous experimental studies have demonstrated the potential of exogenously or endogenously derived stem cells alone, or in combination with *in situ* tissue engineering strategies and/or pharmacotherapeutics in promoting transplant-mediated repair with functional restoration. However, clinical translation of such approaches tends to be confounded by significant challenges, such as allograft rejection and various stem cell based therapies have largely failed to promote significant and/or long-lasting functional benefits in stroke patients. Increasing evidence supports various forms of plasticity triggered after a stroke event and their potential contribution to recovery. This review discusses the crosstalk between microglia and endogenous neuroplasticity in response to brain ischemia with special focus on the engagement of synapses and neural networks and their implications for stroke repair.

DISCUSSION
Neuroplasticity after ischemic injury involves both spontaneous rewiring of neural networks and circuits as well as functional responses in neurogenic niches. These events involve complex interactions with activated microglia, which evolve in a dynamic manner over time. The way microglia contribute towards sculpting neural synapses and networks, suggests that microglia-mediated pro- and/or anti-inflammatory activity may significantly contribute towards spontaneous neuronal plasticity after ischemic lesions, although the underlying mechanisms remain poorly understood. Ability to elucidate as well as to engage such mechanisms in a selective manner, also in the context of microglia-neuron-astrocyte interactions, can be envisaged to play a significant role in harnessing endogenous plasticity to promote stroke repair with functional outcome.

METHODS
An extensive literature search on PubMed was conducted using the keywords “brain ischemia”, “neurogenesis”, “synaptogenesis”, “connectivity”, “CNS development” and “CNS regeneration”.

5. Discussion

The overarching aim of this thesis was to address some of the challenges associated with the complex stroke lesion pathology and repair by combining different treatment strategies. These included *in situ* tissue engineering, stem cell therapy and pharmacological treatment aimed at minimizing lesion sequelae as well as harnessing endogenous CNS repair mechanisms to promote axonal and synaptic plasticity in experimental stroke.

In Paper I and II, we investigated the above in an adult rat MCAo model, one of the main *in vivo* models of transient focal cerebral ischemia. MCAo recapitulates many of the clinical features of ischemic stroke, such as loss of tissue within the ischemic core, BBB disruption, production of ROS, a strong inflammatory response, and variability in the extent/size of stroke lesions as seen in stroke patients. In addition, the neurogenic niches in the SVZ and SGZ are activated in response to ischemia. Our treatment strategy in Paper I involved a novel *in situ* tissue engineering paradigm utilizing co-transplants of NSCs and OECs, while in Paper II, we investigated the effects of NSC transplantation in combination with neuroprotective/anti-inflammatory treatment (*i.e.* minocycline). In Paper III, we compared the effects of the same treatment paradigm as in Paper II, this time in a photothrombotic lesion model using adult rats. The photothrombotic lesion model is another widely used model for inducing reproducible, focal ischemic strokes, mainly in cortical areas. Finally, in Paper IV we reviewed how neurogenesis and neural network remapping after stroke are influenced by inflammation.

The following discussion will focus on the major findings from the experimental studies and main topics related to these findings, including the efficacy of stem cell therapy, limitations of animal models, and factors influencing neurogenesis and plasticity in the mammalian CNS.

5.1 Is co-transplantation a viable tissue engineering paradigm for transplant mediated repair of ischemic stroke lesions?

NSCs are promising reparative candidates for stroke induced lesions because of their multipotency, *i.e.* the capacity to differentiate into neurons and glia (138, 139). However, their survival and integration with host tissue post-transplantation is poor (195). A key hypothesis in Paper I was that co-transplantation of hNSCs with OECs, could increase graft survival and thus lead to improved tissue integration compared to hNSC transplantation alone, as part of a novel *in situ* tissue engineering paradigm. OECs are a special type of glia found in the olfactory mucosa and olfactory bulbs, which surround primary olfactory neurons. Several properties make OECs interesting for stroke repair. These include secretion of neurotrophic and angiogenic factors (*e.g.* GDNF and VEGF), promoting myelination

of large-diameter CNS axons, as well as the fact that OECs transplanted in the CNS following lesioning do not induce an astrocytic response (143-146, 148). Indeed, OECs tend to form pathways by interacting with astrocytic processes that can help bridge a lesion cavity by facilitating axonal growth (196, 197). While there are many reports on the beneficial effects of OECs from spinal cord injury and optic nerve crush injury (144, 147, 198-201), few preclinical stroke studies have used OECs as part of transplant mediated repair. In addition, a recent study demonstrated a minimally invasive and safe procedure for harvesting human olfactory nasal mucosa (202), demonstrating the clinical potential of using OECs as part of transplant-mediated repair of CNS lesions. Taken together, the above suggested that OECs would be highly relevant as part of a novel *in situ* tissue engineering paradigm for stroke repair.

The findings from Paper I showed that OECs in the hNSC-OEC co-culture *in vitro* assays formed network-like structures, which suggested that OECs could potentially be expected to act as a support structure for hNSCs post-transplantation. Although we did find cell graft survival after transplantation to be generally poor, there was increased survival of hNSCs in the co-grafted animals compared to hNSCs alone, as well as evidence of extensive vascular remodelling in the lesion core. Our results suggest that OECs may promote tissue remodelling after stroke exerted through paracrine effects. Thus, Paper I provide the first evidence of a synergistic effect of co-transplantation of OECs and hNSCs in promoting vascular and/or tissue remodelling in a rat model of transient cerebral ischemia.

In the last years, natural and synthetic biopolymers have been extensively studied as a strategy for *in situ* tissue engineering for CNS repair, including stroke (203). The rationale for using biopolymers is to provide structural and molecular cues that support (stem) cell graft survival and/or differentiation and functional integration with host tissue (204, 205). Thus, a range of biopolymers with different rheological properties, including modified poly-(lactic-co-glycolic) acid (PLGA) micro- and nanoparticles, and natural extracellular matrix (ECM), have been extensively applied as *in situ* scaffolds for cell encapsulation or surface attachment substrates, as well as controlled release of neurotrophic factors (203, 204, 206, 207). However, from a clinical perspective, one of the main challenges with using biopolymers is safe intracerebral delivery, especially after complex lesions such as stroke as it may cause further tissue damage (208). Furthermore, depending on the rheological properties of the biopolymer and the method of stereotactic delivery, the relevant procedures may exacerbate injury and/or inadvertently cause secondary damage as a result of injection, leakage of the biopolymer, as well as the need for either dilution of the biopolymer into the extracellular fluid (ECF) of the lesion cavity, or for insertion of a drain to collect the ECF during injection (207). In addition, implantation of such biopolymers tends to result in the creation of an *in situ* micromilieu/microcircuit that does not

functionally integrate with host tissue (204). This highlights the need for alternative approaches to biopolymer-based *in situ* tissue engineering.

5.2 Pharmacological modulation of inflammation in ischemic stroke models

To further build on the hypothesis of combining different approaches for treatment of stroke-induced lesions, we wanted to investigate the possible effects of modulating inflammation in combination with stem cell therapy in our next studies (*i.e.* Papers II and III). Poor cell graft viability due to the inflammatory response remains one of the main challenges in studies of transplant-mediated repair.

In Paper II, we performed 60 min MCAo in rats, and started daily treatment with minocycline immediately after reperfusion, before transplanting hNSCs two weeks after lesioning. Minocycline is a neuroprotective and anti-inflammatory drug already used in the clinic, with BBB protecting properties and the potential to modulate microglia from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype (117, 120). To assess the effects of minocycline on graft survival and lesion sequelae, without possible interference from immunomodulatory drugs, such as cyclosporine, we did not use any other form of immunosuppression. Immunosuppressants have been used in a number of *in vivo* stroke studies (209, 210), but their use may potentially mask positive or negative effects (side effects) when combined with other treatments.

To investigate the effect of minocycline on inflammation, we assessed the expression of one of the main pro-inflammatory and anti-inflammatory cytokines, TNF-α and IL-10, respectively. Both cytokines are secreted by microglia and macrophages, and are upregulated weeks after the onset of ischemia (70, 120). Furthermore, IL-10 may help to downregulate TNF-α by inhibiting cytokine receptor expression and receptor activation (52). We found that acute administration of minocycline after reperfusion and continued for two weeks post-lesioning, *i.e.* up to the point of cell transplantation, significantly modulated pro, but not anti-inflammatory responses, as seen by the downregulation of TNF-α in the minocycline treated group; IL-10 expression was not significantly upregulated in this study. This finding, combined with the fact that we found largely increased (albeit not statistically significant) numbers of surviving transplanted hNSCs, suggests that minocycline treatment may have promoted hNSC survival by partially modulating the inflammatory response and making the lesion microenvironment less hostile. Finally, we assessed the expression of the transcription factor HIF-1α, in the two experimental groups, and found significantly lower expression in the minocycline treated group compared to the hNSCs only group. Although HIF-1α is a key regulator of cell survival and proliferation in response to hypoxic/ischemic conditions (211), under severe hypoxic conditions it may

contribute to increased BBB permeability (212). Our results strongly suggest that minocycline was involved in downregulating HIF-1α activity.

In Paper III, we employed the same treatment strategy as in Paper II, *i.e.* administration of minocycline immediately post-lesioning and continued for two weeks, as a pretreatment before hNSC engraftment, this time in a photothrombotic model of stroke. Previous studies have reported graft survival after photothrombotic lesion (213, 214), but to the best of our knowledge, no studies have combined hNSCs with minocycline in this model. To avoid further damage of intact brain tissue, we targeted the area within the lesion for stem cell engraftment. Injection of transplanted cells in the center of the lesion cavity can potentiate endogenous repair processes in the peri-infarct tissue, however the lack of trophic support and continued inflammation in this area create a hostile microenvironment for engrafted cells (203, 215, 216). Although minocycline may have been involved in modulating the inflammatory response by reducing the observed expression of CD68 in the minocycline treated group, the findings suggest that there was no added benefit of minocycline treatment on the survival of engrafted cells in this stroke model. Indeed, we found overall hNSC survival in both the minocycline treated and untreated groups very poor. If the same combinatory strategy had induced equivalent effects to those observed in Paper II, we would have expected increased hNSC survival in the minocycline-treated group. Furthermore, we observed autofluorescence in the tissue, especially in the lesion area, from cells with the same morphology as microglia/macrophages positive for CD68. This observation is also very similar to the cells that previous studies (213, 214) have reported as surviving grafted MSCs, which may have inadvertently led to a misinterpretation of actual results in those studies. In our study, we have made a conscious effort to eliminate any chance of misinterpretation, by painstakingly excluding all autofluorescence-induced false positives in our analyses of CD68 expression or graft survival.

Finally, the differences in the development of ischemia, and thereby the temporospatial dynamics of lesion sequelae, including inflammatory processes in the two models may be part of the explanation for the differences in the findings between Paper II and III. This emphasizes the fact that effects of different treatment strategies should be interpreted in a context-specific manner, and highlights one of the main challenges in basic and translational research, *i.e.* extrapolating results from different studies and models and making assumptions about potential translational success.

5.3 Harnessing endogenous plasticity

Ischemic stroke leads to increased proliferation of progenitor cells in the SVZ of the ipsilesional lateral ventricle, with subsequent migration of newly formed neuroblasts into the damaged striatum (217). In rodents, this migration represents an ectopic migratory pathway, distinct from the default migratory route of SVZ neuroblasts towards the olfactory bulbs, *via* the rostral migratory stream (142, 160). SVZ activation can be assessed with immunostaining for doublecortin (DCX), a marker for neurogenesis. The neurogenic response to brain ischemia is largely influenced by the extent of tissue damage and upregulation of inflammatory cytokines, with increased proliferation being observed in ischemic lesions in which tissue damage extends to striatal regions closer to the SVZ (154, 155). However, few of the newly formed SVZ neuroblasts manage to migrate extensively and reach the ischemic border, or survive/and or differentiate long-term (164).

In Paper II, we assessed the activation of the neurogenic niches in the lateral ventricles with immunostaining for DCX and observed, in both experimental groups (*i.e.* hNSCs-minocycline and hNSCs-only), increased activation of the ipsilesional SVZ compared to the contralesional SVZ, with subsequent migration of these newly formed neuroblasts towards the injury site. Robust expression of cells positive for DCX was also observed in both frontal hemispheres, irrespective of treatment group, which may denote the presence of migrating or oligodendroglial cells, consistent with an ongoing process of tissue surveillance and synaptic remodelling in the adult brain. Finally, the presence of DCX^+ cells in the perigraft area in the hNSCs-minocycline group was a particularly interesting finding, however whether these cells represent neuroblasts migrating from activated neurogenic niches towards the graft or whether they derive from the hNSC transplant is unclear. The extent to which minocycline may or may not have influenced neurogenesis was beyond the scope of this particular study, however, minocycline did not seem to inhibit SVZ activation. In Paper III, we observed some DCX^+ cells in the perilesional area and along the ipsilesional corpus callosum. The exact origin of these progenitors is unclear. Overall, however, contrary to the findings of Paper II, in Paper III we did not observe any obvious differences in the activation of the ipsilesional vs. the contralesional SVZ. This may be directly related to the fact that photothrombotic lesions are smaller than MCAo-induced ones and do not extend to the striatum (218).

Activated microglia, as well as astrocytes, produce several inflammatory factors, including cytokines and chemokines, which may act as putative chemoattractants for proliferating progenitors (219). Furthermore, long-term accumulation of microglia with a proneurogenic phenotype in the SVZ implies a supportive role of these cells for the continuous neurogenesis observed after stroke (220). However,

if left uncontrolled, the immune response after ischemia impairs progenitor survival and proliferation, and furthermore inhibits repair processes (221). Thus, modulation of the detrimental effects of the inflammatory response *e.g.* pro-inflammatory cytokine production, ROS and NOS, may promote beneficial responses (221).

Based on our experimental findings, as well as the fact that there is increasing consensus in the field that microglia responses can dramatically influence brain plasticity after ischemic stroke, we conducted a review of the relevant literature as part of Paper IV. Specifically, in Paper IV, we discuss the crosstalk between microglia and endogenous neuroplasticity in response to ischemic stroke, and how microglia may be involved in synaptic pruning and network remodelling.

Resident microglia react within 1 h and this response can last for weeks/months after onset of ischemia. Activated microglia constitute an integral part of dynamic responses to brain injury and following ischemic stroke and may determine whether endogenous plastic responses become adaptive or maladaptive in nature. Many of the underlying mechanisms seem to recapitulate fundamental process observed during CNS development, including contribution of microglia in sculpting networks and synapses. Furthermore, such processes tend to involve endogenous neurogenesis as well as neural network remodelling, aspects of which, we have observed in all of our experimental studies (Papers I-III). Elucidation of these mechanisms can be expected to promote our ability to harness endogenous plasticity and thus contribute to the development of improved treatment and rehabilitation paradigms for stroke patients.

5.4 MRI in animal stroke models

MRI is a useful and necessary diagnostic tool in the clinic as well as in basic/translational research of cerebral stroke. In the work presented in this thesis we performed a standard T2-weighted sequence, by which ischemic brain lesions can be identified as hyperintense areas (222). Based on the MRI data we could verify ischemic lesions as well as calculate lesion size by volumetry analysis in the different treatment groups (Paper I and II). In addition to the above, the MRI data enabled the identification of coordinates for cell transplantation in the peri-infarct area (Paper II).

Despite the fact that MRI is a valuable tool in basic/translational research, there are some limitations imposed from the MCAo model itself when it comes to performing MRI on animals with ischemic stroke lesions. In the first hours and days after surgery, the animals are often weak, thus repeated/prolonged exposure to anesthesia, which is necessary for MR scans, may exacerbate brain damage and, on occasions, increase mortality in the acute stage post-lesioning. This consideration

precluded the use of MRI, including DWI, acutely after injury in our studies, which was also in accordance with the recommendations of the relevant ethics committee for the welfare of experimental animals. For this reason, MRI was performed at 7 dpl (Paper I) and 10 dpl (Paper II).

However, to fully capitalize on the information we can acquire from MRI, including extent and lesion severity and delineation of the ischemic penumbra, multiparametric imaging such as, PWI, DWI, MR spectroscopy and functional MRI (223-225) could be implemented in experimental stroke studies. Serial *in vivo* imaging, *i.e.* data acquisition at different time points after lesioning (acute, sub-acute and chronic stage) would also be an advantage for assessing lesion development and possible efficacies of treatment (226, 227). However, apart from site-specific, animal welfare or cost limitations, certain imaging techniques and relevant analyses, as for example DWI/DTI or DTI-tractography, may be technically challenging to perform in rodent models of ischemic stroke because of the relevantly small amount of white matter compared to the human brain. Optimal MRI data acquisition in experimental stroke models may therefore require *ex vivo* imaging (228). On the other hand, this may confound the utility of specific MRI modalities for longitudinal *in vivo* monitoring of experimental stroke animals.

5.5 Methodological considerations

Inherent limitations of experimental stroke models

Extensive knowledge regarding pathophysiological and regenerative processes of ischemic stroke has been derived from preclinical models of ischemic stroke in rodents. MCAo is the most widely used and perhaps the most clinically relevant *in vivo* model for ischemic stroke as it reflects many of the pathological features and heterogeneous nature of stroke lesions seen in clinical patients (170). Despite these advantages, it is important to keep in mind that in the MCAo model, ischemic lesions are induced by surgically inserting a filament into the MCA, thus not reflecting the clinical situation, where an embolus or thrombus stops blood flow to the brain. Ultimately, no matter how clinically relevant a preclinical model may be, it can only recapitulate certain aspects of human stroke.

As mentioned above, variation in lesion sizes in the MCAo model better reflects the clinical situation, but makes it more challenging to analyze and compare results between different treatment groups, or even between animals within the same group. Indeed, in Paper I we observed large variation in the sizes of stroke between rats and between groups, while in Paper II there was less variation in the sizes of stroke lesions, which were generally large. These differences may be attributed to the use of a new type of monofilament in Paper II compared to Paper I (silicone coated vs. prolene filament respectively). This provides excellent scope for refinement of the MCAo model; by either varying the

type of filament, as we have shown in our studies, or by varying the length of occlusion time (*e.g.* 60 vs. 120 min) it is possible to influence the replicability as well as severity of ischemic injuries (229). Additionally, there are many variations of the MCAo model itself, such as the electrocoagulation model (230) and (thrombo)embolic models (231, 232). On the other hand, as the STAIR and STEPS guidelines have highlighted, such differences between studies and laboratories may account for the fact that relatively few findings from preclinical stroke studies have been successfully translated in the clinic (191-194). Furthermore, consideration of factors such as animal acclimatization, social interaction and diet prior to as well as post-lesioning, choice of anesthetics, and peri- and post-operative care, as recently highlighted in the IMPROVE guidelines (233), may significantly improve the robustness of experimental stroke studies.

The photothrombotic lesion model of cortical ischemia is useful for addressing several research questions, especially related to cellular and molecular studies of cortical plasticity (177). This model of focal cortical ischemia, however, only mimics the situation for a small percentage of clinical patients and may therefore be a less relevant preclinical model compared to MCAo. In photothrombotic ischemia, BBB opening occurs at an earlier time point in lesion development and there is a limited penumbra compared to larger vessel strokes (234). The resulting lesions are reproducible, however, they are much smaller in size, compared to MCAo induced lesions. Furthermore, a comparative study of microglial activation in MCAo and photothrombosis suggests that there is a delay in microglial and astrocytic invasion of the ischemic core as well as accumulation of phagocytic microglia after photothrombotic lesion compared to MCAo (235). Despite the differences in the two models, the photothrombotic lesion model can be adapted to induce focal lesions in selected brain areas, as for example demonstrated in a recent study in which the method was adapted to stereotactically lesion the lateral cerebellar nucleus (236).

One of the objectives of this PhD thesis was to compare the efficacy of the same combinatory approach, namely stem cell and pharmacological treatment, in the MCAo and photothrombotic lesion models. As discussed, we did not observe the same effects of combining pharmacological modulation and stem cell therapy in the two different models of ischemic stroke, which once again, highlights the challenge of directly comparing or extrapolating results from one study to another.

Evaluation of treatment efficacy

In our experimental studies (Paper I, II and III), we assessed the effects of treatment at 5 weeks post-lesioning mainly by immunohistochemical analyses of tissue sections, given that a primary goal of

these studies has been to evaluate cell survival and integration with host tissue. In our experience, immunohistochemistry is a relatively straightforward method, which however results in a large amount of data available for further analysis. An added difficulty is presented by the fact that tissue collected from ischemic brains, especially those with large infarcts, is fragile and can be easily damaged by sectioning or during immunostaining, which means that valuable information can be potentially lost during handling. Furthermore, as mentioned earlier, the variability in lesion sizes may be high, both within groups and possibly also between groups of animals. This may pose difficulties in selecting and comparing specific brain regions for analysis. Finally, it is worth considering that when it comes to the survival of exogenously transplanted cells following ischemia, different parameters can influence the fate of these cells (200). These include the timing for transplantation after onset of ischemia, graft location (typically, lesion core vs. peri-infarct area), route of administration (intracerebral vs. intravenous), as well as the use of immunosuppressants. Besides *ex vivo* analyses, few options are available to fully determine the outcome of cell-based therapies, especially within the designated timeline for our studies.

In addition to histological analyses, *in vivo* or *ex vivo* electrophysiology can be used to assess functionality of the engrafted cells and, potentially, determine their integration with host tissue/neuronal circuitry. However, *in vivo* electrophysiological recordings, especially in stroke lesioned animals are invasive and technically challenging, while *ex vivo* electrophysiology of brain slices may only show whether a graft is functional within itself, without necessarily suggesting functional integration with the host tissue.

Sensorimotor and cognitive tests

We chose to perform the Cylinder test, a commonly used test that assesses spontaneous forelimb use during vertical exploration of a tall cylinder (182), in our first two studies (Paper I and II). This test is not biased by motor learning of the animals, as may be the case in other functional tests. In Paper III, we did not assess sensorimotor deficits by the Cylinder test as the cortical area that was targeted with photothrombotic lesioning was the hindlimb region of the sensorimotor cortex. Additionally, given the relatively limited severity of the photothrombotic lesion and keeping in mind the timeline of our study, any induced functional deficits and, by the same token, improved functional outcomes as a result of the treatment would be marginal and thus difficult to assess.

The inclusion of sensorimotor and cognitive tests in animal stroke research is important for evaluating the functional outcome of different treatment strategies. Furthermore, the inclusion of different types

of tests can provide an improved basis for assessment. However, repeated testing of animals in different situations, which may also involve different experimenters, may cause additional stress to already injured animals. In addition, special equipment needed to perform certain tests (*e.g.* rotameter or motor rater), are costly and require appropriate infrastructure within designated testing areas. Lack of availability of such options at the time our studies were conducted, made the use of additional tests not possible.

In rodent models of ischemic stroke, depending on lesion severity, there is a degree of spontaneous recovery observed during the initial weeks post-lesioning, (237). This may partially confound the findings of functional tests conducted in the acute and sub-acute stage post-ischemia. Studies should thus also include long-term (>1 month) monitoring of the animals. This can provide information about the efficacy of treatment on sensorimotor and cognitive deficits beyond the first weeks after ischemic insult (238).

Stroke patients do not only experience functional deficits as a result of stroke, they can also be affected by cognitive deficits such as post-stroke dementia and dysfunctions in executive functions as well as depression and epilepsy (239, 240). However, testing for cognitive deficits and depression in animal models is even more challenging than testing for sensorimotor deficits.

Finally, it is worth considering that intra- and inter-species differences may be a confounding factor in animal performance during individual types of tests. This may influence the validity of certain findings and further complicate the interpretation of results from preclinical stroke models, especially with a view to future clinical translation. If we could improve cognitive abilities in stroke patients, this might also have a positive effect on their lives, which emphasizes the need for including these tests. Although amelioration of functional deficits has been shown after stem cell transplantation (140, 141), we do not know how these interventions affect cognitive abilities.

6. Conclusions

The aim of this thesis was to combine therapeutic approaches to attenuate lesion sequela and promote tissue remodelling after experimental ischemia in the adult rat. Based on the findings from our experimental studies, we can draw the following main conclusions:

(i) Co-transplantation of OECs and hNSCs may be a viable treatment strategy for ischemic stroke lesions due to the synergistic effects observed compared to hNSC transplantation alone.

(ii) Combining minocycline treatment with hNSC transplantation modulates the inflammatory response and promotes graft survival, tissue remodelling and endogenous plasticity.

(iii) Results from our study on photothrombosis highlight one of the main challenges in basic and translational stroke research, namely extrapolating and comparing results from one study and experimental model to another.

(iv) Improving our understanding of the complex involvement of microglia in neuroplasticity after stroke, and how to selectively influence these mechanisms, may play a significant role in harnessing endogenous plasticity to promote stroke repair.

Whether cell replacement is a viable option to include as part of a clinical treatment strategy after ischemic stroke, and how to further promote endogenous repair and plasticity remain to be answered. Based on the findings presented in this thesis, however, the synergistic effect of combining therapeutic strategies, should be the subject of investigation in future research.

7. Future Directions

The number of new incidents and stroke survivors living with disability is only expected to increase in the following years due to an increasing elderly population across the world. The sheer complexity of ischemic lesions, strongly suggests that we need to further improve our knowledge of the molecular and genetic factors that influence stroke pathology and endogenous repair mechanisms in the CNS, which can enable tailored stroke treatment to the individual patient or specific stroke subtypes.

One of the main challenges is the short therapeutic time-window of the few existing treatments for ischemic stroke. Therefore, one of the goals of basic and translational stroke research should be to develop therapies related to pathophysiological events at different stages after onset of stroke in order to widen this window so that more patients can benefit from treatment in the future. One option regarding cell replacement therapy would be autologous transplantation, where cells are harvested from the patient, reprogrammed to a multipotent or specific cell type, and then transplanted back to the patient. This procedure can circumvent the issues regarding graft rejection by the immune system as seen after NSC or ESC transplantation.

Future stroke research will hopefully benefit from new advances in technology such as *in vitro* and *in vivo* reprogramming where *e.g.* glial cells can be converted to neurons *in situ*. However, even if we did manage to promote neurogenesis, either by activating neurogenic niches or through *in vivo* reprogramming, little is still known as to whether these cells would be able to integrate with existing neural networks and create functional connections, or repair damaged neural networks.

The future success of translational stroke research will also depend on developing improved *in* vitro models that can complement and potentially replace animal models. The availability of different cell culture and electrophysiology platforms, such as multielectrode arrays (MEAs) and microfluidic chips, enables us to study functional and structural changes in neural networks closely associated with the ones directly affected by stroke. Various responses related to plasticity after stroke can therefore be studied, such as spontaneous functional recovery, rewiring of neural networks and axonal ramification as well as recruitment of intact synapses after perturbations, mimicking key aspects of stroke related pathology.

Finally, to be able to influence endogenous repair mechanisms beyond the initial activation and proliferation of cells in the neurogenic niches, we need to know more about the molecular mechanisms regulating survival, migration, differentiation and functional integration of these newly formed

progenitors with the host tissue, as well as the mechanisms behind axonal and synaptic plasticity in the context of ischemic stroke.

8. References

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9. Contributions

Papers I-IV are included in the following pages.

9.1 Paper I

9.2 Paper II

Minocycline treatment combined with human neural stem cell transplantation attenuates inflammation and promotes endogenous plasticity after ischemic brain injury

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Keywords
Neuroprotection, neurogenesis, brain tissue remodelling, middle cerebral artery occlusion, stroke

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9.3 Paper III

Pharmacological immunomodulation as a strategy for improving stem cell graft survival after cortical ischemia

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Keywords
Photothrombosis, minocycline, hNSCs, inflammation, neurogenesis

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Manuscript

9.4 Paper IV

Neuroplasticity in stroke recovery. The role of microglia in engaging and modifying synapses and networks

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Abstract
Neuroplasticity after ischemic injury involves both spontaneous rewiring of neural networks and circuits as well as functional responses in neurogenic niches. These events involve complex interactions with activated microglia, which evolve in a dynamic manner over time. Although the exact mechanisms underlying these interactions remain poorly understood, increasing experimental evidence suggests a determining role of pro- and anti-inflammatory microglial activation profiles in shaping both synaptogenesis and neurogenesis. While the inflammatory response of microglia was thought to be detrimental, a more complex profile of the role of microglia in tissue remodeling is emerging. Experimental evidence suggests that microglia in response to injury can rapidly modify neuronal activity and modulate synaptic function, as well as be beneficial for the proliferation and integration of neural progenitor cells (NPCs) from endogenous neurogenic niches into functional networks thereby supporting stroke recovery. The manner in which microglia contribute towards sculpting neural synapses and networks, both in terms of activity-dependent and homeostatic plasticity, suggests that microglia-mediated pro- and/or anti-inflammatory activity may significantly contribute towards spontaneous neuronal plasticity after ischemic lesions. This review discusses the crosstalk between microglia and endogenous neuroplasticity in response to brain ischemia with special focus on the engagement of synapses and neural networks and their implications for grey matter integrity and function in stroke repair.

Introduction
Ischemic stroke accounts for 87 % of all stroke incidents (1) and is caused by embolic or thrombotic obstruction of blood supply to the brain, which triggers a complex molecular cascade including slowed cellular energy metabolism, cell membrane depolarization, excitotoxicity, reactive oxygen species (ROS) production, a complex inflammatory response by activated microglia, and disruption of the blood–brain barrier (BBB), leading to necrotic and apoptotic cell death (2, 3). The severity of focal ischemic injury and lesion sequelae are contingent on the restoration of brain energy metabolism, and largely determined by degree of ischemia, lesion location and size and duration of vessel occlusion (4, 5).

Stroke patients usually demonstrate a degree of spontaneous improvement in the sub-acute phase, which can be further enhanced with appropriate prolonged rehabilitation. The observed functional outcomes appear to be consistent with partial rewiring of surviving neural networks and recruitment of intact synapses, which tends to occur contralateral, but also ipsilateral to the lesion (6-10). Depending on the severity of the stroke and the type of clinical treatment and/or rehabilitation paradigm, these mechanisms may account for spontaneous functional recovery which may be observed in certain stroke patients 30-90 days post-ischemia, especially with regard to alleviation of language and cognitive impairments, but also motor impairments such as voluntary maximum arm extension (4). Furthermore, evidence from animal studies provides valuable insights into the molecular mechanisms of spontaneous recovery after stroke, including release of anti-inflammatory cytokines, angiogenesis, structural remodelling at the axonal, dendritic, and synaptic level, changes in the extracellular matrix (ECM) as well as activation and migration of endogenous neural stem cells (NSCs) (11-13).

In the last decade, increased responsiveness in terms of onset-to-treatment times, improved availability of thrombectomy and thrombolysis in combination with enhanced rehabilitation strategies,

has improved the overall survival rate of stroke patients by 20% (14-17). Despite this progress, stroke remains the leading cause of adult disability, with 50% of survivors suffering from severe sensorimotor and cognitive impairments (18). As a result, ongoing research effort focuses on the development of alternative or complementary approaches.

Numerous experimental studies, including recent studies from our group, have demonstrated the potential of exogenously or endogenously derived stem cells alone, or in combination with *in situ* tissue engineering strategies and/or pharmacotherapeutics in promoting transplant-mediated repair with functional restoration through such diverse mechanisms as neuroprotection, cell replacement, remyelination, tissue/vascular remodelling and *de novo* neurogenesis (19-26). Although highly promising and extremely valuable in elucidating relevant recovery mechanisms at the experimental level, clinical translation of such approaches tends to be confounded by significant challenges, such as allograft rejection as well as the intricacies inherent in trying to create safe, functional *in situ* biointerfaces that could effectively re-establish functional connectivity in multiple foci (27). This is exemplified by the fact that numerous past and ongoing clinical trials of various stem cell based therapies have largely failed to promote significant and/or long-lasting functional benefits in stroke patients (28).

On the other hand, increasing evidence supports various forms of plasticity triggered after a stroke event and their potential contribution to recovery. As mentioned earlier, these plastic responses (29) include rewiring of surviving neural networks and axonal ramification (26), the recruitment of intact synapses post-lesioning (30), as well as the activation of endogenous neuro/gliogenic niches, including germinal layer-derived sites but also ectopic ones, and the migration of neuronal and glial progenitors towards the injury site (31). Such processes are reminiscent of the high level of plasticity observed during development, which reiterates a widely-shared view in regenerative neuroscience, i.e. the principle that regeneration in the adult mammalian central nervous system (CNS) may (partially) rely on the recapitulation of the high degree of neuroplasticity underscoring development (7, 32, 33).

Interestingly, there is increasing consensus regarding a central role of microglia in regulating neuroplasticity. In this review, we discuss endogenous neuroplasticity in response to brain ischemia, with special focus on the role of microglia in engaging synapses and neural networks and their implications for stroke repair.

Microglial-neuron interactions in the uninjured brain

In the adult brain, there is an abundance of resident microglia across all brain regions. There are, however, substantial differences in microglia population densities between different brain areas: more microglia reside in the grey matter compared to white matter, while particularly large numbers can be found in the substantia nigra, basal ganglia, hippocampus and olfactory telencephalon, as opposed to the cerebellum and brainstem (34). In the uninjured brain, resident microglia are characterized by a highly branched ramified phenotype and contribute to homeostasis by scanning their microenvironment and being in apposition with synapses without disturbing neural networks (35-39). Specifically, histological evidence from experimental animal studies has shown that microglia engulf presynaptic and postsynaptic elements and that their processes display a high level of motility, alternately contacting and retracting from pre- and postsynaptic terminals (29, 40-42). It is not known whether this interplay is involved in experience dependent synaptic plasticity or elimination of

synapses. Further, the exact molecular cues and pathways that attract microglia towards synaptic structures remain largely unknown (40). Similarly, highly branched microglia with radial processes can be found in the neuropil, while along axon tracts, microglia processes may be extended and aligned parallel or perpendicular to axon fibers (34, 43). The striking differences in the distribution and phenotypic characteristics of resident microglia in the brain indicate high sensitivity to microenvironmental inputs and, likely, distinct functions in the maintenance of brain homeostasis.

Experimental evidence strongly supports the view that microglial arborizations correlate with both increased growth as well as elimination of dendritic spines, but also high responsiveness to neuronal activity (29, 44). For example, ATP released by neurons during neurotransmission has been shown to enhance surveillance by microglial processes (41, 45-48) and to stimulate microglial process outgrowth. The latter however, may be predominantly/selectively controlled by glutamatergic AMPA, NMDA, and kainate receptor activation on microglia processes (45, 47). Furthermore, it has been shown that tumor necrosis factor α (TNF-α) mediates synaptic scaling, i.e. a feedback mechanism whereby consistent electrical output is facilitated by scaling of synaptic strength through the insertion or deletion of postsynaptic AMPA receptor (49, 50). This results in microglial process outgrowth or retraction from synapses, a response that may differ markedly between different brain regions, as for example process outgrowth in the hippocampus and cortex *versus* retraction in the striatum (48, 50). It follows that ATP-mediated communication between resident microglia and neurons may be region specific. This communication is characterized by the reciprocal nature of microglia-neuron interactions (42), effectively establishing a feedback loop that regulates neuronal activity. This supports the view that surveillance of the brain by resting state microglia is a highly active process. However, its exact implications on synaptic function and role in plasticity remain to be elucidated.

Microglial responses to injury

Following pathological alterations in the brain, such as ischemic insult, resident microglia respond to changes in neuronal integrity, synaptic inputs and activity by rapidly switching from a ramified to amoeboid morphology and migrating towards the injury (39, 51). Specific interactions between microglia and neurons are shaped by an intricate dynamic pattern of environmental cues, resulting in transient changes in the exact nature of microglial activity, which can influence the evolution of stroke lesion pathology in the acute, sub-acute, and chronic stages in a positive or detrimental manner (35, 37, 52).

Activated microglia are antigenically indistinguishable from recruited macrophages (53, 54). Microglia polarization in response to injury includes the pro-inflammatory M1 (classic) and anti-inflammatory M2 (alternative) phenotype (55). Induction of M1 polarization involves a host of different signaling pathways including interferon γ (IFNγ)-mediated activation of signal transducer and activator of transcription 1 (STAT1) factor, which in turn triggers ROS and nitric oxide (NO) production and the secretion of pro-inflammatory cytokines, including TNF-α, interleukin-1β (IL-1β), and IL-12 (52, 56). Furthermore, Toll-like receptor 4 (TLR4) activity can also induce M1 polarization through the formation of an activation complex, which includes members of the interferon regulatory factor family (IRF), which regulate inducible nitric oxide synthase (iNOS) secretion and major histocompatibility complex (MHC) II activity (52, 57, 58).

On the other hand, M2 polarization is mediated by exposure to IL-4 and IL-10 or IL-13 (59) and involves the upregulation of factors such as non-TLR pattern recognition receptor Dectin-1, dendritic cell-specific ICAM-grabbing non-integrin **(**DC-SIGN), mannose receptor, and scavenger receptors A and B (60). M2 microglia have a distinct function from M1 microglia and can ameliorate tissue damage through secretion of neuroprotective factors, including GDNF and BDNF (61, 62), as well as anti-inflammatory cytokines, such as IL-10 and TGF-β (63-65).

Activated microglia infiltrating the lesion site, together with other components such as reactive astrocytes, and ECM molecules, especially chondroitin sulfate proteoglycans (CSPGs) contribute to the formation of the glial scar (66-68). For example, M2 type microglia promote ECM deposition and also prevent its degradation by inducing the secretion of FIZZ 1 protein and the heparin-binding lectin Ym1, respectively (69, 70). Although the glial scar constitutes a chemical and physical barrier inhibiting axonal outgrowth, it has a critical role in limiting damage during the acute phase after injury by sealing the lesion site, restoring homeostasis, preserving spared tissue, and regulating immune responses (71, 72). Beyond their contribution to glial scar formation in acute stroke pathology, activated microglia constitute an integral part of dynamic responses to brain injury and, following ischemic stroke, they may determine whether endogenous plastic responses become adaptive or maladaptive in nature.

Microglia and synaptic remodeling – lessons from development

A highly relevant perspective is the role of microglia during CNS development, especially with a view to synaptic remodeling. The first microglia that appear during embryogenesis are derived from myeloid progenitors and are the only glial cells of the CNS at this stage, preceding both astrogliogenesis and oligodendrogliogenesis (40, 73). Thus, in the absence of astrocytes, microglia play an active role in early embryonic synaptogenesis, while during later stages of pre- and postnatal development, microglia become a key regulator of synaptic pruning (40, 50, 74) and, in this way, promote neural circuit refinement. CX_3 chemokine ligand 1 (CX_3CL1), also known as fractalkine, can be either secreted or membrane bound and its receptor, CX_3CR1, is exclusively expressed by CNS microglia (75, 76). Although the exact mechanism by which microglia promote synaptic maturation is not known, it is likely that CX_3CL1 secreted by neurons provides a cue that attracts microglia and binds CX_3CL1 through CX_3CR1 (76, 77). It has been reported that deficits in microglial function that affect CX_3CL1/CX_3CR1 binding can lead to developmental disorders or induce neurotoxicity (78-81).

Apart from the above, the classical complement cascade is another set of molecules implicated in synaptic remodeling during development (50). In the postnatal brain, resident microglia express complement receptor 3 (CR3), which binds to C3 protein localized, along with C1q, in a subset of immature synapses. The upstream regulator of C1q, is TGF-β (82, 83). It has been proposed that C1q serves as a tag for synapses that need to be eliminated (40, 82). Thus, expression of complement proteins at developing synapses is crucial for their elimination through engulfment by phagocytic microglia. This process largely depends on neuronal activity, as microglia preferentially phagocytose less active presynaptic inputs (50). The contribution of microglia in sculpting neural circuits in the postnatal brain is both activity dependent and complement dependent, however the latter only occurs in the absence of neuroinflammation (50, 84).

Neural network plasticity in response to cerebral ischemia

Neural networks and circuits are highly interconnected, thus damage induced by a brain injury such as focal cerebral ischemia will inadvertently affect loci distal to the original injury site (85). This effect, originally described as diaschisis (86), explains how neuronal loss in one brain region affects neuronal excitability and impairs function in remote brain regions (87). This process is characterized by evolution of the related pathology and dissipation of the injury in the brain in a spatiotemporal manner. Conversely, diaschisis may explain the manner in which intra- and interhemispheric neural network remodeling occurs in response to injury, constituting evidence of spontaneous or experience-dependent plasticity (87). As a result, gain of function could be attributable to the partial restoration of neural networks previously involved in impaired function or alternatively, to a compensatory or substitution mechanism, which involves recruitment of other networks (87-89).

Apart from clinical findings, much of the evidence of network remodeling in response to brain ischemia is derived from animal models by application of a range of molecular, neuroanatomical, neurophysiological, and neuroimaging techniques, including functional magnetic resonance imaging (fMRI), which have provided valuable insights into morphology-activity relationships and dynamic changes at the network and synaptic level (90, 91).

One such example is the manner in which ischemic injury affecting the primary motor (M1) and/or somatosensory (S1) cortex results in early reorganization of topographic representations of spared neural circuits within the perilesional area, characterized by a redistribution of neuronal receptive fields (RF) (87, 92-94). Furthermore, this unmasking of latent synaptic inputs is related to reorganization of thalamocortical networks and involves inputs that inhibit or promote cortical neuron excitability (87, 95). Relevant experimental findings showed that despite the fact that the emerging neural networks were characterized by loss of selectivity, i.e. they processed information from multiple limbs instead of a single contralateral limb, experience-dependent plasticity through learning of specific motor tasks significantly contributed to functional recovery at the chronic stage post-ischemia (87).

Changes in excitatory and inhibitory neurotransmission within the ischemic core and perilesional area are consistent with the evolution of ischemic pathology over time and can thus influence perilesional remapping acutely but also at the chronic stage after ischemic stroke. For example, experimental evidence suggests that hyperexcitability in the sensorimotor cortex (SMC) proximal to the lesion is observed acutely after brain ischemia, peaking at 7 days post-lesioning, and thereafter gradually declines over several months (96). This effect has been attributed to a downregulation of GABAergic inhibition mediated by a transient reduction in the binding density of $GABA_A$ receptor and/or $GABA_A$ receptor subunits at the infarct core and surrounding tissue (87, 97-99).

Similarly, a reduction in the binding density of NMDA, AMPA and kainate receptors was observed after ischemic injury affecting S1, which peaked at 30 days post-lesioning, at which stage an upregulation of NMDA receptor binding was observed in the perilesional area (87, 100). Furthermore, the relevant findings suggest that alterations of inhibitory and excitatory inputs at the core and perilesional area, especially during the first week after injury, occur in an opposite manner. Moreover, in tandem with perilesional remapping, morphological changes can also be observed with increased dendritic spine turnover as well as plastic changes in dendrite arborization. Interestingly, this form of plasticity was found to be more pronounced in cortical regions distal to the infarct area, compared to the perilesional

area (87, 92, 101).

The fact that microglia contribute towards sculpting neural synapses and networks, both in an activity- and complement dependent manner, suggests that microglia-mediated pro- or anti-inflammatory activity may significantly contribute towards spontaneous neuronal plasticity after ischemic lesion. One such example is evidence from experimental studies supporting that microglial CR3 activation by inflammatory stimulus can trigger long-term synaptic depression (LTD) in surrounding neurons *via* NADPH oxidase, one of the main mediators of neurotoxicity in stroke (102). Specifically, superoxide production from NADPH oxidase induced LTD *via* activation of PP2A and GluA2-mediated AMPAR internalization (102). This indicates that an enhanced neuroinflammatory response in microglia can rapidly modify neuronal activity and modulate synaptic function. Furthermore, another study revealed that activity-dependent connections between microglia and synapses are markedly prolonged after cerebral ischemia, with contact between microglial processes and neuronal synapses maintained for ~1 h, compared to ~5 min in the uninjured brain, and subsequent disappearance of the presynaptic bouton and synaptic elimination (41).

Further elucidation of such mechanisms may provide important insights as to the manner in which compromised synaptic efficiency may result in functional deficits after stroke. Additionally, such insights may clarify the potential role of microglia in detecting the functional state of affected synapses and attempting to preserve function or eliminate them (41). Conversely, selective targeting of such responses, as for example by AMPA receptor removal (103), may determine whether homeostatic plasticity can be maintained by sustained synaptic input or whether synaptic strength can be redistributed in a manner that favors the wiring of coincidently active pathways, i.e. *via* the formation of Hebbian synapses (45, 104-106).

Endogenous neurogenesis

Another form of plasticity in which microglia may play an instrumental role in the context of stroke repair is endogenous neurogenesis. It has been established that neurogenesis, a term which encompasses the generation of all cells of neural lineage, i.e. neurons, astrocytes, and oligodendrocytes, continues throughout adult life. In the brain, the main neurogenic niches generating neural progenitor cells (NPCs) are the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus. Furthermore, relatively recent evidence increasingly supports the view that adult neurogenesis also occurs in several other CNS regions including the spinal cord, neocortex, cerebellum, striatum, amygdala, substantia nigra and hypothalamus (71, 107-112).

Cerebral ischemia, activates both neurogenic niches and non-neurogenic brain regions. Interestingly, such reactive responses seem to have a functional rather than constitutive character. Extensive experimental evidence from animal models of brain ischemia has demonstrated that NPCs generated in the ipsilesional SVZ, instead of migrating to the olfactory bulb through the rostral migratory stream, i.e. their regular migratory pathway, start migrating towards the infarct site. Similarly, post-mortem tissue from stroke patients obtained 5-15 days post ischemia revealed NPC proliferation and migration towards the infarct from the ipsilesional SVZ. Remarkably, in cases where such progenitors reach the perilesional area, they can terminally differentiate into medium spiny neurons, as evidenced in a number of animal studies (71, 113, 114). This suggests that the neurogenic niche response may play a role in functionally integrated cell replacement (71). The extent of SVZ-derived NPC proliferation and

migration can be positively associated with the severity of the lesion. Furthermore, this response seems to be sustained at the chronic stage post-lesioning (115). Strikingly, NPC proliferation at the chronic stage is restricted to those regions directly affected by stroke (109).

Earlier studies demonstrated that endogenous neurogenic responses can be detrimentally affected by local inflammation mediated by activated microglia (35, 116, 117). Specifically, *in vivo* and *in vitro* experiments have shown that factors such as TNF-α, IFN-γ, IL-1, and IL-6, suppressed neurogenesis and also inhibited NSC survival, an effect that could be partially controlled through pharmacological modulation of the inflammatory response, as for example by IL-6 neutralization or administration of minocycline (117-121). However, a more complex profile of the role of microglia in neurogenesis has been emerging, suggesting that instead of being detrimental to endogenous neurogenesis, activated microglia may indeed be beneficial for NPC proliferation and integration into functional networks supporting stroke recovery.

For example, it has been shown that stimulation of microglia with low levels of IFN-γ can promote early neurogenesis after stroke (122). Furthermore, it has been reported that at the chronic stage (16 weeks) after ischemia, microglia within the ipsilesional SVZ exhibited a ramified or intermediate morphology (suggestive of an M2 phenotype), compared to the amoeboid morphology (M1 phenotype) of microglia found in the perilesional striatum. Additionally, there was an upregulation in the numbers of SVZ microglia expressing IGF-1 (35). This indicates that the prolonged presence of such microglia within the neurogenic niche can be supportive of sustained NPC proliferation, a process that may effectively promote tissue remodelling after stroke through prolonged availability of migrating NPCs towards the infarct site.

Similarly, microglia can play a crucial role in NPC migration towards the infarct through the secretion of chemokines such as stromal cell-derived factor 1α (CDF-1α) whose receptor, CXC4, is highly expressed in NPCs (123). In this manner, CDF-1α upregulation in response to ischemia may thus fuel NPC proliferation and migration (35, 124). The above findings suggest that selective modulation of microglia or microglia subtype activation may positively influence short- and long-term neurogenic responses. Furthermore, considering that activated microglia secrete cytokines and growth factors such as TNF-α and BDNF, they may also influence morphology-activity relationships, including excitatory and inhibitory synaptic transmission as well as dendritic spine arborization and thus promote functional integration of newly generated NPCs within the lesion hemisphere (35, 50, 125, 126).

Conclusions

Neuroplasticity after ischemic injury involves both spontaneous rewiring of neural networks and circuits as well as functional responses in neurogenic niches. Such events involve complex interactions with activated microglia which evolve in a dynamic manner over time. Although the exact mechanisms underlying these interactions remain poorly understood, increasing experimental evidence suggests a determining role of pro- and anti-inflammatory microglial activation profiles in shaping networks and synapses. Ability to elucidate as well as to engage such mechanisms in a selective manner, also in the context of microglia-neuron-astrocyte interactions, can be envisaged to play a significant role in harnessing endogenous plasticity to promote stroke repair with functional outcome.

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