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Fine morphological alterations during brain injury and recovery analyzed with intravital microscopy

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Academic Dissertation

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To my family

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I. Paveliev M*, **Kislin M***, Molotkov D, Yuryev M, Rauvala H, Khirug L. Acute Brain Trauma in Mice Followed By Longitudinal Two-photon Imaging. J. Vis. Exp. 2014, e51559.

The candidate designed, performed experiments and analyzed the data together with MP, prepared the video together with DM, MP, MY and LK, wrote the manuscript together with MP, HR and LK.

II. Paveliev M, Fenrich K, **Kislin M**, Kulesskaya N, Kuja-Panula J, Kajander T, Määttänen A, Kari E, Mugantseva E, Ahonen-Bishopp A, Khiroug L, Ihalainen P, Riekkola M, Peltonen J, Rougon G & Rauvala H. HB-GAM (pleiotrophin) reverses inhibition of neural regeneration by the CNS extracellular matrix. Submitted manuscript.

The candidate performed most of the *in vivo* experiments on brain trauma and analyzed effect of HB-GAM on dendritic regeneration and plasticity.

III. Kremneva E*, **Kislin M***, Kang X, Khiroug L. Motility of astrocytic mitochondria is arrested by Ca^{2+}-dependent interaction between mitochondria and actin filaments. Cell Calcium. 2013, 53(2), P. 85-93.

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IV. Lihavainen E, **Kislin M**, Toptunov D, Khiroug L, Ribeiro AS. Automatic quantification of mitochondrial fragmentation from two-photon microscope images of mouse brain tissue. Submitted manuscript.

The candidate performed *in vivo* two-photon microscopy experiments and wrote the manuscript together with EL, LK and AR.

V. **Kislin M**, Pryazhnikov E, Lihavainen E, Toptunov D, Rauvala H, Ribeiro A, Khiroug L. Quantitative intravital microscopic analysis of neuronal mitochondria morphology in the cortex of anesthetized mice following ischemic and traumatic injuries. Manuscript.

The candidate substantially contributed to the experimental design, performed *in vivo* two photon microscopy of neuronal mitochondria on anesthetized and awake animals, participated in the analysis of the results and wrote the manuscript together with LK.

VI. **Kislin M**, Mugantseva E, Molotkov D, Kulesskaya N, Khirug S, Kirilkin I, Pryazhnikov E, Kolikova J, Toptunov D, Yuryev M, Giniatullin R, Voikar V, Rivera C, Rauvala H, Khiroug L. Flat-Floored Air-Lifted Mobile Homecage: A New Method for Head Fixation of Awake Behaving Mice During Microscopy or Electrophysiology. J. Vis. Exp. 2014, e51869.

The candidate substantially contributed to the experimental design, planned and performed most of *in vivo* two-photon microscopy, behavioral experiments, participated in BOLD signal acquisition experiments together with DM and wrote the manuscript together with LK.

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ABSTRACT

Acute brain trauma and ischemia are severe injuries that have no adequate treatment to date. In vivo two-photon microscopy allows studying longitudinally the process of injury development and brain recovery. This thesis summarizes the work on: i) animal model of acute brain injury (Study I) and role of extracellular matrix in neuronal recovery and plasticity (Study II), ii) investigation of mitochondria during physiological/pathological Ca^{2+} elevations *in vitro* (Study III) and further implementation of quantitative microscopic analysis of neuronal mitochondrial morphology *in vivo* (Study IV), iii) mitochondrial damage and recovery in animal models of acute neurodegenerative disorders in the neocortex of anesthetized mice (Study V), iv) a novel approach for awake head-fixed recordings (Study VI). The results described in this work provide novel approaches for intravital morphological analysis of neurons and of their mitochondria, increase our understanding of pathogenesis after traumatic and ischemic injury in neocortex of rodents, and enable the development of novel therapies for CNS injuries.

ABSTRAKTI

Sekä akuuttinen aivoisten trauma että iskemiä ovat eritäin vakavia vammoja, ja keinoja niiden parantamiseen ovat puutelisiä. Vammojen kehittämisen ja aivoisten parantamisen mekanismit tutkimisen on mahdollinen tuplafotoni mikroskopian avulla. Tämä väitöskirja on perustettu kuudella julkaisutella ja kertoo viidestä asiasta: i) akuuttinen aivoisten trauman koe-eläin malli (Tutkimus I) ja soluväliaisten matriksin rooli hermosolujen parantamisessa ja plastisuudessa (Tutkimus II), ii) in vitro tutkimus mitokondrian roolista fysiologiassa/patofysiologiassa kalsiumin nostamisessa (Tutkimus III) sekä in vivo mitokondria morfologian analysointi keinoen kehittäminen (Tutkimus IV), iii) mitokondrian vahingoittaminen ja parantaminen neurodegenerativisten sairauksien malleissa hiirten neokorteksissa anestesian alla (Tutkimus V), iv) uusi keino in vivo rekisteröintien varten ilman anestesia (Tutkimus VI). Tutkimuksen tulokset antoivat uudet keinot mitokondrian analysointien hermosoluissa, ne parantavat myös meidän ymmärtäminen post-traumateisten ja post-iskemiään vammojen hiirten neokorteksissa. Tutkimustulokset tulevat merkittävällä tavalla lisäämään ymmärrystä aivohalvauksen patofysiologiasta ja edistää uusien terapiamahdollisuuksien löytämistä.

ABBREVIATIONS

$[Ca^{2+}]_i$ – intracellular calcium concentration

2-VO - occlusion of common carotid arteries

4-VO - occlusion of four-vessel (two vertebral and two common carotid arteries)

AD – Alzheimer disease

AMPA – α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AQP – aquaporin (channel)

ATP – adenosine triphosphate

BBB – blood-brain barrier

CBF - cerebral blood flow

CCA – common carotid artery

CCI - cortical contusion injury

CNS – central nervous system

CS - chondroitin sulfate

CsA - cyclosporin A

CSD - cortical spreading depression

CSPG – chondroitin sulfate proteoglycan

CT - computed tomography

DAI - diffuse axonal injury

Drp1- dynamin-related protein 1

DWI - diffusion-weighted magnetic resonance imaging

ECM – extracellular matrix

EEG – electroencephalography

EG(C or Y)FP – enhanced green (cyan or yellow) fluorescent protein

ER - endoplasmic reticulum

FLL - focal laser lesion

FPI - fluid percussion injury

GABA – gamma-aminobutyric acid

GAG – glycosaminoglycan

GFAP – glial fibrillary acidic protein

HPC - hemorrhagic progression of a contusion

i.v. – intravenous

i.p. - intraperitoneal

ICP - intracranial pressure

IMM – inner mitochondrial membranes

IMS - intermembrane space

IV2PM - *in vivo* two-photon microscopy

Kir – potassium inward rectifying (channel)

MCAO - middle cerebral artery occlusion

mGluR – metabotropic glutamate receptor

MPD - mild photodamage

mPTP - mitochondrial permeability transition pore

MRI - magnetic resonance imaging

mtDNA – mitochondrial deoxyribonucleic acid

NKCC – neuronal potassium-chloride cotransporter

NMDA – N-methyl-D-aspartic acid

NVU - neurovascular unit

OGD - oxygen/glucose deprivation

OMM - outer mitochondrial membranes

OPA1 - optic atrophy factor 1

OXPHOS - oxidative phosphorylation

PBBI - penetrating ballistic-like injury

PET - positron emission tomography

PID - peri-infarct depolarization

PNNs - perineuronal nets

PTSD - post-traumatic stress disorder

RBPS - Rose Bengal photosensitization

TBI - traumatic brain injury

TIA - transient ischemic attack

TIM - protein translocase of the inner mitochondrial membrane

TOM - protein translocase of the outer mitochondrial membrane

tPA - tissue plasminogen activator

REVIEW OF LITERATURE

1. The Brain in traumatic and ischemic injury

1.1. Clinical background

Traumatic and ischemic brain injuries are among the leading causes of mortality and long-term disability and have been identified as important worldwide public health problems that pose an enormous socio-economic burden.

Traumatic brain injury (TBI) is caused by the impact of external physical forces that produce temporal or permanent impairment of cognitive abilities and/or physical functioning. The leading causes of TBI are motor vehicle accidents, falls, assaults, job-related injuries and sport activities. Each year over 10 million people are affected by TBI, around 1.5 million of those die, it is the number one cause of coma and among the survivors it commonly causes lifelong disabilities, making dramatic personal impact and society load (Roozenbeek, Maas, and Menon 2013). In most epidemiologic studies there are three age related peaks: in early childhood (under the age of 5), in young adults (the most active population), and in the elderly (over the age of 75). Each year, in the US alone, the annual economic burden from TBI is more than $60 billion. A cumulative result of past TBI shows that a considerable proportion of the population is suffering from permanent TBI-related disability. Currently, no pharmacological treatments have been proven to protect against the harmful consequences of TBI.

Stroke is the second most common cause of death and a leading cause of disability in adults worldwide (WHO, 2013). Stroke can be caused by a blockage of blood flow to the brain in the case of ischemic stroke or a blood vessel rupture in the case of hemorrhagic stroke and in both cases it is characterized by an abrupt loss of brain function. Approximately 17 million people have strokes each year and around 6.2 of those die shortly after the stroke. Two thirds of strokes occur in people over 65 years old. The estimated cost of strokes is more than $36.5 billion per year, in the US alone, 33 million of people live with stroke in their anamnesis (Feigin et al. 2013; Go et al. 2014). Unlike TBI, early thrombolysis or thrombectomy are available for ischemic stroke patients as treatments. The thrombolytic treatments with tissue plasminogen activator (tPA) has been proven effective in treating stroke when given within 4.5 hours after ischemic onset

(Cronin 2010). Unfortunately, this therapy is available only for a limited group of patients due to the narrow therapeutic time window (which is often missed) (Tansy and Liebeskind 2013). Although stroke and traumatic brain primarily affect different age groups, both result in a significant number of individuals with long-term or even permanent deficits.

Traumatic and ischemic injures of the brain arise from very different initial events. TBI generally results from external mechanical impact and in a minority of cases from an object penetrating the skull and meninges. The damage after TBI can be localized to the sites of penetration/impact (open/closed-head type of TBI) or can be more widespread if fragments of bone enter the brain or the brain is slammed inside the skull. These initial events of TBI cause direct and immediate damage that may lead to disability or death. The three generally accepted assessment approaches are: the Glasgow Coma Scale, Post-Traumatic Amnesia and Loss of Consciousness, which are used to categorize the severity of TBI at the acute phase into mild, moderate and severe. The ratio of mild to moderate to severe traumatic brain injuries is estimated to be 22:1.5:1, so mild brain injuries represent the majority (from 75% to 95%) of cases (Andriessen, Jacobs, and Vos 2010). It is possible to segregate four major pathoanatomic consequences following TBI: hematomas, including epidural, subdural, and parenchymal, then contusions, subarachnoid hemorrhage, and diffuse axonal injury (Saatman et al. 2008) although they can be present together in different proportions.

The classification of strokes is clearer and includes three major categories: ischemic, hemorrhagic stroke and transient ischemic attack (TIA). The most common form of stroke is ischemic, it constitutes about 87% of all cases (Go et al. 2014). Ischemic stroke generally results from cerebral thrombosis, which is obstruction of a blood vessel by a blood clot forming locally and from cerebral embolism, which is a blood clot formed in the heart or large arteries of upper chest and neck. In rare cases ischemic stroke results from systemic hypoperfusion commonly due to heart failure. Ischemic stroke can be focal or global as well as permanent or transient. The duration and localization of ischemic stroke and size of infarction strongly affect the prognosis of clinical outcomes. Near 75% of ischemic stroke survivors' disabilities' are severe enough to reduce their employability. Hemorrhagic stroke is induced by bleeding from a ruptured vessel and represents about 10% of all cases. Aneurysms and arteriovenous malformations are major blood vessel diseases that cause hemorrhagic stroke. A

greater risk of death arises from bleeding, however it deprives affected tissues of a blood supply and creates pressure on the brain. Despite this, the consequences can be less severe and recovery may be more complete for a person who survives hemorrhage than for those who experience ischemic stroke. Transient ischemic attack is frequently called a mini-stroke, it is caused by transient episodes of cerebral thrombosis that induces neurologic dysfunction caused by ischemia without acute infarction. When TIA is over and the clot dissolved, it usually causes no permanent injury to the brain (Siket and Edlow 2013). However, it should be taken into account that about one third of patients after TIA experience ischemic stroke within a year.

The outcomes from stroke and TBI depend on two substantially different mechanisms: primary damage, occurring at the moment of impact, and secondary damage that represents consecutive pathological processes initiated at the moment of injury with delayed clinical presentation. The primary impact in traumatic and ischemic injures initiates a wide range of secondary injury mechanisms that critically participate in diseases pathogenesis. The severity of the secondary mechanisms of TBI and ischemic stroke directly depends upon injury severity or the location of the primary impact. Secondary injury in TBI and ischemic stroke are similar to some extent and can lead to a spectrum of pathological changes that substantially influence the patients' health statuses.

The current abilities to block TBI or ischemic stroke-induced events are very limited. Despite this unmet clinical need and considerable investments, the current failure rate in clinical trials of drugs in this area is catastrophically high, with nine out of ten trials either failing or being aborted. Novel pharmacological therapies such as anti-oxidant treatments, glutamate blockers and physiotherapy, such as constraint-induced movement therapy, have shown promising effectiveness in pre-clinical studies, but have limited clinical success or are not widely used. This situation imposes an enormous financial burden on taxpayers and forces major pharmaceutical companies to withdraw from drug discovery against traumatic and ischemic injury. Further research has to be performed to expand our understanding of the complex pathophysiology of these injuries with the aim of developing novel drugs and effective therapeutic strategies for patients suffering from these devastating diseases.

1.2. Consequences of traumatic and ischemic injury

1.2.1. Functional deficit

Survivors after traumatic and ischemic injury experience physical, cognitive and psychosocial deficit that disturb their emotional, mental and social performance in their daily lives (Ponsford et al. 2014). During recovery after TBI and stroke the wide spectrum of disorders that affect life quality can occur and last for a long period or even persist permanently. The disorders faced by survivors just after injury strongly depend on the duration and location of the brain damage, its severity and the medical support received post-injury. Long-term post-injury outcomes depend on multiple factors, such as pre-injury socio-demographic characteristics, injury severity variables, post-injury personal factors and post-injury environmental factors.

Pre-injury risk factors such as biological (non-modifiable factors) - age, gender, genetic predisposition, race/ethnicity and familial history; physiological factors(modifiable) - hypertension, carotid disease, diabetes, dyslipidemia (elevated cholesterol and triglycerides), sleep apnea, intracranial atherosclerosis, transient ischemic attack, valvular heart disease, atrial fibrillation, aortic arch atheroma, sickle cell disease, depression, brain infections and chronic inflammation; lifestyle factors (modifiable) - obesity, physical inactivity, smoking, heavy drinking, high sodium intake, as well as their comorbidity are known from epidemiological studies to strongly contribute to prognosis for recovery following stroke and TBI (Ankolekar et al. 2012; Kuklina et al. 2012). Relatively effective preventive strategies are available to control certain risk factors has been established in recent years (Kernan et al. 2014). In some cases application of risk assessment tools can reduce as much as 80% of all recurrent strokes. However, risk factors often co-occur in individual patient, this complexity makes development and implementation of optimal prevention strategy a challenging task.

Several indicators are used to predict long-term outcomes in TBI and stroke survivors during the first few weeks and months after the injury. The location and size of the infarct determines the type of impairment to some extent since certain areas of the brain control specific functions. For example, brain stem damage caused by TBI can affect vegetative functions like breathing, swallowing, blood pressure and heart rate, whereas ischemic stroke that affected vascular territory

in the frontal lobe can change survivor’s emotional responses and judgments on environment and daily activities and affect memories and understanding of abstract concepts. There are three general types of impairment that can arise following stroke and TBI: physical, cognitive and emotional/behavioral deficits.

The interactions between post-injury deficits are complex and unfortunately understood only partially. One of probable consequences of TBI and ischemic stroke is physical weakness. Musculoskeletal perversions that affect mobility are the most prevalent symptoms and they are present in up to 79% of stroke patients (Cheng, Leys, and Esquenazi 2013) and in 40% of patients after TBI (Jang 2009). The neurological damage affects multiple systems with impacts on motor functions that result in mobility loss, hemiparesis, spasticity, gait disturbance, skilled movement loss, apraxia, ataxia, involuntary movement and poor balance. In most cases patients suffer from more than one physical disability. Around 60% of stroke patients show sensory deficits (Schabrun and Hillier 2009) that disturb their abilities to perceive and discriminate sensations of pain, temperature, pressure and vibration, as well as their abilities to accurately locate body parts in space. Moto-sensory deficits determine just a portion of post-injury impairments. Commonly, in TBI and ischemic stroke patients other physical problems such as incontinence, speech and swallowing disorders can occur. Often epilepsy becomes a residual disorder in the post-injury period and in some cases can persist for the rest of the patients’ lives. The frequency of seizures ranges between 2.3% and 14% in ischemic stroke (Menon and Shorvon 2009) and from 2% in population-based studies to 14–30% in patients with severe TBI (Christensen 2012). Seizures in the setting of TBI and ischemic stroke will furthermore worsen the prognosis of injury.

Cognitive deficits are a common consequence of traumatic and ischemic injury and its prevalence ranges from 20% to 80% of the survivors. Memory and learning, attention and communication are to a greater or lesser extent affected by amnesia, agnosia, aphasia, apraxia, dysopsia, executive dysfunction and mood disorders. Memory deficits are the most enduring cognitive impairment and are particularly difficult to treat. The underlying mechanisms of cognitive impairment are not well known, however pathogenesis is likely to involve vascular cognitive impairment by itself or by Alzheimer’s disease (AD). There is evidence from autopsy studies suggesting that around half of the patients with cognitive deficit have attributions from both (Jellinger 2007). Many patients develop mild cognitive

impairment and some meet the clinical criteria of dementia. In particular, approximately 10% of patients develop new dementia after their first stroke, whereas 30% developed dementia after a recurrent stroke (Pendlebury and Rothwell 2009).

The survivors that face physical or/and cognitive impairments usually experience frustration and depression, or in case of TBI - post-traumatic stress disorder (PTSD). Depression is found in 31% of patients in the acute period after a stroke (Hackett and Pickles 2014). The frequency of PTSD varies dramatically along epidemiological studies, mainly due to debates that PTSD and TBI do not co-occur (Harvey et al. 2003). In general, PTSD ranges from 3 to 30% in civilian patients and from 12% to 89% in military patients and veterans (Bahraini et al. 2014). Mood disorders are individually complex and when combined with stroke and TBI they create significant assessment challenges and lead to worse cognitive and physical outcomes (van de Port et al. 2006). Depression and PTSD need to be carefully diagnosed and treated accordingly in order to improve functional recovery. Recently, a new aspect of antidepressant medications has arisen after fluoxetine (a selective serotonin reuptake inhibitor) was shown to improve the functional recovery of patients with hemiplegic stroke (Gainotti et al. 2001; Paolucci 2013). This opens up a new field in post-injury rehabilitation through a neuronal target.

During rehabilitation, therapeutic modalities and exercise, along with medication interventions are targeted on lingering symptoms including motor and cognitive deficits and aimed to improve the functional status of the survivors so that they can independently perform personal care and regular daily activities and ideally can return to work and other social interactions. At present, post-injury rehabilitation is realized by the support of multidisciplinary teams in neurovascular units for stroke or by neuroscience units for TBI that organize packages of individualized care. However, neuropsychological and physical deficits are usually addressed in the rehabilitation setting as separate issues. Typically, more attention is given to physical and occupational rather than cognitive therapy. There are several task-specific and practice-based therapies that are designed to boost mobility, coordination and durability. One of them is constraint-induced movement therapy - a treatment for mild and moderate upper extremity motor dysfunction and is often beneficial for acute or sub-acute stroke patients, although the effect does not persist in some cases and a limited number of clinical studies has been carried out in TBI (Thrane et al. 2014). Another rehabilitation

therapy is functional electrical stimulation aimed to contract weakened muscles in a precise manner in order to resemble functional tasks. Clinical studies have shown that this therapy can prevent or reduce shoulder subluxation early after stroke, but it does not reduce pain or improve upper arm motor function (Vafadar, Côté, and Archambault 2015). Electromyogram biofeedback is also used to improve affected arms via the delivery of auditory or visual stimuli from electrodes placed on the skin. However, the long-term efficacy of this therapy is unclear. At present there are many physiotherapies for individuals with TBI and stroke that recruit a high variability of methods with different training intensities, some of them have demonstrated beneficial outcomes on motor function even with a negative effect on tone and increased pain (Saunders, Greig, and Mead 2014). For example, motor recovery can be improved with clinically proven effectiveness by meaningful task-specific training (Arya et al. 2012). It can be followed with botulinum toxin A administration in selected groups of patients that have developed spasticity (Teasell et al. 2012).

So far, there is no treatment with clearly proved efficacy for post-stroke or post-TBI cognitive impairment. Some of the anti-dementia treatments like cholinesterase inhibitor, donepezil (Rockwood et al. 2013) or NMDA receptor antagonists like memantine (Wilcock 2003) have been shown to improve some cognitive functions significantly, but due to the controversial results in the preclinical and clinical studies, further research is needed to define the efficacy of these treatments for trauma or ischemia induced cognitive deficits. However, there are currently several possible treatments undergoing preclinical trials. Some of them attempt to restore reduced cognition, others try to overcome or compensate for reduced function.

Rehabilitation after traumatic and ischemic injuries is a complex process that is now lacking adequate management or well-timed therapy and suffers from the accessibility of neurorehabilitation services (Mendelow et al. 2008). There are many questions concerning the treatment of stroke and TBI patients which have not been studied yet. A better understanding of injury mechanism and recovery will promote the development of rehabilitation systems in a more logical and targeted manner.

1.2.2. Pathophysiology of stroke and TBI

There have been many neuropathological studies on autopsy or postmortem samples that provide clinico-pathological correlations in patients following stroke or TBI. Clinical examination of the primary injury was reported for the first time after the advent of X-ray computed tomography (Gudeman et al. 1979). A major contribution to our understanding of brain pathology after the injury has been provided by neuroimaging, including computed tomography (CT) and magnetic resonance imaging (MRI) (Lövblad and Baird 2006), and electrophysiological approaches like quantitative, topological electroencephalography (EEG) (Haneef et al. 2013), magnetoencephalography and magnetic source imaging. In the last decade, positron emission tomography (PET), single proton emission tomography, functional and diffusion-weighted MRI (DWI) have been used to identify functional correlations between the structural changes (Hunter et al. 2012; Schaefer, Grant, and Gonzalez 2000). Transcranial Doppler and angiography with contrast in CT and MRI provide additional data on vessel abnormalities, including narrowing or occlusion, embolus, atherosclerosis, arteriovenous malformations and aneurysms, and regions with low cerebral blood flow (CBF). These methods are especially critical in understanding the progression of TBI (Bouzat, Oddo, and Payen 2014). Laboratory tests of blood, urine and cerebrospinal fluid samples have allowed the identification of a large number of injury biomarkers (Zetterberg, Smith, and Blennow 2013). This palette of approaches helps to identify key pathological consequences and also drive clinical diagnosis, prognosis and acute treatment decisions (von Kummer, Dzialowski, and Gerber 2015).

Human brain tissue typically suffers from multiple pathologies following stroke and TBI. Propagation of injury in both cases arises from similar pathophysiological cascades. Many of the cellular and molecular responses are due to common secondary injuries, such as ischemia, rather than reflecting the primary tissue response, such as mechanical axonal damage (Silver, McAllister, and Yudofsky 2011). Below is a description of the key encompassing factors of individual pathological processes seen in TBI and stroke.

Contusion

TBI results in a contusion when the region of injury receives kinetic energy from the primary impact that causes shearing of the tissues. This immediately ruptures cells in the injury region and microvessels (Kurland et al. 2012). On a CT scan, a

contusion generally appears as a hemorrhagic lesion, but it can be distinguished from a laceration or hematoma since the pia mater remains intact and blood is intermixed with brain tissue. Excitotoxicity and microvascular dysfunction are two main consequences of contusion. In the parallel to secondary injury, protective mechanisms are activated, but these are found to be improper, like the inflammatory response that serves to clear tissue debris, but begins to overproduce free radicals causing propagation of the tissue injury (O'Connell and Littleton-Kearney 2013). The main drivers of secondary injury that lead to functional deficits after contusion are: tissue ischemia and vasogenic edema, further contributing to extravasation. Additionally, loss of the structural integrity of surrounding microvessels induces in hemorrhagic progression of a contusion (HPC) - expansion of the hemorrhagic lesion. Approximately half of patients with contusions demonstrate HPC on serial CT scans within the first 12 hours or late in 3–4 days after head trauma. HPC usually occurs after a patient's hospitalization, so they are in clinical conditions where it is possible to prevent this conscience. Around 19% of patients with large contusions develop HPC that requires surgical decompression (Alahmadi, Vachhrajani, and Cusimano 2010). Novel treatments are needed to prevent HPC, but currently only a few molecular events have been suggested for progressive microvascular failure. Therefore, clinical solution for contusion would not appear in near-term outlook.

Diffuse traumatic axonal injury

While the pathophysiological consequences of TBI are heterogeneous, one of the most common processes occurring in 40-50% of TBI patients hospitalized with closed head injury is diffuse axonal injury (DAI) (Povlishock and Katz 2005). Axons appear to be especially vulnerable to injury and DAI can be detected in the corpus callosum, brainstem and in internal capsule (Silver, McAllister, and Yudofsky 2011). Because of the low resolutions capacities of conventional CT and MRI, they cannot identify DAI. In the majority of cases, DAI is accompanied by small hemorrhages and can be predicted from CT scans or MRI images that are more sensitive to blood components. In the clinical setting, usage of the DWI mode of MRI now increases the diagnosis of DAI directly. Quantitative diffusion tensor tractography also looks promising (Bazarian et al. 2007), although still controversial (Ilvesmäki et al. 2014), for the acute and long-term follow-up of TBI patients with DAI. So far, conventionally diagnostic confirmation of DAI can be done by histopathological examination. Primary axotomy - axonal degeneration

induced by the primary impact, is considered to be a relatively rare event. Mainly DAI is initiated by perturbations in axonal transport and the cytoskeleton that result in axonal swelling followed by secondary disconnection and Wallerian degeneration. Previously, DAI has been suggested to appear immediately or within months after TBI, but recent clinical evidence has demonstrated that it can be delayed for years (Chen et al. 2009). DAI may have a role in mild cognitive impairment or dementia after TBI, because it can induce development of Alzheimer-like pathologies (Johnson, Stewart, and Smith 2012). Degenerated axons are one of the major therapeutic targets in TBI that potentially may ameliorate chronic neurodegeneration.

Brain Edema

Cerebral edema describes brain swelling following strokes and TBI. It is a major cause of long term disability and mortality. There are four major types of brain edema: i) cytotoxic - related to intracellular sustained water accumulation due to cellular injury; ii) vasogenic - extracellular water accumulation resulting from blood–brain barrier (BBB) breakdown; iii) osmotic- caused by imbalances between the blood and tissues; iv) and hydrocephalic edema/interstitial - related to an obstruction of cerebrospinal fluid outflow (Unterberg et al. 2004). Cytotoxic edema is cell swelling induced either singly or by combination of the following mechanisms: plasma membrane permeability to ions, failure of the ion-pumps, and sustained uptake of hyperosmotic solutes. Vasogenic edema is characterized by plasma protein enrichment resulting from increased permeability of the capillary induced by primary mechanic impact or ischemic stroke. Ischemic stroke and TBI induce both cytotoxic and vasogenic edema. However, it has been recognized that after TBI edema happens predominantly due to BBB disruption, since on CT scans initially hyperdense hemorrhagic lesions develop a hypodense halo around the hemorrhagic focus. However, this view has been criticized since contrast enhanced CT and MRI studies reveal only minimal or moderate BBB leakage (Bullock et al. 1990) and clinical data have revealed no evidence of BBB leakage within the first 24 hours after injury (Maeda et al. 2003), supporting the concept of cellular brain edema. During the initial 2–3 days following TBI or ischemic stroke brain edema can progress from the core and spread to healthy tissue. It remains a crucial factor in the acute phase because patients are at risk of developing secondary damage. It may increase intracranial pressure (ICP), decrease cerebral perfusion and brain oxygenation, and eventually lead to brain

herniation and patient death. Even today, brain edema is still treated only symptomatically. Surgical decompressive craniectomy or osmotherapy are used mainly to decrease ICP. The most common treatment strategy is administration of the osmodiuretics, mannitol and hypertonic saline, which have well-documented transient ICP-reducing effect. Nevertheless, there are strong side effects related to rebound increase in ICP after withdrawal, electrolyte disturbances and renal and pulmonary failure (Grände and Romner 2012). The outcome has been poorly studied, so it is only possible to speculate on the osmotherapy efficacy. In principle, brain edema by itself is reversible, but its treatment is still challenging.

Blood brain barrier disruption

One of unique features of brain vasculature is the BBB that maintain neuronal homeostasis via regulation of the brain parenchyma composition (Davson 1976). Endothelial cells with tight junctions and the ensemble of astrocytes, pericytes and microglia form a relatively stable structure that is continuously modified by different external and internal factors. BBB provides a barrier, restricts migration of blood cells into the brain and facilitates active transport across it. Dysfunction of BBB has been identified as a common feature in stroke and TBI patients that can occur immediately as a direct consequence of endothelial damage and/or sub-acutely within several days (Schoknecht and Shalev 2012). BBB becomes permeable while brain edema progress, this facilitates immune cells and serum components entering the brain, thus promoting inflammation as well as ion imbalance that increases excitability and can lead to epileptogenesis (Schoknecht, David, and Heinemann 2014). From another side, BBB opening can trigger vasogenic edema and an increase of intracranial pressure, hence promoting secondary damage. The role of BBB dysfunction in acute pathophysiology of stroke and TBI is generally recognized, but there are only few studies highlighting its long-term effects. Assessment of BBB permeability is limited in hospitalized patients since it requires invasive techniques. Mainly it is monitored by the albumin quotient in samples collected simultaneously from the cerebrospinal fluid and serum (Reiber and Peter 2001). In addition, contrasting imaging techniques can provide mapping and progression of the permeability. At present, dynamic contrast-enhanced MRI and dynamic contrast-enhanced CT are used to detect BBB disruption. Both methods involve i.v. injection of the contrast agents gadolinium or iodinated contrast, and voxel-wise measurement over the time of the MR signal or attenuation coefficient in CT scans (Kassner, Mandell, and Mikulis

2011). They can predict relatively accurately and promptly the transformation of BBB leakage to cerebral hemorrhage. This happens in 30-40% of stroke patients and causes severe clinical outcomes (Balami et al. 2011). Thrombolytic treatment with tissue plasminogen activator is an additional risk. Although, contrast imaging provides an assessment of individual patient risk and allows the adjustment of clinical decisions in acute treatment, the development of new medications is still needed. Preventing BBB dysfunction in the acute phase after stroke and TBI is needed to increase the number of patients that can be treated with thrombolysis and minimize its adverse effects.

Intracranial hemorrhage

Intracranial hemorrhages are the least treatable and often the cause of fatal consequences after stroke and TBI. They can be epidural, subdural, subarachnoid or intracerebral. Clinical studies have reported that epidural hemorrhage rarely appears in stroke patients and can be seen in 0.2% to 4% of TBI cases, mainly after fracture of temporal bone that leads to laceration of middle meningeal artery or vein, striping the dura from the skull and blood clot formation. This type of hemorrhage causes only minimal direct damage to the brain; nevertheless it increases intracranial pressure and promotes compression on cortical blood vessels and on the brainstem. Therefore, decompressive craniectomy needs to be performed in time to prevent severe consequences (Evans 2006). Subdural hemorrhage has been reported in about 5% of all TBI patients with rupture of bridging veins that connect cortical veins with dural sinus or contusions with cortical vessel damage. The results of subdural hemorrhage are more extensive than in epidural hemorrhage, because there is no mechanical barrier for the blood and it can easily spread. Subarachnoid hemorrhage can be found in approximately 5-6% of all stroke patients, mainly caused by a ruptured cerebral aneurysm or rarely by vascular malformation (Johnson et al. 1996) and it has been found in CT scans of 33-60% TBI cases (Greene et al. 1996). Investigations show similarities between traumatic and aneurysmal subarachnoid hemorrhage suggesting that a similar therapeutic approach can be beneficial. The main target for interventions is the vasospasm (Armin, Colohan, and Zhang 2006). If it is severe enough, it can impair cerebral blood flow and causes a secondary ischemic stroke. Currently only nimodipine, L-type voltage-gated calcium channel blocker with good blood–brain barrier permeability, has clinically proved its efficacy in treating vasospasm (Barker and Ogilvy 1996; Young et al. 2015). There are a few options to prevent

aneurysm re-bleeding and actually no agents to ameliorate the harmful effects of subarachnoid hemorrhage. Intracerebral hemorrhage, often called microbleeds, results in blood leaks from small vessels and capillaries is present in 5% of all stoke patients, two-thirds of whom had hypertension in their anamnesis (Sutherland and Auer 2006), and in 16% of TBI patients they are commonly present in their frontal and temporal lobes (Evans 2006). The clinical picture ranges from asymptomatic to fatal. It is one of the most disabling forms of hemorrhage. Damage mainly arises from mechanical brain injury caused by blood (Sutherland and Auer 2006). Treatment (if any) and prognosis of intracerebral hemorrhage strongly depends on its size (less than 10 mL – good recover; over 60 mL – coma or mortality) and location as well as the patient's state. However, there are no specific therapies improving the outcome after intracerebral hemorrhage (Sahni and Weinberger 2007).

Ischemia

Pathophysiologically, ischemia is defined as a reduction in blood flow and appears in 87% of stroke and 30-60% of TBI patients. There are three major types of ischemic strokes: i) large-artery atherosclerosis, ii) cardioembolism, iii) small-vessel occlusion (Adams et al. 1993). Clots usually tend to form at or near an atherosclerotic plaque. Sometimes plaque deposits can block the flow of blood by their growth that can obstruct the blood vessel lumen. In cases of transient ischemic attack clots tend to form at times when blood pressure is low during sleep or early in the morning. Embolic stroke is caused by clots that form in one part of the body and then breaks away and reaches an artery in the brain or a vessel leading to the brain. Clot formation can be directly induced by primary traumatic impact and secondary ischemic stroke can occur within hours of TBI. Normal CBF is approximately 50-60 ml/100g/min. In response to clotting, local vasodilatation, redirection of the blood to the collaterals and increased oxygen and glucose consumption try to compensate for the drop in CBF. However, a 60% reduction of CBF disturbs brain metabolism, depresses neuronal activity and induces functional silencing. A CBF of less than 10ml/100g/min results in irreversible neuronal injury. Notably, time is the critical component of the ischemic stroke progression since some cells may survive if interventions are implemented rapidly. The concept of an ischemic penumbra, arising from animal experiments by Astrup and co-authors (Astrup et al. 1977), has formed current approaches to acute ischemic stroke assessment. Accordingly, hypoperfused

tissue may be mapped by CBF thresholds into three regions: i) infarct (core), where irreversible necrosis happens; ii) oligemia - region where cells will survive even without reperfusion; iii) ischemic penumbra that may survive only with prompt reperfusion (Heiss 2000). Actually, as it has been demonstrated in clinical studies, the region with dysfunctional but still viable neurons is relatively large in the acute phase after a stroke (Woodruff et al. 2011). Without proper reperfusion the volume of the ischemic penumbra decreases with a corresponding increase in the size of the infarcted core. Since the publication in 1995 of the results from the National Institute of Neurological Disorders and Stroke trials on intravenous recombinant tPA for acute ischemic stroke (Troke and Roup 1995), the use of tPA has become standard therapy for patients within the first 4.5 hours of stroke onset with a significant improvement in neurologic recovery. The tPA treatment does not affect the rate of mortality and systemic hemorrhages, but intracranial hemorrhage has occurred in 6.4% of the treated patients versus 0.6% in a placebo group. This poses an urgent clinical need to develop individual patient risk assessment. The CT scan is now obligatory to exclude the presence of a hemorrhage that would exclude the use of tPA. In addition, CT angiogram can be used to identify large vessel stenosis or occlusion, MRI – for visualization of possible infarcted areas and prognosis of thrombolysis outcomes and cerebral angiography – for comparing the need for surgical interventions or intra-arterial thrombolysis (Fugate and Rabinstein 2014). Intravenous thrombolysis is not only effective in clinical trials, but has also become a real-world daily practice over the past two decades, although it increases acute therapy costs and requires stroke unit care. In general tPA changes our notion of ischemic stroke victims as hopeless cases and it actually produces economic benefits by reducing societal and health care costs (Johnston 2010).

In summary, the pathophysiology of traumatic and ischemic injury involves a number of mechanisms leading to a broad spectrum of functional deficits. The emergency and early management phase tries to minimize secondary injury by stabilizing the patients' hemodynamics, breathing capability and intracranial pressure, limiting secondary ischemia and hemorrhage and preventing brain edema. The lack of assessment approaches does not allow us to obtain accurate neurological information, hence limiting appropriate patients selections for further existing interventions with approved efficacy. Meta-analysis of

randomized controlled trials confirms the benefits of neurovascular or neuroscience unit care, but still access to them is restricted. As it has been reviewed above, ischemic and traumatic pathophysiological features can appear simultaneously in stroke and TBI patients, and their interactions mainly predict the clinical outcomes. The search during the last decades for a ‘‘magic bullet’’ drug for the treatment of stroke or TBI has totally failed. Taking into account clinical disease pattern, the results of pre-clinical trials and animal research, probably the treatment in stroke and TBI must target several coexistent pathological mechanisms in individual patients.

2. The rodent brain as a model system for ischemic and traumatic injury

Clinical unmet needs stimulate research that can facilitate our understanding of the pathophysiological phenomena of stroke and TBI at the molecular, single cell, network, and organismic levels and the development of new therapeutics to maintain, restore or improve the health of the patients. So far, many of the basic principles and mechanisms underlying pathogenesis, natural compensation and functional deficits have remained unclear. Many of the essential questions can not be addressed in humans therefore appropriate model systems are needed.

The ideal model for stroke and TBI research should have a sufficient number of features that reflect the complexity of the human brain, risk factors common in patients and human response to injury. Presently, there are no computerized or *in vitro* models available for this purpose. Rodents, lagomorphs, cats and non-human primates are often used in stroke and TBI related research. Model organisms should not only demonstrate the ability to have injuries similar to those seen in humans, but should in addition be easy to breed, house and maintain as well as to manipulate. In this regard, rats and mice are valuable model systems since more than 85% of their genomes are identical to the human genome (Bejerano et al. 2004). Furthermore, their cerebrovascular anatomies and basic physiologies show remarkable similarities with those of humans (Yamori et al. 1976). Nevertheless, during evolution the brain changes its structure to suit novel functional needs. Thus, the human neocortex shows a dramatic increase in surface area by means of a highly convoluted shape and increased number of cortical columns that apparently provide more powerful computational capacity (Herculano-Houzel

2009). In addition, there are several differences in vascular anatomy between rodents and humans. For example, instead of an anterior communicating artery rats have an azygos anterior cerebral artery formed from two proximal anterior cerebral arteries, they have bigger posterior communicating arteries that are present in both sides, and have 4-5 times more collaterals between their anterior and middle cerebral arteries compared to human anatomy (Lee 1995). Interestingly, there are some differences in cerebral vascular anatomy between different strains which then reflect on animal sensitivity to ischemia. For example, BALB/C mice are more sensitive to MCAO, since the infarct volume is significantly larger compare to C57BL/6 and SV-129 (Majid et al. 2000), BDF and Swiss Webster mice (Barone et al. 1993) since this train have a larger territory supplied by middle cerebral arteries. In models of global ischemia, C57BL/6 is more sensitive than other strains (Yang et al. 1997). As a consequence, results obtained in the model organism should be carefully verified. Moreover, recently it has been recommended to perform experiments with healthy adult male animals and then extent research to female and aged, overweight, diabetic, hypertensive animals which exhibit risk factors well-known to affect long-term outcomes (Fisher et al. 2009). Use of an inadequate model may lead to misinterpretation of the results and the extrapolation from animal models to humans can be unreliable. At present, preclinical research aims to understand the pathogenesis and its relations to the primary impact and injury progression as well as identify the underlining mechanisms and suggest therapeutic management of stroke and TBI by means of *in vitro* and *in vivo* animal models.

2.1. Animal models of stroke

Starting form pioneering work by Hill and coauthors (Hill et al. 1955), a number of animal models have been developed to reproduce different types of stroke and their clinical symptoms. Each model of stroke triggers specific pathological and compensation mechanisms. Therefore, understanding the specific features of each model is essential for interpretation of the results. Here a brief introduction to those most relevant to the present study and frequently used *in vivo* stroke models is given.

2.1.1. Global ischemic models

Global ischemia occurs when cerebral blood flow is fully blocked by cardiac arrest or occlusion of the four major arteries entering to the Circle of Willis (Traystman 2003) and causes ischemic injury in vulnerable brain areas. Global ischemia can be complete and reversible. Complete global ischemia can be induced by euthanasia via 100% CO_2 positive-pressure ventilation or cardiac arrest due to potassium chloride (KCl) injection. They rapidly decrease EEG activity (Cartner, Barlow, and Ness 2007) and can be used to study the onset of the injury and as a reference for end–point histology. There are several reversible global ischemia models, namely, occlusion of four-vessel (4-VO, two vertebral and two common carotid arteries), two vessel (2-VO, only common carotid arteries) and asphyxia cardiac arrest. They have been widely employed to investigate the mechanisms underlying brain damage after transient global ischemia. The 4-VO model was originally developed by Pulsinelli and Brierley (Pulsinelli and Brierley 1979) for awake rats, but it can be also utilized in anesthetized animals. Ten, 20, or 30 minutes of the 4-VO induce a severe reduction of the blood flow (<3% of control) in the neocortex, striatum, cerebellum and hippocampus. After reperfusion the brain experiences hyperemia for 5 - 15 minutes and cerebral hypoperfusion that can last for 24 hours in some brain structures (Pulsinelli, Levy, and Duffy 1982). Neurons have been found to be damaged after reperfusion in the striatum (30 minutes), hippocampus (3-6 hours) and posterior neocortex (1-3 days). The 4-VO has been used in many studies and is well validated. However, the results are highly variable in the 4-VO model due to its surgical difficulty and it has a success rate of about 50-75% (Traystman 2003).

As an alternative approach the two-vessel (2-VO) model is used. This model has been developed by Eklof and Siesjo (Eklof and Siesjo 1972) for characterization of cerebral energy state following incomplete ischemia. In the 2-VO model, both the ischemia and reperfusion are almost immediate, blood flow is decreased to less than 5% of the control in the cortex, thalamus and midbrain and is followed by long-term hypoperfusion (Kågström, Smith, and Siesjö 1983). Regarding our study it is important to mention that one week after reperfusion, neuronal damage has been found in the neocortex of animals exposed only to 4 minutes occlusion (Smith, Auer, and Siesjo 1984). In general, the 2-VO model is similar to the 4-VO, but requires a simpler surgical preparation. In humans global ischemia is not frequent, in most cases stroke affects only 4.5-14% of one hemisphere (Carmichael 2005). Therefore, results from global ischemia models can be directly

extrapolated to patients after a cardiac arrest episode or to a small proportion of stroke patients. However, its relevance to other clinical situations is questionable.

2.1.2. Middle cerebral artery occlusion (MCAO) models

MCAO model has been developed by Robinson and coauthors (Robinson et al. 1975) as a model of focal ischemia that induces a decrease in blood flow by 80% in brain territory supplied by middle cerebral arteries. MCAO results in either a permanent or temporary occlusion. This model has been used extensively due to its relevance to human thromboembolic stroke and thrombolysis (Traystman 2003). Several different types of MCAO models, exist such as cauterization or electrocoagulation via craniotomy (Tamura et al. 1981), photothrombosis (Markgraf et al. 1993), thromboembolic (Zhang et al. 1997) and the most widely used intraluminal filament technique (Longa et al. 1989). MCAO allows experimental access to the concept of an ischemic penumbra. In transient filament MCAO, the striatum experiences severe ischemia and thus it can be recognized as the ischemic core while the neocortex within the MCA territory is a region with delayed neurodegeneration after reperfusion creates an ischemic penumbra (Takagi et al. 1995). The infarct volume following transient filament MCAO is large and relevant to patients group with high mortality rate. The cerebral infarct can be limited to the neocortex in three-vessel occlusion version of MCAO (Yanamoto et al. 1998). The thromboembolic type of MCAO is induced by injection of thrombin that results in clot formation (Zhang et al. 1997; Orset et al. 2007). This model provided unique opportunity to validate thrombolytic treatment safety and efficacy and to study reperfusion injury in clinically relevant conditions. There are several concerns with MCAO models: i) subarachnoid hemorrhage consistently occurs and leads to decreased CBF; ii) hypothalamic damage is present (rarely in humans), when occlusion is 60 minutes or longer; iii) after surgery animals may suffer from difficulties in eating; iv) animals produce complex deficits that prevent specific access to function recovery (MacRae 2011).

2.1.3. Rose Bengal photothrombosis model

The photothrombosis model was developed by Watson and coauthors (Watson et al. 1985) to produce highly localized focal ischemia by the means of a photosensitized dye (Rose Bengal). When irradiated by light through the intact or

thinned skull or through a cranial window the dye undergoes photochemical reaction which leads to production of singlet oxygen that initiates damage of the vascular endothelium and clot formation. The size of ischemic core is highly reproducible and can be adjusted by dye concentration, light intensity and duration (Wang et al. 2010). Many ischemic pathological events seen in other animal models have been also observed after photothrombosis. However, as in the MCAO model there are several concerns with : i) free radicals not only stimulate platelet aggregation, but also directly damage microvasculature and brain parenchyma; ii) it is hard to induce controlled reperfusion, nevertheless spontaneous reperfusion can occur; iii) the ischemic penumbra is relatively small and hard to identify.

2.1.4. Single penetrating arteriole occlusion

Recently, model of targeted insult has been developed by Nishimura and coauthors (Nishimura, Schaffer, and Friedman 2006) to produce three aspects of stroke: i) extravasation due to BBB destruction; ii) vessel rupture to produce hemorrhage; iii) intravascular clot for mini-ischemic stroke. These models utilize nonlinear optics, namely two-photon microscopy, to induce ionization within a microliter volume leading to limited damage. In addition, Rose Bengal photosensitization with a laser light source targeted to selected microvessels on the cortical surface can be used to form an intravascular clot in a single vessel (Schaffer et al. 2006). Penetrating arterioles and veins represent a critical bottleneck in the flow to the cortex. Occlusion of this terminal branch of surface network causes a severe decrease in the blood flow of downstream capillaries and leads to neuronal death within the penetrating vessel territory (Nishimura et al. 2007; Nishimura et al. 2010). It has been shown that the neurodegeneration following occlusion of a single penetrating arteriole in the somatosensory cortex leads to measurable behavioral impairments in rats (Shih et al. 2013). The mini-strokes in rodents appear to be a highly controlled and reproducible model favorably relevant to TIA and silent stroke in humans. Moreover, they match human microinfarcts in geometry and show the ability to induce a local inflammatory response.

2.2. Animal models of TBI

A number of models have been developed to induce brain trauma in rodents. At present, there are several well established models: weight-drop injury, impact acceleration injury, blast injury, fluid percussion injury (FPI), and cortical contusion injury (CCI). However, no single animal model covers the spectrum of events occurring in TBI patients. This section briefly introduces the most frequently used animal models of TBI.

2.2.1. Controlled cortical impact

A controlled cortical impact (CCI) model for focal contusions has been developed by Ligthall (Lighthall 1988) in ferrets and adapted for the mice (Smith et al. 1995) to recapitulate clinical brain injury with skull deformation and related cortical compression. The CCI model utilizes devises that can produce highly controlled time, velocity, depth of pneumatic or electromagnetic impact and deliver it to the brain. CCI requires accurate unilateral craniotomy lying most often between the bregma and lambda; much attention is paid to the dura that should remain intact to meet brain trauma characteristics closely. This model mimics cortical tissue loss, acute subdural hemorrhage, axonal injury, concussion and BBB dysfunction (Smith et al. 1995; Lighthall, Goshgarian, and Pinderski 1990). Neurodegeneration can be observed not only in the cortical region, but also in the hippocampus and thalamus (Hall et al. 2005). The CCI model produces persist deficits in cognitive, motor and sensory functions. There are two main concerns with the CCI model: the nature of damage in the hippocampus and assessment of the functional consequences (Onyszchuk et al. 2007).

2.2.2. Fluid percussion injury

The fluid percussion injury (FPI) model has been developed in rats by Dixon and coauthors (Dixon et al. 1987) to reproduce clinical features of TBI without skull fracture. To produce its primary impact the FPI model utilizes a pendulum to strike the piston of a liquid filled reservoir that result in the generation of a fluid pressure pulse directed to the intact dura of a test animal through a craniotomy (McIntosh et al. 1987). FPI models can be divided into midline, parasagittal and the most frequently used lateral based on the location of the craniotomy. This model replicates several pathophysiological consequences of human TBI such as a combination of focal cortical contusion, diffuse subcortical neuronal injury,

intracranial hemorrhage, brain swelling and progressive grey matter damage (Xiong, Mahmood, and Chopp 2013). There are several disadvantages in the FPI model: it is less controlled than CCI and a small shift in the craniotomy site is associated with marked differences in the outcomes; compared with other models, FPI has a high rate of mortality due to brainstem-compromised prolonged apnea (Cernak 2005).

2.2.3. Penetrating injury

At present, there are few stab-type (Ghirnikar, Lee, and Eng 1998) or penetrating ballistic-like (PBBI) (Williams et al. 2005) animal models of brain injury available to mimic humans open-skull or penetrating TBIs, but none of them is in routine use (Xiong, Mahmood, and Chopp 2013). The PBBI model utilizes a pellet accelerated by a modified air-rifle that hits a probe. This impact results in marked white and grey matter damage, brain edema, cortical spreading depression, inflammation and can result in sensorimotor impairment depending on the trajectory of penetration (Cernak et al. 2014).

2.3. Modeling stroke and TBI *in vitro*

In addition to animal models, there are also a variety of *in vitro* cell culture, acute and organotypic tissue preparations available to model some components of stroke and TBI damage. The *in vitro* models might be useful in the assessment of cellular and molecular mechanisms or for high throughput drug screenings. The key value of *in vitro* experiments is that they allow the design of more appropriate *in vivo* experiments.

2.3.1. Stretch injury

The cell stretch model has been developed by Ellis and coauthors (Ellis et al. 1995) in cultivated astrocytes as a simple, reproducible model of controlled strain-related injuries. This model is extremely interesting when adapted for axonal dissection in the cultured primary neurons. This model not only gives a simulation of aspects axotomy, but also in controlled conditions allowing the study and manipulation of the secondary injury.

2.3.2. Oxygen/glucose deprivation

Oxygen/glucose deprivation (OGD) is a commonly used *in vitro* model for ischemia in mature cortical cultures, and in acute and organotypic brain slices (Newell et al. 1995). This model also simulates reperfusion since glucose-free and anoxic (oxygen is depleted with nitrogen) medium can be replaced with normal culturing medium. OGD typically produces both necrotic and apoptotic neuronal death. It has been shown that Ca^{2+} plays a key role in triggering cell death.

2.3.3. Excitotoxicity

During ischemia, ATP depletion induces neuronal depolarization that results in excessive synaptic release of glutamate and its accumulation in the extracellular space. Typically cultivated mature neurons are transiently exposed (5-10 minutes) to 300μM glutamate and 10μM glycine in Ca^{2+} - containing buffer (Choi 1985). Excitotoxicity as well as OGD induces neuronal death. Necrosis mainly happens in an acute phase due to rapid Na^{+} influx, osmotic imbalance and cell swelling. While apoptosis is delayed, it involves over-activation of NMDA-receptors, which drives the accumulation of Ca^{2+} in neurons and causes mitochondria damage.

2.3.4. Ionomycin

Ionomycin is the Ca^{2+} ionophore that permeates the plasma membrane in a concentration dependent manner. Increased intracellular concentration of Ca^{2+} can mimic glutamate toxicity and induce delayed degeneration by apoptosis (Castillo and Babson 1998).

3. Role of different cell types and extracellular tissues in the rodent brain

The brain is characterized by functional networks of different cells, including neurons and glia, structurally supported by the extracellular matrix. The original concept of brain function relies mainly on neurons wired together as electronic components that convey and compute activity. An increased number of experimental lines of evidence eventually modified our notion of the brain. Now it is obvious that along with neurons there are several cell types and structures maintaining brain function. In pathophysiological contexts, cellular and extracellular components of the brain are considered as the neurovascular unit

(NVU). Initially it was described as a "metabolic coupling" of the regional neuronal activity to blood flow (Harder, Zhang, and Gebremedhin 2002), presently it incorporates all types of neurons and astrocytes, microglia cells, oligodendrocytes, basal lamina, smooth muscular cells, pericytes, endothelial cells and the extracellular matrix (Muoio, Persson, and Sendeski 2014). Each element of the NVU seems to have a specific and active role in adaptations to physiological and pathological stimuli; nevertheless, they are coupled with one another through complex cell–matrix-cell interactions and communication via paracrine factors. The spatial organization of the NVU is still being debated, generally the cortical column or patch is recognized as a single NVU. Our knowledge of each component and their multifactorial interactions is limited (Kowiański et al. 2013), but understanding the NVU is crucial for understanding various diseases.

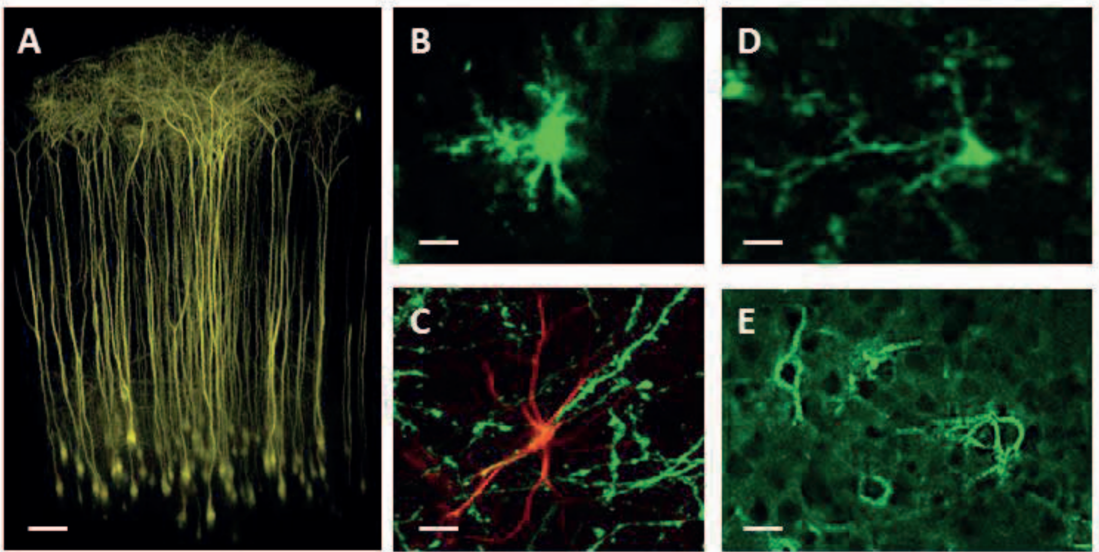

Figure 1. Different types of brain cells. A. Pyramidal neurons (yellow) of cortical layer V sending their apical dendrites to cortical surface in living brain of Thy1-YFP mouse. Scale bar, 50µm. B. Astroglial cell (green) in neocortex of GFAP-GFP mouse. C. Astrocyte stained with anti-GFAP antibodies (red) contacting dendritic spines (green). D. Microglia (green) with ramified process probing its territory in intact cortex of CX3CR1-EGFP mouse. E. Perineuronal nets (green) stained with wisteria floribunda agglutinin (WFA) in cortical sections from adult mouse neocortex showing punctate labelling that surrounds the soma and proximal dendrites of neurons. B.-E. Scale bar, 10µm.

3.1. Neurons

The histological studies of Theodor Schwann, Albert von Kölliker, Franz Nissl, Camillo Golgi, Santiago Ramón y Cajal, Auguste Forel, Alexzander Dogel' and a host of successors have shown that the neuron is the structural and functional unit of the brain. Neurons are highly specialized electrically excitable cells with three distinctive compartments: soma or cell body, axons and dendrites. Neurons in the brain serve to transform incoming information into specific patterns of electrical activity. There are millions of neurons in the central nervous system and they can be classified based on their morphology (Uni-, Bi- or multipolar, anaxomic), type of released neurotransmitter (dopamine-, serotoninergic etc.), electrophysiological properties (fast, burst, regular, late, irregular spiking etc.) or function (On and Off types of visual neurons; sensory, motor or interneurons) (Sharpee 2014). Generally they are divided into glutamatergic excitatory and GABAergic inhibitory neurons, or principal and interneurons. Specific junctions originally called synapses by Cajal allow them to communicate with one another through either electrical or chemical signals (García-López, García-Marín, and Freire 2007). Moreover, neurons can utilize several neuropeptides (substance P, endorphins etc.), gaseous messengers (nitric oxide and carbon monoxide) and lipid mediators (e.g., arachidonic acid, endocannabinoids) to modulate ongoing neuronal activity and information flow.

Assemblies of neurons can be organized into anatomical (for example the basal ganglio-thalamo-cortical circuit) and functional (myotatic reflex) circuits that form the bases for information processing. The connections in neuronal circuits exhibit in divergent, convergent and feedback patterns. Neurons represent the information in the form of action potential patterns over a given time. They combine and integrate information that they receive mostly from excitatory and partially from inhibitory sources. These signals transform either into excitatory or inhibitory post-synaptic potentials, which then interact at the level of single neurons. Integration may take various forms and originally was recognized as the algebraic summation of post-synaptic events. The traditional view of the neuronal computational properties stems from the "somatocentric" perspective where dendrites are recognized as passive cables. Experimentally, neuronal integration has been addressed by measuring somatic membrane potential and the frequency of action potentials initiated in the axon in response to stimuli (Jack, Noble, and Tsien 1975; Koch, Poggio, and Torre 1983). In this concept, a key role in the integration is delegated to the inhibitory inputs that act as a gatekeeper holding

excitation away from the soma. However, there are several lines of evidence showing that inhibitory interneurons have specific domains on the dendritic tree of the principal neurons. In addition, synaptic inputs can be nonlinearly amplified due to voltage-dependent conductance of the dendrites or initiation of local dendritic spikes. At present, the main neuronal decision - action potential generation has been vicarious to preceded and prepared in the dendrites (Sjöström et al. 2008; Branco and Häusser 2010). Remarkable advances in optical and genetic technologies have promoted our understanding of the nature of dendrites, principles of signal computation in the various forms of behavior.

For this study the morphology of dendrites needs to be highlighted. Dendritic trees appear in all shapes and sizes and are highly variable not only in morphology, but also in the molecular composition of receptors, transporters and ion channels. This diversity reflects neuronal function and its connectivity (Migliore and Shepherd 2005). The majority of inputs from other neurons arrive at the dendritic spines, which are a well-studied place in the brain for the plasticity that underlies learning and memory. Since postsynaptic activity depends on the dendritic arbor properties, synaptic plasticity is indirectly affected by spine location in the dendritic tree. For example, inherent properties of dendrites determine the helical ordering of the spines in the Purkinje cells. Apparently, this is needed to maximize the interaction with climbing and parallel fibers as well as with stellate cell axons (O'Brien and Unwin 2006). Notably, dendritic morphology remains relatively static in the adult brain. Nevertheless, the electrical properties of dendritic branches (Sjöström et al. 2008), local translation (Kindler and Kreienkamp 2012) and vesicular trafficking (Hanus et al. 2014) can be modified rapidly in an activity-dependent manner suggesting that there are specific principles of dendritic learning.

3.2. Glia

Glial cells were first described by Rudolf Virchow, later Golgi and Cajal suggested that they provide structural, metabolic and homeostasis supply for neurons. During the last decades, several experimental observations have shown that glia cells undertake many other important functions in the brain (Verkhratsky and Butt 2013). Glia cells are abundant in the central nervous system and according to different estimations the glia to neuron ratio is 0.35 in the neocortex of rodents.

Glia is a common term for non-neuronal brain cell, including astrocytes, oligodendrocytes, microglia, radial glia and NG2 cells. The ratio of major glia cells - astrocytes: oligodendrocytes: microglia - is estimated to be 10 : 5 : 1 in healthy adult rodent brain.

Astrocytes are electrically silent cells that show great morphological diversity, classically, they have been divided into protoplasmic and fibrous subtypes. Other highly specialized subtypes of astrocytes have been characterized, including the Bergmann glia of the cerebellum and the Müller glia of the retina. Probably astrocytes are also diverse functionally (Matyash and Kettenmann 2010). Astrocytes actively participate in cell-cell communication since they are directly in contact with neurons, oligodendrocytes, microglia, endothelial cells and pericytes. These interactions suggest that they are key elements of the NVU. Astrocytes are connected to each other and possibly to oligodendrocytes via gap junctions permeable to Ca^{2+} and other small molecules (Parpura and Verkhratsky 2012). Astrocytes are tightly metabolically coupled with neurons - glucose from the blood is transported into the end-feet of the astrocytes via glucose GLUT-1 type transporters and then metabolized by glycolysis to lactate that can be transfer from astrocytes to neurons in an activity-dependent manner (Magistretti 2011). Astrocytes can affect local microcirculation as well as the properties of the BBB in response to increased neural activity (Takano et al. 2006). The 'tripartite synapse' concept (Araque et al. 1999) suggests that astrocytes can modulate neuronal activity via entrapment of synapse terminals by peripheral processes, thus controlling neurotransmission by active transport (Anderson and Swanson 2000).

Microglial cells are resident monocytes that invade the brain during embryogenesis and serve as professional phagocytes in the brain parenchyma. The microglia is a highly branched type of cell, with fine peripheral processes with a high motility rate in their resting states (Nimmerjahn, Kirchhoff, and Helmchen 2005). Ramified or amoeboid morphology reflects the microglial phenotype, phagocytic and pro-inflammatory, respectively. In contrast to astrocytes, microglial cells do not form durable contacts between each other and are territorially organized, so that each cell occupies distinct territories around 30 - 60 µm in diameter. During brain development, they actively remove apoptotic cells and can be involved in synapse removal during development (Stevens et al. 2007) and probably can participate in synapse pruning during critical periods development in the postnatal brain (Paolicelli et al. 2011). In the adult brain,

microglial cells not only remove cell debris, they can recognize signals which tell them to remove parts of cells, leading to the dendritic arbor remodeling or full removal (Rappert et al. 2004). In addition, microglia phagocytose several molecules such as myelin and amyloid deposits thus contributing to the pathologies of Alzheimer disease and in several demyelinating disorders (Kettenmann et al. 2011). Moreover, microglia can react to acute injury by rapid processes extension towards a lesion site or extravasation from blood vessels, subsequent migration and injury healing (Davalos et al. 2005).

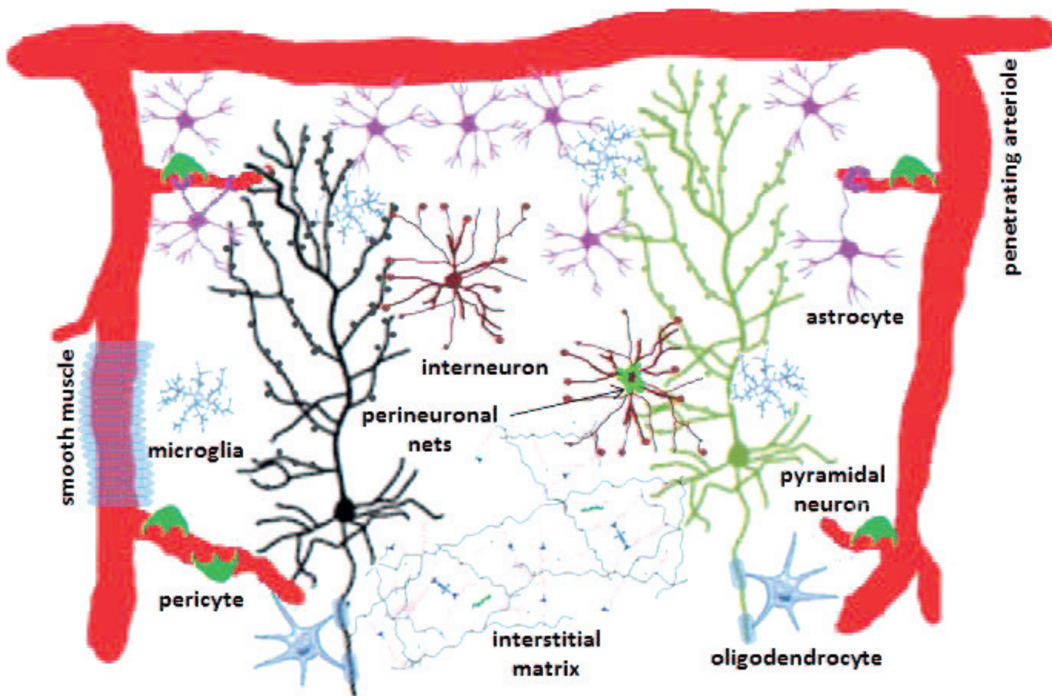

Figure 2. A schematic representation of the neurovascular unit. Cellular components of the NVU: neurons, astrocytes, microglia, oligodendrocyte, pericytes and endothelial cells. The major compartments of the ECM in the brain: interstitial matrix and perineuronal nets. Concept of NVU incorporates role of each element in adaptation to physiological and pathological stimuli.

Oligodendrocytes are mainly myelin-forming cells that extend many processes to several neurons and form multispiral cellular membrane based sheath around axons (Baumann and Pham-Dinh 2001). These structural properties mainly mean they have the same functions as oligodendrocytes. They actively participate in the development and regulation of axons, triggering sodium channels clustering at the

node of Ranvier, and thus supporting rapid action potential propagation. In the adult brain oligodendrocytes metabolically support axons (Morrison, Lee, and Rothstein 2013) and participate in the stability of neuronal circuitry by inhibiting axonal plasticity and growth, furthermore restricting and impairing regeneration after the injury. The dysfunction of oligodendrocytes may cause many neurodegenerative diseases like multiple sclerosis, leukodystrophies like Pelizaeus–Merzbacher disease and demyelinating neuropathies (Bankston, Mandler, and Feng 2013).

3.3. Extracellular matrix

The extracellular matrix (ECM) fills a large, approximately 10-20%, of the brain volume and forms a dense network of proteins and glycans (Ruoslahti 1996) that promote three-dimensional organization of brain cells and control local diffusion properties of brain parenchyma. The ECM is produced intracellularly and secreted almost by all brain cells. During brain development the composition of the ECM undergoes rapid turnover (Meyer-Puttlitz et al. 1995; Rauch 2007) leading to significant changes in its structure. The ECM is a source of paracrine factors that trigger, promote and guide cell proliferation and differentiation as well as the growth of peripheral processes (Bandtlow and Zimmermann 2000). When the active process of brain development is near to completion, the composition of the ECM starts to stabilize. This shifts the function of ECM towards structural maintenance and regulation of NVU plasticity (Dityatev and Schachner 2003). In the adult brain there are three types of ECM: basal lamina, interstitial matrix and the perineuronal nets (PNNs).

The basal lamina is a sheet-like layer that serves as an anchor for the endothelial cells and as a boundary between the blood and brain parenchyma. The basal lamina is secreted and its composition is controlled by endothelial cells and astrocytes. It consists of structural elements, mainly type IV collagens and elastin, specialized proteins such as laminins, entactin, fibronectin and vitronectin, and of proteoglycans like agrin and perlecan (Yurchenco, Amenta, and Patton 2004; Baeten and Akassoglou 2011). Dystroglycan and integrins are the two main receptors/adhesion proteins that form an anchor between the basal lamina and the cytoskeleton of astrocytes and endothelial cell. In addition, these proteins can regulate intracellular signaling pathways and the expression of tight junction

proteins. Thus, the matrix proteins can allow cells to adapt to environmental changes and to modify BBB permeability (Tilling et al. 2002).

The interstitial matrix is likely to be similar in structural organization to the proteoglycan component of cartilage. It contains proteoglycans, hyaluronan, tenascins and link proteins that together form a dense extracellular network (Rauch 2007). Proteoglycans consist of a core protein and negatively charged glycosaminoglycan (GAG) chains of repeating disaccharide units sulfated at various positions. Based on chemical nature of GAGs, proteoglycans are divided into chondroitin sulfate proteoglycans (CSPG), heparan sulfate proteoglycans, dermatan sulfate proteoglycans or keratan sulfate proteoglycans. CSPGs are the most abundant type of proteoglycan and in the brain are found by aggrecan, brevican, neurocan, versican, phosphacan and neuron-glial antigen 2 (Kjellén and Lindahl 1991). Chondroitin sulfate glycosaminoglycan chains are post-translationally added to the protein core and consists of a varying number of disaccharide repeats containing N-acetylgalactosamine and glucuronic acid. In the developing brain chondroitin sulfate chains have been shown to bind the heparin-binding growth factors such as HB-GAM and midkine (Rauvala 1989; Muramatsu 2002; Deepa et al. 2002; Nandini and Sugahara 2006; Sirko et al. 2010). A transmembrane protein tyrosine phosphatase sigma (PTPσ) has been suggested to be a cellular receptor for CSPGs since its Ig-like domain binds with high affinity to chondroitin sulfate –GAGs (Shen et al. 2009; Fry et al. 2010). Alternatively, CSPGs can interact with contactin-1 or modulate integrin activation through indirect interaction (Mikami, Yasunaga, and Kitagawa 2009; Afshari et al. 2010). Although, the direct intracellular mechanism and functional consequences remain unclear, chondroitinase ABC (an enzyme that degrades CSPGs) treatment has been extensively used to demonstrate the key role of the chondroitin sulfate (CS) chains of the CSPGs as inhibitors of regeneration and plasticity in the adult nervous system (Gogolla et al. 2009; Pizzorusso et al. 2006).

The perineuronal nets (PNNs) are composed of the same molecules as the interstitial matrix, but form a condensed layer of mesh-like matrix that surrounds a select subpopulation of neuronal cell bodies, proximal dendrites and probably synapses. The PNNs are often detected by lectin - wisteria floribunda agglutinin (WFA) that selectively labels N-acetylgalactosamines beta 1 residues of glycoproteins (Kwok et al. 2011). WFA-positive staining around neurons can be detected relatively late in the postnatal brain. Experimental results have

demonstrated that PNNs stabilize synapses and prevent the damage of neurons by free radical and buffer ions (Dityatev and Schachner 2003). Typically in the adult brain PNNs are associated with GABAergic parvalbumin interneurons that strongly contribute to functional plasticity and learning (Donato, Rompani, and Caroni 2013). Thus PNNs seem to be critical regulators of adult brain plasticity.

4. Mitochondria

Mitochondria are essential organelles in eukaryotic cells. They are involved in metabolism of amino acids, lipids, steroids, heme and iron-sulfur clusters, calcium and redox homeostasis and they act as gatekeepers of cell life and death by controlling apoptotic signaling pathways. The process of discovery and identification refers to earliest intracellular structural studies during the middle-1800s that describe so called “granules” in the different cell types. In 1898, Carl Benda coined the term Fadenkorper (German) or mitochondria from the Greek μίτος, "thread", and χονδρίον, "granule" that arises from their presence during spermatogenesis (Ernster and Schatz 1981). Ideas of mitochondria structure were originally based on Janus Green B staining introduced by Leonor Michaelis in 1900. However, the real beauty of mitochondrial nature was recognized in 1952 with the pioneering electron microscopy images of mitochondria (Palade 1952; Sjostrand 1953). Important mitochondrial functions within the cell have been described by many researchers for over a century. Albert Lehninger and Eugene Kennedy discovered that mitochondria are the site of oxidative phosphorylation (OXPHOS) (Kennedy and Lehninger 1948) and Peter D. Mitchell described how the diffusion of hydrogen ions across membranes is related to ATP production during respiration in his chemiosmotic theory (Mitchell 1961).

Unlike many other intracellular organelles, mitochondria and plant plastids are not synthetized *de novo* and are only built up from progenitors by fission. The endosymbiotic theory suggests that this is due to the initial capture of free-living aerobically respiring prokaryotic organisms by primitive cells. Mitochondria through their evolution as intracellular symbionts established efficient self-regulating energy production and in delegated to their host cells transcription and translation of the majority of their proteins (Henze and Martin 2003). More than 99% of mitochondria proteins (> 1000) are nuclear-encoded, possibly due to gene

transfer that happened during evolution, but still mitochondria have their own circular double-stranded genomes that contain approximately 16,600 base pairs and 37 tightly packed genes: 22 tRNAs, 2 rRNAs and 13 structural proteins for OXPHOS (Anderson et al. 1981). In fact, mitochondrial DNA (mtDNA) encodes the protein synthetic machinery and the remaining extremely hydrophobic 13 subunits of the oxidative phosphorylation machinery.

Mitochondria are double-membrane organelles and compose of the outer and inner mitochondrial membranes (OMM and IMM), the intermembrane space (IMS) and matrix. OMM has some similar features with plasmalemma, like protein : lipid ratio - 1:1; it contains porins, such as voltage-dependent anion channels, which makes it permeable to small molecules such as adenine nucleotides, creatine phosphate, cations, coenzyme A and a number of other metabolites as well as to tRNA and peptides up to 3 kDa, the diffusion of which is driven by concentration gradients. The OMM contains protein machinery that controls mitochondrial shape, their motility and signaling between the mitochondria and the rest of the cell. The OMM and IMM communicate with each other at contact sites that have been well known for a long time from electron microscopy data, but their functional role and molecular composition are still a subject of ongoing research (Reichert and Neupert 2002; Horvath et al. 2015). The IMM has some prokaryotic membranes properties like a high 4:1 protein:lipid ratio and cardiolipin enrichment (approximately 20% of the total lipids). The IMM has a larger surface area than the OMM due to cristae formation via infolding or invagination of the IMM (Mannella 2006). The electron transport chain is found in the IMM and it pumps protons across the IMM towards the IMS, generating an electrochemical gradient – the driving force for ATPase synthase that phosphorylates ADP to ATP. Thus, the IMM is the actual site of the OXPHOS but it is IMS dependent. According to high resolution electron tomography, the IMS can be structurally subdivided into two compartments: the peripheral IMS and the intracristae space. They are connected via small connections formed by cristae junctions (Herrmann and Riemer 2010). The mitochondrial matrix, the organelle’s “cytoplasm”, is a unique biochemical environment within a cell which carries out key metabolism reactions. It contains mtDNA, ribosomes and plenty of enzymes that are often organized into metabolons (Clarke and Masters 1975; Kurganov and Lyubarev 1988) permitting the oxidization of substrates or *de novo* synthesis in a cyclic manner (Scheffler 2001).

Mitochondria have a sophisticated system of membrane transporters since they actively exchange different ions and metabolites. Moreover, the bulk of mitochondrial proteins are encoded by genomic DNA and translated in the cytosol. The protein import is a bottleneck for adequate mitochondria function. Most mitochondrial proteins are synthesized as pro-proteins with 20-40 amino acid N-terminal targeting sequence, which contains a positively charged amino acid motif (Omura 1998; Habib, Neupert, and Rapaport 2007). Thus, mitochondrial protein import and insertion into the IMM require a mitochondria membrane potential that activates the channel protein and has an electrophoretic effect on mitochondria targeted proteins. The targeting signals allow proteins to be recognized by cytoplasmic molecular chaperone and directed and sorted into the appropriate import pathways and specific mitochondrial compartments. In the experimental setting, construction of hybrid protein sequences are often delivered into mitochondria protein of interest, for example green fluorescent protein. The protein sequence is fused with a mitochondrial targeting signal derived from the subunit 8 of human cytochrome c oxidase (COX-8) (Rizzuto et al. 1995), rarely from E1 alpha pyruvate dehydrogenase and Mn-superoxide dismutase for matrix targeting or from the C-terminus of glycerol-phosphate dehydrogenase (Porcelli et al. 2005) for mitochondrial intermembrane space targeting. The import pathways of mitochondrial proteins are only partially understood (Endo and Yamano 2010). Nevertheless, all of them utilize ATP molecules and require membrane potential, therefore protein import can occur only in healthy mitochondria, this point is important for the present study (Paschen and Neupert 2001; Neupert and Herrmann 2007). Any defect in protein import may lead to mitochondrial disorders like deafness-dystonia syndrome (Binder et al. 2003), Mohr-Tranebjaerg syndrome (Bauer et al. 1999) and Jensen syndrome (Tranebjaerg et al. 2001).

4.1. Mitochondria life cycle and metabolism

Mitochondria regulate their own semi-autonomous “life-cycle” both in dividing cells and post-mitotic neurons. This involves three main stages: biogenesis, fission/fusion and mitophagy. The number of mitochondria varies between different cell types and reflects their actual metabolic consumption (Moyes and Hood 2003). Initial observations (Lewis and Lewis 1915; Biesele and Tobioka 1956) and live cell microscopy studies in the 1980s (Johnson et al. 1981; Chen 1988;

Farkas et al. 1989) remarkably changed our understanding of the mitochondria since they demonstrated the complex dynamics of this fascinating organelle.

It is hard to identify the starting point of mitochondria biogenesis since there are formed from the preexisting mitochondria. In mitochondria case, biogenesis can be defined as division, an increase in number, that is accompanied with growth, an increase in size and mass (Jornayvaz and Shulman 2010). Therefore mitochondrial proteins as well as mtDNA should be expressed and imported replicated in a highly coordinated manner to facilitate the formation of two or more new mitochondria by division of a single progenitor. Upon cell division mtDNAs demonstrate mitotic segregation and approximately five mtDNAs per mitochondrion are randomly split. Mitochondria density within a cell is tightly controlled by several signaling pathways and specific transcription factors, like the peroxisome proliferator-activated receptor gamma coactivator-1 family of transcriptional coactivators (Scarpulla, Vega, and Kelly 2012). Presently, it is also hypothesized that there are some internal mitochondria factors that can trigger biogenesis (Lee and Wei 2005). The site of mitochondria biogenesis is still under debate. However, it is generally assumed that the biogenesis site is located near the cell body since protein import and mtDNA replication have been shown to occur perinuclearly (Davis and Clayton 1996). Moreover, it has been shown that healthy mitochondria in axons predominantly move anterogradely (Miller and Sheetz 2004). However, several experimental lines of evidence propose local mitochondria biogenesis. Thus for example, nuclear-encoded mitochondrial proteins can be translated locally in the axon (Gioio et al. 2001), and replication of mtDNA as well as mitochondria division can occur far away from the soma in distal axon (Amiri and Hollenbeck 2008).

The key point of the mitochondrial division is fission. The protein machinery of mitochondria fission is well characterized and it recruits large versatile GTPases from dynamin superfamily members that are typically involved in membrane scission events (Schmid and Frolov 2011). The main regulator of fission is dynamin-related protein 1 (Drp1). Experimental data suggest that fission starts with accumulation of mitochondrial fission protein 1 on the OMM. Next, it recruits mitochondrial division protein 1 from the cytosol. When this protein becomes membrane-associated, it nucleates the assembly of Drp1–GTP oligomers that form spirals around mitochondria. Finally, GTP hydrolysis of the Drp1–GTP oligomers severs both the OMM and IMM in the classical manner known for

dynamins (Westermann 2010; van der Bliek, Shen, and Kawajiri 2013). It has been demonstrated that Dpr1 self-assembles can be initiated at the positions where the endoplasmic reticulum (ER) contact mitochondria, suggesting an active role for the ER in fission (Friedman et al. 2011). The nature of IMM division during mitochondria fission at present has been only surmised from electron microscopy data and the protein machinery that mediates IMM separation has not been elucidated.

Two mitochondria can join to form a single mitochondrion: this process is called fusion. Although membrane fusion is one of the basic processes that happens in cells, the protein machinery realizing it is quite diverse (Martens and McMahon 2008). In the case of mitochondria, simultaneous fusion of the OMM and IMM is required. Several lines of evidence suggest that mitofusins 1, 2 - membrane-anchored dynamin family GTPases are responsible for fusion of the OMM, while optic atrophy factor 1 (OPA1) is required for IMM fusion (Westermann 2010; van der Bliek, Shen, and Kawajiri 2013). Following fusion, mitochondria solutes, metabolites and proteins are redistributed in the matrix leading to optimization of the mitochondrion's bioenergetics. Furthermore, fusion can be recognized as a protective mechanism that allows the repair of damaged mitochondria by intermixing their components with healthy mitochondria allowing their damaged proteins to be proteolysed and mutant mtDNA repaired.

Mitochondria have a limited lifespan, and it is about 24-30 days in rat brain and 9-10 days in the liver (Gross and Rabinowitz 1969; Menzies and Gold 1971). On one hand mitochondria have essential metabolic function, on the other their dysfunction determines cell death (de Grey 2005; Green and Reed 1998). Damaged mitochondria should be quickly recognized and removed to avoid harmful consequences. Mitophagy mediates the selective removal of damaged mitochondria, moreover it assures the steady-state turnover of mitochondria (Kim, Rodriguez-Enriquez, and Lemasters 2007; Youle and Narendra 2011). Typically, elongated mitochondria undergo asymmetric fragmentation into healthy tubules and small depolarized globules which are suitable for encapsulation. Then, PTEN induced putative kinase 1 (PINK1) recruits cytoplasmic E3 ubiquitin ligase – parkin that actively ubiquitinylates mitochondrial proteins. These trigger mitochondria engulfment by autophagosomes and further fusion with lysosomes for proteolytic degradation (Eiyama and Okamoto 2015). The site of the mitophagy like the biogenesis is still under debate. Some experimental

results suggest that the degradation occurs near the cell soma where the majority of lysosomes are located since depolarized mitochondria in axons predominantly move retrogradely (Miller and Sheetz 2004) and autophago-lysosmes maturate during this translocation (Maday, Wallace, and Holzbaur 2012; Sheng 2014). However, other experiments suggest that mitophagy happens locally due to the arrest of motor proteins (Wang et al. 2011) or even through local transmitophagy, where clustered mitochondria in axons are expelled to neighboring astrocytes and then degraded in lysosomes (Davis et al. 2014).

During the interplay between biogenesis and mitophagy, mitochondria perform well-studied metabolic reactions, including oxidative phosphorylation, Krebs (tricarboxylic acid) cycle, beta-oxidation of fatty acids, heme synthesis and gluconeogenesis. It has been shown that mitochondria function can be modulated through calcium accumulation (Szabadkai and Duchen 2008). Calcium can increase ATP production by enhancing pyruvate uptake and the activity of the Krebs cycle enzymes (McCormack, Halestrap, and Denton 1990) as well as by direct effect on ATP synthase activity (Jouaville et al. 1999). On the other hand, mitochondria can serve as cytosolic calcium sinks since they have a huge capacity to accumulate calcium (Dhalla 1969). Under resting conditions cytosolic calcium concentration is remarkably low (50 nM) due to activity of calcium binding proteins and vigorous Ca^{2+} efflux into the extracellular space and intracellular stores (Ghosh and Greenberg 1995; Gilabert 2012). Mitochondria can take up to 1-5 µM of calcium into the matrix and are able to withhold a 100-fold gradient across the IMM using a mitochondrial membrane potential dependent mechanism (Nicholls and Chalmers 2004). Mitochondria spatiotemporally regulate global and local cytosolic calcium concentration (Tinel et al. 1999). Therefore, they tune one of the key intracellular signaling pathways, this is highly significant for all cells and particularly for neurons since calcium concentration regulates gene expression, controlling vesicle secretion and electrical activity (Brini et al. 2014). Up to now, the specific molecular nature of the mitochondrial calcium uptake remains unknown, although several routes have been proposed (Santo-Domingo and Demaurex 2010; Olson, Chalmers, and McCarron 2012). Calcium can enter into mitochondrial through voltage-dependent anion-selective channels in the OMM and in the IMM through Ca^{2+} uniporter (MCU) (Kirichok, Krapivinsky, and Clapham 2004), high-affinity Ca^{2+}/H^{+} exchangers (LetM1) (Zotova et al. 2010) or uncoupling proteins (UCP2 and 3) (Kapùs et al. 1991; Gunter et al. 2000). In addition, mitochondrial ryanodine receptor type 1 has been speculated to participate in

calcium uptake, although this channel is not found in the IMM (Salnikov et al. 2009). It has also been suggested that several pathways of calcium uptake co-exist in the mitochondria to deal with the different concentrations during cytosolic calcium elevations (Olson, Chalmers, and McCarron 2012; Graier, Frieden, and Malli 2007). In the mitochondria matrix calcium is buffered in a pH dependent manner by the formation of insoluble xCa^{2+}-xPO_4^{x-}-xOH^- complexes (Nicholls and Chalmers 2004). The major route for calcium extrusion into the surroundings from mitochondria is through an xNa^+/Ca^{2+} exchanger that is coupled to the H^+ gradient across the IMM through a Na^+/H^+ exchanger. At present, it is generally accepted that calcium mediates bidirectional signaling aimed to tune mitochondria metabolic performance accordingly to actual needs (Szabadkai and Duchen 2008).

4.2. Mitochondria morphology, dynamics and trafficking

Mitochondria display a very complex architectural organization that is dynamic and varies highly between cell types and within an individual cell as well as under different physiological and environmental conditions. Historically, mitochondria morphology has been studied with conventional transmission electron microscopy that generates two-dimensional (2D) image from a single thin section of an object with a complicated geometry. This approach is imprinted in our mind and we usually think of mitochondria as static organelles with a stable morphology. In addition, it leads to misinterpretation of actual mitochondria morphology since in most cases it is impossible to accurately trace a single mitochondrion. In the last decades, serial block-face scanning electron microscopy and electron tomography have permitted 3D reconstruction of mitochondria (Perkins et al. 1997; Mannella 2006). Recently, cryo-electron tomography has made it possible to overcome artifacts induced by fixation, dehydration and staining (Costello 2006). Furthermore, live cell imaging and super-resolution optical techniques have increased our comprehension of mitochondrial complexity and dynamic behavior.

Mitochondria at can elongate or shorten in their resting state. Morphological changes in mitochondria are dynamically controlled by constantly ongoing fusion and fission. In a single cell mitochondria can be presented both as small globules and short tubes that are functionally independent and electrically isolated from each other and as large interconnected filamentous networks (Park et al. 2001; Collins et al. 2002). It has been suggested that large mitochondrial networks are

beneficial for oxidative phosphorylation and calcium buffering (Skulachev 2001; De Giorgi, Lartigue, and Ichas 2000). The key members of the fusion/fission machinery have been briefly described above. Several protein modifications like phosphorylation, sumoylation, s-nitrosylation, O-glycosylation and ubiquitylation have been proposed to regulate the proteins of the fusion/fission machinery, thus affecting mitochondria morphology (Knott et al. 2008). Disruption of this machinery leads to neurodegenerative disease like Charcot-Marie-Tooth subtype 2A peripheral neuropathy (Sajic 2014), optic atrophy (Alavi and Fuhrmann 2013) and strongly contributes to amyotrophic lateral sclerosis (Bruijn, Miller, and Cleveland 2004), Huntington (Costa and Scorrano 2012), Parkinson’s (Scarffe et al. 2014) and Alzheimer's diseases (Readnower, Sauerbeck, and Sullivan 2011; Schon and Przedborski 2011; Lin and Beal 2006). The fission/fusion equilibrium directly reflects current metabolic and homeostatic needs. Unbalanced fission leads to mitochondria fragmentation whereas disturbed fusion results in mitochondria elongation (Karbowski and Youle 2003).

There is a growing number of indications suggesting a bidirectional relationship between mitochondrial morphology and function (Picard et al. 2013; Wang, and Yoon 2015). For example, OXPHOS inhibition by rotenone (Frank et al. 2012), antimycin A (Barsoum et al. 2006), 3-nitropropionic acid (Liot et al. 2009) and chemical uncouplers (Ishihara et al. 2003) induce mitochondrial depolarization that triggers the cleavage of OPA1, resulting in disrupted mitochondrial morphology (Ishihara et al. 2006). Furthermore, mitochondria can elongate in response to starvation or rapamycin-induced autophagy (Gomes, Di Benedetto, and Scorrano 2011; Rambold et al. 2011). Thus, mitochondrial morphology indicates the energetic states of cells (Lyamzaev et al. 2004; Benard et al. 2007). On the other hand, mitochondria alterations introduced in cells by knock-down or overexpression of fission/fusion proteins affect key mitochondrial functions. Cells lacking mitofusin 2 have reduced mitochondria membrane potential, lower oxygen consumption and less active OXPHOS. In contrast, overexpression of the same protein significantly improves mitochondrial metabolism (Pich et al. 2005). Knock-down of OPA1 or Drp1 have a similar mitochondrial phenotype as in mitofusin 2 with a lower mitochondrial membrane potential and decreased respiration rates, although its overexpression does not affect mitochondria (Chen, Chomyn, and Chan 2005). Blockage of fission leads to decreased mitophagy and results in accumulation of damaged mitochondria that start to overproduce reactive oxygen species and induce oxidative stress (Parone et al. 2008). Notably,

when fission and fusion are blocked simultaneously mitochondrial morphology remains intact. However, reduced respiration can still be observed (Okamoto and Shaw 2005). Different mitochondrial morphologies and the ability of mitochondria to rapidly alter their morphologies have generated much speculation (Friedman and Nunnari 2014). When comparing experimental results from mitochondrial experiments, it is extremely important to control experimental conditions and be careful in the extrapolation of data from yeast to mammalian cultivated cells and further to *in vivo* situations.

The cytosolic localization of mitochondria is not random; they move where higher ATP amounts are required or where cytosolic Ca^{2+} elevations need to be regulated. Mitochondria move rapidly from one cellular region to another in a complex surveillance-like fashion, and their movement can be accompanied with fission/fusion events (Twig et al. 2010). Mitochondrial motility is mediated by motor proteins stepping along cytoskeletal elements (Morris and Hollenbeck 1995). Three large super-families of molecular motors have been identified: kinesins – for anterograde movement toward the (+)-end of microtubules; dynein - for retrograde toward (−)-end; and myosins – for movement along actin. Mitochondrial anterograde transport mostly relies upon kinesin superfamily protein 5 (KIF5) motors that attach through the Milton adaptor protein and the Miro OMM receptor in *Drosophila* (Sheng 2014). In mammals, there are Trak1 and Trak2, instead of Milton, that can mediate both KIF5- and dynein-driving bi-directional transport of mitochondria (van Spronsen et al. 2013). In addition, there are Miro1 and Miro 2; each containing GTPase and Ca^{2+}-binding (EF-hands) motifs. In parallel to the Miro/Milton complex, other proteins have been shown to associate with kinesins and mitochondria: syntabulin, FEZ1 and RANBP2 (Lovas and Wang 2013).While mitochondria bind to KIFs via adaptors, for dynein it is not entirely clear which (if any) adaptors are involved. Although mitochondria are able to travel along actin, little is known about actin motors or their mitochondrial adaptors. The exact mechanism of the transition between immobility and transport remains unknown. Presently, it has been proposed that Miro at sites of high intracellular calcium promotes mitochondrial detachment from microtubules (Da Silva et al. 2014; Sheng 2014; Lovas and Wang 2013).

Mitochondria morphology has particular features in neurons since they are post mitotic cells that have highly distinctive compartments. Mitochondria in neurons are involved in the biosynthesis of neurotransmitters, regulation of intracellular

Ca^{2+} signaling, acting as a sink and storage of Ca^{2+}, and they are important for synaptic communication and neuronal plasticity (Chan 2006; Mattson, Gleichmann, and Cheng 2008). In dendrites and axons mitochondria are usually orientated longitudinally and may be several microns long. Pioneer electron microscopy studies described mitochondria with “extraordinary length” - up to 9 µm in the dendrites of adult cat neocortex (Pappas and Purpura 1961). In contrast, mitochondria in axons are usually much shorter and non-significantly differ from somatic mitochondria in length. Notably, mitochondria form really long filaments as it has been demonstrated in cultivated fibroblasts. Their lengths can be there as long as 50 µm (Amchenkova et al. 1988). Serial electron microscopy of hippocampal slices from adult rats and ground squirrels and subsequent 3D reconstructions of dendrites and mitochondria revealed that mitochondria are present in filamentous forms of at least 36 µm in length (Popov et al. 2005). In contrast to dendritic processes, the mitochondrial population of the axonal processes in the same hippocampal regions have been shown to exist only in the form of discrete bodies, no longer than 3 µm in length (Popov et al. 2005). From what have been seen in vitro primary cultivated neurons, mitochondria in dendrites are more generally believed to be present only as discrete bodies. The dynamic nature of mitochondria and the remarkable complexity of dendrites and axons have highlighted several fundamental questions and opened up a new era of mitochondria research in neuroscience. Where the focus is no longer kept on metabolism and homeostasis *per se*, but on mitochondria as part of an integrated subcellular system. Adaptation and transfer of our knowledge regarding mitochondria to the *in vivo* situation is an ongoing mission in many laboratories.

5. Mechanisms of ischemic and traumatic brain injury

The brain is a highly metabolically active organ which requires a constant supply of oxygen and glucose to maintain its function. Early observations of the mechanisms of ischemic and traumatic brain injury focused mainly on biochemical and physiological changes. They revealed that primary impact accompanied with circulation interruption results in: i) inhibition of the electron transport chain in mitochondria and decreased cell level of ATP (Reichelt 1968; Broniszewska-Ardelt

and Jongkind 1971; Yatsu, Lee, and Liao 1975; Siesjö and Ljunggren 1973); ii) acidosis due to accumulation of lactate and markedly decreased intra- and extracellular pH (Ljunggren, Norberg, and Siesjö 1974; Siesjö 1988; Smith, von Hanwehr, and Siesjö 1986); iii) losses of intracellular potassium and a large influx of calcium into cells (Prenen et al. 1988; Dienel 1984; Hossmann and Grosse Ophoff 1986; Yanagihara and McCall 1982; Hansen 1985); iv) release of glutamate and excitotoxicity (Choi 1985; Hahn, Aizenman, and Lipton 1988; Meldrum and Garthwaite 1990; Olney and Sharpe 1969; Novelli et al. 1988; Palmer et al. 1993; Vespa et al. 1998; Faden et al. 1989); v) increased free fatty acids and arachidonic acid, activated lipid peroxidation and free radical production (Yoshida et al. 1984; Flamm et al. 1978; Bazán and Rodríguez de Turco 1980; Majewska, Strosznajder, and Lazarewicz 1978; Kuwashima et al. 1978; Bazán 1976); and vi) cytokine synthesis and inflammation (Garcia and Kamijyo 1974; Yamasaki et al. 1995; Minami et al. 1992). Moreover, it has been demonstrated that initial pathogenesis and non-selective loss of all cell types happens in the core or focal tissue and can spread further and increase damage to penumbral or perifocal tissue (Memezawa et al. 1992; Hossmann 1994; Astrup, Siesjö, and Symon 1981). Metabolic stress and ionic perturbations are generally accepted major contributors damage seen in cerebral ischemia and brain trauma. During the last decade, it has become clear that pathogenesis is far more complex than has been previously suggested. Subsequent research has shown the action and interaction of many factors contributing to loss of cellular integrity and tissue destruction. Below the specific aspects of pathophysiological events which occur following ischemic and traumatic brain injury are described.

5.1. Cellular and microenvironment events in infarct of ischemia and brain trauma

Studies *in vitro* and in laboratory animals allow us to identify the key reasons for energy production failure. In ischemic core or trauma foci, interstitial pO_2 and glucose metabolic rates are found to be near zero leading to ATP and phosphocreatine depletion (Folbergrová et al. 1992). Tissue anoxia or severe hypoxia (15–10 mm Hg pO_2) is the main initial consequence of ischemic and traumatic brain injury (Rose, Neill, and Hemphill 2006). These conditions immediately affect neuronal functioning since the majority of ATP is consumed by plasma membrane ATPases which ensure ionic gradients during resting

membrane potential and action potential prorogation (Erecinska and Silver 1989). Typically, the mammalian brain subjected to global or focal injury demonstrates rapid suppression of EEG activity within 10–20 seconds of onset (Hossmann and Olsson 1970; Siesjö 1981; Murphy et al. 2008). Probably this happens as a result of energy failure due to anoxia and hypoglycemia that immediately leads to dissipation of cellular ionic gradients (Hansen 1984; Jiang, Xia, and Haddad 1992; Zandt et al. 2013).

The initial pathophysiological process following ischemic and traumatic brain injury is often called anoxic depolarization. It includes energy failure, loss of electrolyte balance, neuron and glia depolarization and water influx. The parenchymal concentration of ATP decreases dramatically in ischemic core or trauma foci during the first 5 min of injury (Folbergrová et al. 1992) and then during the initial hours stabilizes at 10-20% of the baseline level (Welsh, Marcy, and Sims 1991; Folbergrová et al. 1995; Katsura et al. 1993). Creatine kinase can restore ATP levels from phosphocreatine and ADP. However, it is not efficient and phosphocreatine concentration shows a similar decrease as ATP. Furthermore, glucose that reaches the core or the focal tissue undergoes glycolysis with the production of ATP, thus supporting membrane pumps for a longer time. However, this is accompanied by an increased production of NADH and 10-fold accumulation of lactate causing intra- and extracellular acidosis to approximately pH 6.6 (von Hanwehr, Smith, and Siesjö 1986; Nedergaard et al. 1991). Acidosis reduces ion fluxes through cellular membrane channels (Moody 1984), thus it can be classed as one of the adaptive mechanisms that reduces energy requirement. Cells can tolerate low pH only for several minutes (Hoxworth et al. 1999); prolonged acidosis disturbs cell function and can trigger edema (Yao and Haddad 2004).

The early events are now well-studied in acute hippocampal and cortical slices where anoxic depolarization is mimicked by induction of oxygen/glucose deprivation or ouabain treatment, which inhibits Na^+/K^+-ATPase. Initially, neurons subjected to OGD demonstrate adaptive compensation through increased membrane permeability to potassium ions and suppression of voltage-dependent Na^+ and Ca^{2+}-channels (Tanaka et al. 1997). The cells have increased extracellular concentrations of potassium hyperpolarized membranes that can be detected in electrophysiological recordings as a substantial drop of membrane resistance, reduced cell excitability and firing (Fujiwara et al. 1987; Krnjević and Leblond

1989; Yamamoto, Tanaka, and Higashi 1997). Sooner or later, the activity of Na^+/K^+-ATPase decreases in affected cells and causes loss of ionic gradients across the plasma membrane (Hansen 1984). At this time, an electrochemical gradient drives substantial sodium, calcium and chloride ion influx into the cell resulting in gradual depolarization followed by rapid major depolarization that approaches 0 mV (Rader and Lanthorn 1989; Silver and Erecińska 1990; Jiang, Agulian, and Haddad 1992). Very likely, ion influx occurs through several pathways. Depolarization reverses the operational direction of the Na^+/Ca^{2+} exchanger since it is driven by the electrochemical Na^+ gradient. This together with failure of ATP-dependent Ca^{2+} extrusion causes massive Ca^{2+} uptake resulting in micromolar intracellular levels of this ion (Xie et al. 1995; Stys and Lopachin 1997). In addition, the sodium–potassium–chloride co-transporter 1 (NKCC1) becomes deregulated and contributes to excessive Na^+ accumulation (Sheldon et al. 2004). Moreover, the potassium–chloride co-transporter 2 (KCC2) cannot effectively extrude chloride; thus, its intracellular concentration increases leading to deterioration in the strength of GABAergic hyperpolarization through the $GABA_A$ receptor (Rivera et al. 1999; Kahle et al. 2008). These change neuronal excitability and the excitatory/inhibitory balance. As a result, anoxic injury leads to extracellular accumulation of glutamate, GABA, aspartate, alanine and ammonia (Katayama et al. 1991; Pocock and Nicholls 1998). Increase of extracellular glutamate concentration to toxic levels has been found in human beings, especially after focal parenchymal contusions (up to 50-fold) (Bullock et al. 1998), and in rats during transient ischemia (Benveniste et al. 1984). The following mechanisms of glutamate release in ischemic and traumatic brain injury have been suggested: i) opening of potassium-induced voltage-gated ion channels resulting in intracellular Ca^{2+} elevation (Drejer et al. 1985) or ii) Ca^{2+} release from intracellular stores (Katchman and Hershkowitz 1993) that induces massive exocytosis of glutamate vesicles; iii) the reversed operation of glutamate transporters (Rossi, Oshima, and Attwell 2000).

Presently, it is generally appreciated that extracellular glutamate accumulation and Ca^{2+} overload are essential pathophysiological mechanisms of ischemic and traumatic brain injury mediating a significant portion of the cell damage (Lipton 1999; Andriessen, Jacobs, and Vos 2010; Kostandy 2012). Glutamate is the key excitatory neurotransmitter in the mammalian brain that can activate several ionotropic (NMDA, AMPA, kainate) and metabotropic glutamate ($mGluR_{1\text{-}8}$) receptors with specific intracellular consequences in neurons and glia cells. The

neurotoxin property of glutamate was first shown several decades ago (Lucas and Newhouse 1957; Rothman 1985; Olney and Sharpe 1969). These initial observations eventually formed the basis for the "excitotoxicity hypothesis" where glutamate mediates neuronal damage through neuronal excitation (Rothman and Olney 1986). Subsequent *in vitro* studies have suggested a dose – response relationship; high concentrations immediately cause necrosis whereas low concentrations induce delayed apoptosis (Choi 1985). Furthermore, it has been shown that in hypoxia and hypoglycemic conditions neurons become more sensitive to lower concentrations of glutamate (Kimura, Katayama, and Nishizawa 1999). The excitotoxicity hypothesis has been strongly supported by preclinical evaluation of the glutamate receptor antagonists that demonstrated strong neuroprotective effect and significant reduction of the infarct size in animal models of stroke and TBI (Gill, Foster, and Woodruff 1987; Myseros and Bullock 1995; Rao et al. 2001; Marklund et al. 2006; Durukan and Tatlisumak 2007). Unfortunately, clinical trials have failed to demonstrate any effectiveness of these agents (Muir and Lees 1995; Hoyte et al. 2004; O'Collins et al. 2006; Chacon et al. 2008).

In the ischemic core or trauma foci, the process of glutamate re-uptake is found to be suppressed (Camacho and Massieu 2006). The extracellular concentration of glutamate is strictly regulated by high affinity ATP-dependent sodium-cotransports (Storck et al. 1992; Danbolt, Storm-Mathisen, and Kanner 1992): glutamate-aspartate transporter and glutamate transporter 1, can both be located in neurons as well as in astrocytes. Under conditions of limited energy production these transporters can additionally mediate the release of glutamate since they follow Na^+ gradient (Nicholls and Attwell 1990; Rossi, Oshima, and Attwell 2000). Prolonged accumulation of glutamate in the synaptic cleft and subsequent spillover to extracellular spaces lead to hyperactivation of glutamate receptors and sustained depolarization of plasma membranes removes the voltage-dependent Mg^{2+} block of NMDA receptors (Mayer, Westbrook, and Guthrie 1984; MacDonald, Xiong, and Jackson 2006). It is believed that this ligand-gated cation channel is a major route for excessive Ca^{2+} influx in ischemic and traumatic brain injury (Salinska, Pluta, and Lazarewicz 1989; Faden et al. 1989; Monyer et al. 1992).

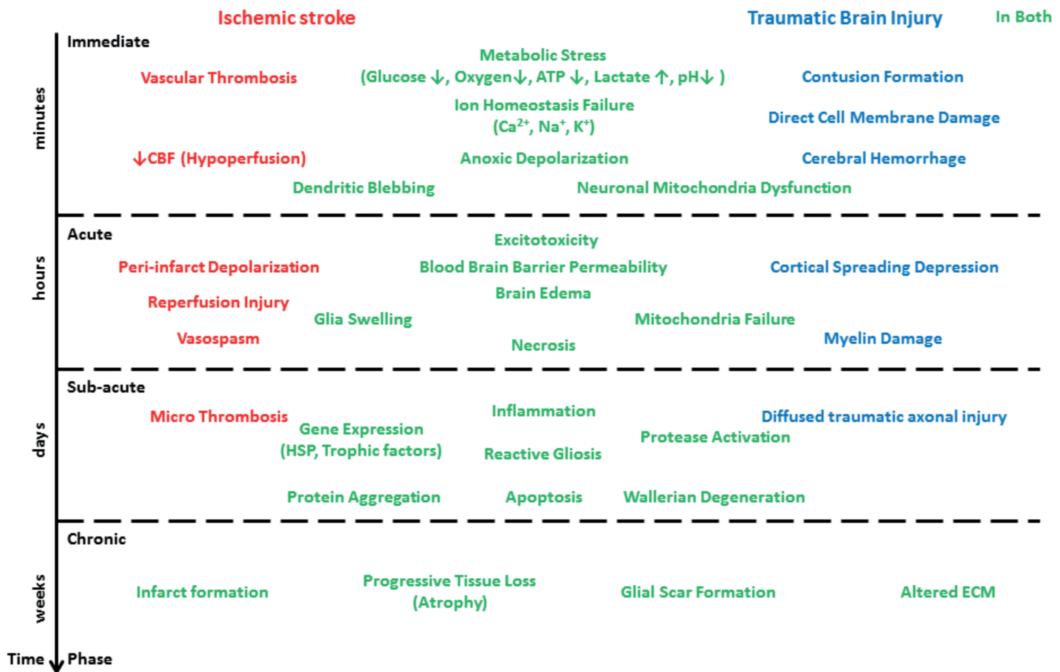

Figure 3. Key pathophysiological events triggered by stroke and TBI. Specific and common (green) features of ischemic (red) and traumatic brain (blue) injury presented during immediate, acute, sub-acute and chronic phases of injury progression. (Modified from (Bramlett and Dietrich 2004)).

The rise in intracellular Ca^{2+} seems to play a major role in linking excitotoxicity with cell death (Choi 1985; Isaev et al. 2008). When Ca^{2+} enters neurons through NMDA receptors, it can directly activate neuronal nitric oxide synthase which starts to produce nitric oxide (Sattler et al. 1999). In addition, it also triggers the activity of membrane phospholipase, protein kinases and cyclooxygenase which contribute to the production of free radicals (Lafon-Cazal et al. 1993) and membrane damage (Won, Kim, and Gwag 2002). Moreover, intracellular Ca^{2+} can be deregulated further by its release from intracellular stores induced via metabotropic glutamate receptor activation (Opitz and Reymann 1991) or Ca^{2+}-induced Ca^{2+} release (Llano, DiPolo, and Marty 1994). Delayed calcium deregulation has been suggested to amplify neuronal death in glutamate-induced excitotoxicity (Alano et al. 2002; Horn et al. 2002; Khodorov 2004; Abramov and Duchen 2008). Interestingly, neurons have differences in glutamate receptor expression and in Ca^{2+} homeostasis regulation which results in specific patterns of Ca^{2+} elevation during and following ischemic and traumatic brain injury leading to

mosaic and selective regional vulnerability (Kirino and Sano 1984; Mitani et al. 1994; Schmidt-Kastner and Freund 1991). It is clear that there are additional pathways by which Ca^{2+} overload may occur in ischemic and traumatized neurons (Zündorf and Reiser 2011). High cytotoxic Ca^{2+} levels have been suggested to overstimulate catabolic enzymes, calpains and other proteases (Yokota et al. 2003; Roberts-Lewis et al. 1994), calcineurins, and endonucleases causing cytoskeletal breakdown (Waxman et al. 1993), DNA fragmentation (Charriaut-Marlangue et al. 1995), mitochondrial (Starkov, Chinopoulos, and Fiskum 2004; Chinopoulos and Adam-Vizi 2006) and endoplasmic reticulum dysfunction (Paschen 1996; Bodalia, Li, and Jackson 2013) and finally cell death. Although numerous events have been identified and characterized at the molecular and cellular levels, a way to stabilize brain Ca^{2+} homeostasis has not yet been found. Further elucidation of interactions of pathological processes in different neural and glial cell types may allow Ca^{2+} signaling to be targeted.

5.2. Cellular and microenvironment events in penumbral and perifocal tissue

Pathology is less drastic in the penumbral and perifocal tissue, although it still leads to infarct. The blood supply to the region is reduced but constant (Dunn et al. 2001), thus energy metabolism is less severely altered - levels of ATP and phosphocreatine are reduced to approximately 50–70% of baseline level. Moreover, PET and autoradiography studies have demonstrated that in the penumbral and perifocal tissue blood flow is lowered, but constant interstitial pO2 is maintained (Liu et al. 2004), whereas glucose metabolism during the first hours is significantly increased (Nedergaard, Jakobsen, and Diemer 1988; Back, Zhao, and Ginsberg 1995; Belayev et al. 1997; Kuge et al. 2001). However, it drops to the same level as in the core after 3.5 hours (Yao et al. 1995). Lactate accumulation has been detected within the penumbra. It is several times lower than in the core regions (Folbergrová et al. 1995) indicating mitochondria dysfunction. The total glutamate content in the tissue is unchanged at the unset of injury, further it is a gradually elevated and reaches a maximum in one day (Stoffel et al. 2002; Bonova et al. 2013). Notably, there are normal ionic gradients and no signs of anoxic depolarization, therefore there is no EEG in this region (Strong, Venables, and Gibson 1983; Zhang et al. 2013). Multiple mechanisms have been identified over the past few decades that are responsible for triggering and continuously

stimulating molecular and cellular events leading to injury spreading from the core to the penumbra (Moskowitz, Lo, and Iadecola 2010).

Initial excitotoxic events, mainly associated with massive release of glutamate and ionic imbalance, have negative impact at the tissue level further promoting ischemic and traumatic injury. During the hours and days following the injury onset repeated vasogenic, cytotoxic edema and anoxic depolarization-like events occur spontaneously in experimental focal cerebral ischemia or TBI, as well as in various human brain injuries (Unterberg et al. 2004; Simard et al. 2007; Hartings et al. 2009; Lauritzen et al. 2011; Z. Bere et al. 2014).

Originally a wave of electrical silence lasting several minutes was described following interruption of the cerebral circulation (Leao 1947). Initiated from the border of the ischemic core or trauma foci (Hartings et al. 2003) anoxic depolarization-like events termed peri-infarct depolarizations (PIDs) propagate through the surrounding brain area that is partially metabolically and functionally compromised (Nedergaard and Hansen 1993; Mies, Iijima, and Hossmann 1993; Fabricius et al. 2006; Oliveira-Ferreira et al. 2010). PIDs are characterized by potential recordings as cortical direct current shifts of around 20 mV propagating slowly at the speed of 3–5 mm/min associated with massive disturbance of membrane ion homeostasis. PIDs may also spread out into healthy brain tissue, where they are recognized as cortical spreading depression (CSD). As described above, anoxic depolarization results in irreversible damage since the affected cells are metabolically arrested and cannot repolarize, leading to cell death within minutes. In cases of cortical spreading depressions, abrupt and deep depolarization is innocuous and can even happen as migraine aura without metabolic compromise (Dreier 2011). Presently, PIDs are proposed to promote cell damage by diminishing available energy (Heiss 2012).

PIDs are suggested to be triggered by high levels of extracellular glutamate and more favorably by high potassium. The slow rate of PIDs propagation indicates that they are probably mediated by chemical diffusion; the underlying mechanism and the diffusion pathway are still under debate (Pietrobon and Moskowitz 2014). PIDs induce transient changes in regional cerebral blood flow ranging from transient hyperemia to transient hypoperfusion (Chuquet, Hollender, and Nimchinsky 2007; Farkas et al. 2011; Lückl et al. 2012; Bere et al. 2014). During the depolarization wave propagation the metabolic rate of the penumbral and

perifocal tissue markedly increases, most likely due to the considerably enhanced energy demands of the activated ion exchange pumps (Somjen 2001). A mismatch between metabolic needs and reduced glucose and oxygen supply leads to phenomenon termed spreading ischemia (Dreier 2011). As it has been reported in animals models of stroke and TBI, 3–4 hours following the onset of injury PIDs promote acute injury to the neuronal circuitry (Risher et al. 2010) and expands the size of the infarct (Takano et al. 1996; Hartings et al. 2003; Hartings et al. 2011). Inhibition of initiation and propagation of PIDs with AMPA and NMDA receptor antagonists are found to be protective *in vitro* and in the animal models (Sakowitz et al. 2009; Eikermann-Haerter et al. 2015), but still cannot be addressed in humans.

Another pathological consequence at the tissue level is development of cerebral edema. Brain swelling following ischemic and traumatic injury can mainly occur with water influx from the vasculature, whereas cytotoxic edema represents a shift of water from the extracellular to intracellular compartments that can be accompanied by shrinkage of the extracellular space. It is generally accepted that cytotoxic edema is induced by an increase in the intracellular concentration of osmotic active solutes (especially Na^+), failure of the active ion-pumps, mainly Na^+/K^+-ATPase and extracellular hypoosmolality (Kimelberg 1995; Unterberg et al. 2004). Although all neuronal and glial cell types swell to maintain osmotic neutrality, the astrocytes contribute most to brain swelling due to unique composition of their plasma membrane transporters (Saadoun and Papadopoulos 2010). In astrocytes water transport is facilitated through specific water channels, aquaporins, which allow osmotic gradient dependent bidirectional water flux (Agre 2006). Aquaporin 4 (AQP4) is the most abundant water channel in the brain (Nagelhus and Ottersen 2013) and is mainly located on astrocytic endfeet surrounding blood capillaries (Papadopoulos and Verkman 2013). Interestingly, AQP4 is often found to be co-localized with Kir4.1 K^+-channels suggesting co-regulation of water and K^+ homeostasis. AQP4 has been shown to be involved in cerebral edema development in various animal models of stroke and TBI (Shenaq et al. 2012; Akdemir et al. 2014; Katada et al. 2014). At present there are no known AQP4 activators or inhibitors, however knock-out mouse lines demonstrate reduced infarct size following ischemia (Katada et al. 2014). Cytotoxic edema not only disrupts neurons and glia cells but also ultimately affects the permeability and structure of the BBB. Vasogenic edema occurs following BBB disruption and this leads to leakage of water that is accompanied by

plasma proteins and electrolytes into the brain parenchyma (Unterberg et al. 2004; Khanna et al. 2014). The regulation of matrix metalloproteinase expression and activation is complex and tightly controlled, and loss of this control has been identified as potentially playing a critical role in the pathophysiology of the BBB in stroke and TBI (Seo et al. 2012; Chaturvedi and Kaczmarek 2014). However, there is still limited knowledge regarding the complex interaction between BBB damage, secondary brain injury and their temporal relationship.

5.3. Neuronal structural changes

Neurons have a complex and highly polarized morphology. Pathological events at cellular and tissue levels affect neuronal morphology. Perhaps the earliest structural changes in the ischemic core or trauma foci can occur within the first minutes of ischemic and traumatic brain injury. Dendrites are particularly vulnerable to injury possibly due to their high concentration of excitatory glutamate receptors. Dendritic injury are seen as “beads-on-a-string”, focal swellings along the dendritic arbor separated from each other by thin segments (Olney, Fuller, and De Gubareff 1979; Ikonomidou et al. 1989; Shengxiang Zhang et al. 2005). These structural changes have been called “blebbing” or “beading” of dendrites to describe a gross structural change and distinguish it from with the localized swelling -“varicose” typical of normal dendrite structures. Presently, dendritic blebbing is a well-studied attribute of neuronal pathology and has been found following different pathologies like ischemia (Hori and Carpenter 1994; Zhang et al. 2005), brain trauma (Castejón 1998; Sword et al. 2013), epilepsy (Wong and Guo 2013) and even in Parkinson’s (Mattila et al. 1999) and Alzheimer’s diseases (Dickson and Vickers 2001; Hall et al. 2000). Dendritic blebbing was first described by Cajal and recognized as a fixation artefact (García-López, García-Marín, and Freire 2007). For a long time the nature of blebbing remain unclear in electron microscopy studies. Morphological abnormalities in dendrites have been observed in cultured neurons (Hasbani et al. 1998; Ikegaya et al. 2001; Park, Bateman, and Goldberg 1996) and in acute brain slices (Obeidat, Jarvis, and Andrew 2000) by the means of Golgi staining and membrane-bound dyes following glutamate treatment and oxygen-glucose deprivation. Finally, blebs were confirmed to be a real morphological changes (and unlikely to be artifacts) in response to pathological stimuli in a series of *in vitro* and *in vivo* studies (Obeidat, Jarvis, and Andrew 2000; Kirov et al. 2004; Andrew et al. 2007; Zhang and Murphy

2007; Brown, Wong, and Murphy 2008; Murphy et al. 2008; Liu and Murphy 2009; Risher et al. 2010) that recruit two-photon microscopy and genetically modified mice that express GFP (Feng, Mellor, and Bernstein 2000), known for its remarkable stability over a wide pH range.

There are several mechanisms proposed to describe dendritic blebbing. The formation of blebs can result from osmotic shock initiated by anoxic depolarization following primary ischemic or traumatic impact. It has been shown *in vitro* during excitotoxicity that hyperosmotic saline can prevent dendritic blebbing whereas an increase the intracellular concentrations of Na^+ and Cl^-, but not of Ca^{2+} (Hoffmann and Dunham 1995), causes fast morphological changes (Greenwood et al. 2007). Structural studies indicate that cytoskeleton perturbations, like loss of microtubule-associated protein 2 (Matesic and Lin 1994), can be accompanied with bleb formation (Purpura et al. 1982; Gisselsson, Matus, and Wieloch 2005). Presently, dendritic blebbing is generally considered as an early feature of neuronal dysfunction that precedes cell death (Takeuchi et al. 2005). Although histological studies demonstrate co-localization of blebs with apoptotic and necrotic markers (Enright, Zhang, and Murphy 2007), the power of dendritic beading as indicator of neuronal cell fate is questionable because of its reversible nature. Alternatively, dendritic blebbing can be recognized as an adaptation or a cellular protective mechanism that localizes the consequences of ion stress and excitotoxicity, thereby protecting the rest of the neurons. However, in this case the surviving neurons would compromised synaptic transmission until they regain their dendritic morphologies as blebs disrupt signal propagation (Li and Murphy 2008).

Cell swelling is also a common feature following ischemic and traumatic brain injury (Steiner et al. 2012; Jayakumar et al. 2014). It is usually accompanied with loss of brain interstitial space. Ultrastructural studies have demonstrated the clumping of nuclear chromatin, mitochondria cristae remodeling, microtubule disruption and ribosome detachment from the endoplasmic reticulum in the swollen cell, preferentially in astrocytes (Jenkins, Becker, and Coburn 1984). Later as cellular damage progresses further (Wei et al. 2004), it is possible to detect in the electron micrographs flocculent densities in mitochondria indicating calcium overload, and also lysosome ruptures that precede catastrophic autolysis and necrosis (Hawkins et al. 1972). However, at the same time astrocytes and microglia cells do not exhibit any features of apoptosis and necrosis, instead

numerous cytoplasmic vacuoles can be found, probably indicating the activation of glial cells (Aggoun-Zouaoui et al. 1998).

Morphological abnormalities such as dendritic blebbing and astrocyte swelling in penumbral and perifocal tissue occur simultaneously with PIDs and CSD propagation (Murphy et al. 2008; Risher et al. 2010; Sword et al. 2013). A minimal level of blood flow appears to be necessary in order to maintain structural integrity and prevent the onset of blebbing; however, ionic stress during PIDs and CSD is sufficient to trigger reversible blebs formation (Murphy et al. 2008; Risher et al. 2010). Notably, several iterations of rapid dendritic morphology recovery after blebbing result in the formation of persistent blebs reflecting the accumulation of PIDs-induced damage. Hence, irreversible dendritic abnormalities can be consider in penumbral and perifocal tissue as an indicator of the final steps during transition towards the infarct core. The reported CSD effects on dendritic morphology in health normoxic tissue are highly variable (Takano et al. 2007), but they do not probably induce long-lasting dendritic injury (Sword et al. 2013).

5.4. Mitochondria dysfunction

Substantial experimental evidences from *in vitro* and *in vivo* animal models of stroke and TBI indicate that mitochondrial dysfunction plays a key role in the pathophysiology of acute neurodegeneration (Kushnareva et al. 2005). Mitochondria dysfunction is typically heterogeneous and can occur both in the acute and sub-acute phases of ischemic and traumatic injury.

Early classic ultrastructural studies (Lehninger 1962; Blondin and Green 1969; Dodson et al. 1974; Garcia et al. 1978; Petito 1986; Pluta and Gajkowska 1984) have demonstrated that mitochondrial morphology is altered following ischemic brain injury. Mitochondria are found to be moderately or severely swollen, many of them exhibit an electron-lucent matrix and some have poorly defined or dilated cristae. Mitochondrial swelling is a hallmark ultrastructural change occurring after cerebral ischemia and it is recognized as the earliest sign of cell damage (Solenski et al. 2002; Radenovic et al. 2011). *In vitro* assessment of mitochondrial swelling by means of light scatter is widely performed in suspensions of isolated mitochondria. Our present knowledge about brain mitochondrial swelling has been derived from observations in suspensions of isolated mitochondria containing both neuronal and glial mitochondria (Malamed 1964; Lifshitz et al.

2003; Nukala et al. 2006; Li et al. 2014). From this type of experimental preparation it has been suggested that swelling has a colloidal-osmotic nature when it is triggered by Ca^{2+}. In addition, it has been shown that mitochondrial volume and shape is regulated by K^{+}-fluxes (Blondin and Green 1969). Changes in mitochondrial morphology is often recognized as one of the most important indicators of the opening of the mitochondrial permeability transition pore (mPTP) (Hunter, Haworth, and Southard 1976). This pore is composed of the inner membrane adenine nucleotide translocator, the outer membrane voltage-dependent anion channel and matrix cyclophilin D. The formed pore is permeable to molecules less than 1500 Daltons and allows these to flow freely between the cytoplasm and mitochondrial matrix. mPTP contributes to the release of apoptosis-inducing molecules and to metabolic failure of mitochondria (Zoratti and Szabò 1995; Halestrap 1989). Induction of the mPTP might transform mitochondria from elongated to rounded structures (Brustovetsky, Li, and Brustovetsky 2009) and disrupt their interactions with other intracellular elements, for example, with the cytoskeleton (Rintoul et al. 2003).

Mitochondria dysfunction is well-studied in excitotoxicity by the means of fluorescence microscopy. The exposure of cortical neurons to glutamate results in a massive intracellular increase of Ca^{2+} (Abramov and Duchen 2008; Pivovarova and Andrews 2010), rapid inhibition of oxidative ATP production, mitochondrial Ca^{2+} overload and depolarization of mitochondrial membrane potential (Starkov, Chinopoulos, and Fiskum 2004; Halestrap 2006). Notably, Ca^{2+} uptake is mediated by a ruthenium-red-sensitive uniporter located in the IMM (Nicholls and Budd 2000) and it is driven by a mitochondrial membrane potential (180mV). Several approaches like mild uncoupling (Shabalina and Nedergaard 2011) have been utilized to manipulate mitochondrial membrane potential in regard to intracellular Ca^{2+} regulation in neurons. Presently, a physiologically relevant therapeutic strategy is a subject of ongoing research. Along with homeostatic and metabolic changes, mitochondria exhibit disruption in motility and morphology during excitotoxicity.

Mitochondrial movement in axons and dendrites have been found to be impaired following Ca^{2+} elevation (Chang and Reynolds 2006). Although 30–50% of glutamate-treated neurons proceed to apoptosis, mitochondria recover their motility in proximal axons within minutes and in dendrites within hours (Rintoul et al. 2003). Mitochondria arrest has been shown to be accompanied by significant

change in mitochondrial shape from an elongated to a rounded/fragmented shape (Isaev et al. 1996; Rintoul et al. 2003; Pivovarova et al. 2004; Greenwood and Connolly 2007; Brustovetsky, Li, and Brustovetsky 2009). Typically, alterations in mitochondrial morphology take the form of fragmentation. This is likely to be a protective response when fragmented mitochondria are utilized by mitophagy or pathological when the OMM is ruptured or the mPTP opens leading to toxic consequences. These live cell microscopy data have been confirmed by ultrastructural studies that demonstrate swelling of mitochondria following glutamate treatment in cortical neurons (Sohn, Kim, and Gwag 1998) and in HT4 cell line derived from hippocampal neurons (Tirosh et al. 2000). There are lines of evidence suggesting that mitochondria can spontaneously undergo reversible remodeling (Shalbuyeva et al. 2006). Glutamate-dependent mitochondria arrest can be mimicked just by blocking OXYPHOS with respiratory chain inhibitors (Rintoul et al. 2003). The remodeling of mitochondrial morphology, such as shortening and rounding, has been reported to be dependent either upon specific Ca^{2+} influx through NMDA receptors (Rintoul et al. 2003; Pivovarova et al. 2004) or upon Na^{+}-driven water influx (Greenwood and Connolly 2007). Moreover, it has been proposed that the collapse of mitochondrial membrane potential can by itself trigger swelling and other ultrastructural changes in mitochondria. In addition, glutamate application to cultivated cells can causes swollen varicosities to form in the cytosol, which could influence mitochondrial morphology (Greenwood et al. 2007). These two events are connected temporally, spatially and functionally *in vitro* following glutamate application. However, the mechanistic details and functional consequences of this phenomenon remain unclear.

Much evidence has been obtained *in vitro* suggesting that the opening of the mitochondrial permeability transition pore (mPTP), which induces matrix swelling with inner membrane remodeling and eventual outer membrane rupture, both result in the release of apoptogenic proteins. The key molecule released from mitochondria is cytochrome c (Li et al. 1997; Green and Reed 1998), which forms an apoptosome complex in association with apoptosis protease-activating factor 1. Further, it activates procaspase-9 followed by proteolytic caspase cascade. Other IMS pro-apoptotic proteins, like Smac/DIABLO and Omi/HtrA2, antagonize inhibitors of caspases, thus facilitating apoptosis progression (Du et al. 2000; Srinivasula et al. 2001; Hegde et al. 2002). Moreover, several proteins released from mitochondria, such as apoptosis inducing a factor and endonuclease G, can

mediate DNA fragmentation and chromatin condensation in a caspase-independent manner (Li, Luo, and Wang 2001). Thus, mitochondria are a hub of apoptosis signaling during excitotoxicity. There are relatively few reports on altered mitochondrial activities following traumatic brain injury. There is general support for the hypothesis generated from the ischemia literature on early mitochondrial alterations is relevant to the pathogenesis of delayed neural cell death and can be directly applied to traumatic brain injury situation (Lifshitz et al. 2003).

Ameliorating mitochondrial dysfunction, and specifically inhibition of mPTP opening, has been suggested as a protective strategy against ischemic and reperfusion injury (Halestrap 2006), although its exact role, contribution to the injury and timing are unclear. *In vitro* studies on primary cultivated neurons suggest that mitochondrial dysfunction is induced by glutamate excitotoxicity and occurs during ischemia onset (Abramov and Duchen 2008). However, there is other evidence for the neuroprotective action of mitochondria protecting component cyclosporin A (CsA), which suggests that mitochondrial dysfunction occurs during reperfusion or after a period of ionic stress (Domañska-Janik et al. 2004).

In animal models of stroke and TBI, mitochondria dysfunction has been manly addressed using end point histological and biochemical approaches (Schinzel et al. 2005; Matsumoto et al. 1999). To the best of our knowledge, no longitudinal studies that monitor neuronal mitochondrial morphology within living brain have been performed during and following ischemic or traumatic brain injury. Previously, less direct methods such as NADH fluorescence (Mayevsky and Chance 2007) and rhodamine 123 fluorescence (Liu and Murphy 2009) time-lapse imaging have been used to monitor onset and acute changes in mitochondria following ischemia. This approaches allowed to report for the first time the *in vivo* dynamics of mitochondria depolarization with high temporal and spatial resolution as well as to demonstrate the effects of CsA treatment.

Mitochondrial calcium overload and subsequent dysfunction remain to play an important role in the pathology progression and affect post-injury recovery, thus raising interest in this process as a target for novel treatments in stroke and TBI. However, the underlying mechanisms are controversial and their relevance to *in vivo* conditions remains unknown. Future research should be aimed at

understanding when and where mitochondrial dysfunction actually occurs in stroke and TBI, and how it can be rescued.

5.5. Reperfusion injury

In a clinical situation, reperfusion of thrombus-occluded arteries within 1-6 hours after stroke onset can be promoted by the means of tissue plasminogen activator (Khatri et al. 2009). In ischemic stroke cases where thrombolysis is not applicable, spontaneous reperfusion can occur and its rate increases gradually with time. Accordingly, 80% of patients with cortical infarcts exhibit reperfusion within the first 2 weeks after a stroke (Barber et al. 1998; Jorgensen et al. 1995). These clinical situations have been mainly addressed in transient MCAO and 2-VO animal models.

The duration of ischemia is the key factor that determines the outcomes of the reperfusion. If global ischemia lasts for 1-2 minutes or transient MCAO for 2.5-30 minutes and is followed by adequate reperfusion, the cellular metabolism gradually normalizes with subsequent recovery of ion gradients across the plasma membrane (Ekholm et al. 1993; Pedrono et al. 2010). Reperfusion after prolonged global (10-15 min) or focal (30 min, 1h and 2h) ischemia is accompanied by multifocal hypoperfusion, known as a “no-reflow phenomenon” (Ames et al. 1968; Hase et al. 2012; Bai and Lyden 2015). Recently, it has been suggested that pericyte constriction contributes significantly to long-lasting decrease in blood flow as well as BBB disruption following reperfusion (Yemisci et al. 2009; Hall et al. 2014). Blood flow is markedly reduced (30-50% of normal flow) for 6-24 hours after 10 minutes global ischemia, and the brain tissue remains metabolically compromised in this time (Crumrine and LaManna 1991). Even when the supply of oxygen and metabolites returns to their normal states, the process of cell damage continues.

Reperfusion injury is mainly driven by secondary excitotoxicity, vasogenic edema and inflammation. Although extracellular glutamate concentration has been found to reduce rapidly (Benveniste et al. 1984), there are several lines of evidence which suggest delayed glutamate release (Phillis, Song, and O’Regan 1997; Lang et al. 2011). Moreover, administration of glutamate antagonist after reperfusion onset has been shown to prevent ion gradient impairment, CSD and cell death (Sakowitz et al. 2009; Eikermann-Haerter et al. 2015). Reperfusion can

significantly promote mitochondria dysfunction via Ca^{2+} overload and mitochondria fragmentation mediated by the upregulation of the cyclophilin D protein, mitochondrial membrane potential failure or by free radical damage (Li et al. 2012). With blood flow recovery, the immune system is heavily triggered by the release of cytokines and cell death-associated molecular patterns (Lambertsen, Biber, and Finsen 2012). Leukocytes actively influx into the ischemic area and facilitate the inflammatory response. In addition, they can initiate re-occlusion of the vessels thus contributing significantly to reperfusion injury. Furthermore, reperfusion can cause microhemorrhages and vasogenic edema since endothelial cells, pericytes and astrocytes are injured or killed during the ischemia episode (Heiss 2012). Thus, even after successful thrombolysis or recolonization, pathological processes can continue and lead to poor outcomes.

5.6. Gliosis

The ability to repair brain damage following ischemic and traumatic injury mainly determines long-term outcomes. Although the brain has a limited capacity to regenerate, tissue repair and replacement by proliferation and local migration of cells as well as spontaneous dendrite and axon sprouting take place approximately 1 – 2 weeks in animal models of stoke and TBI. However, this process typically fails due to an inhibitory environment promoted by gliosis and chondroitin sulfate proteoglycans (Silver and Miller 2004; Burda and Sofroniew 2014).

The main cell types involved in attempts to quickly restore homeostasis in injured tissue are astrocytes, microglia, and NG2-positive oligodendrocyte progenitor cells (Fawcett and Asher 1999). Furthermore, in injuries with damaged meninges, several connective tissue cells are recruited. Typically, part of the response is referred to as astrocytes activation and called reactive gliosis or astrogliosis (Eng and Ghirnikar 1994; Sofroniew and Vinters 2010). Reactive astrogliosis in the adult brain is generally characterized by morphological changes in astrocytes as well as by upregulation of intermediate filament proteins like glial fibrillary acidic protein (GFAP) and vimentin (Eng 1985; Pekny et al. 1999; Brenner 2014). It has been suggested that propagation of these changes in astrocytes are mediated by signaling via gap junctions (Gangoso et al. 2012) and accompanied by altered expression of many proteins (Rosario Hernandez et al. 2002; Zamanian et al.

2012). In severe injuries reactive astrogliosis is accommodated with hypertrophy of astrocyte processes and expression of another intermediate filament proteins like nestin and synemin (Eliasson et al. 1999). These intermediate filaments are routinely used as astrogliosis markers (Ostergaard and Jensen 2013). Reactive astrocytes react differently to an injury (Milos Pekny and Nilsson 2005; Sofroniew 2009), some become hypertrophic or polarized toward the lesion site while others, preferentially juxtavascular astrocytes (Bardehle et al. 2013), proliferate and migrate to the injury border. Probably, to isolate damaged tissue form the healthy ones, a glial scar is formed from hyperfilamentous astrocytes that are tightly packed. Glial scars are characterized by limited extracellular space and many gaps and tight junctions between neighbor astrocytes that form a physical border (Fawcett and Asher 1999; Silver and Miller 2004).

Reactive astrocytes are known to be a major source of CSPGS following ischemic and traumatic brain injury (McKeon et al. 1991). Several growth factors and cytokines stimulate expression of ECM proteins, like CSPGS, mainly aggrecan, brevican, neurocan, versican and phosphacan (McKeon, Jurynec, and Buck 1999; Jones, Margolis, and Tuszynski 2003), semaphorin 3 (De Winter et al. 2002) and ephrin-B2 (Bundesen et al. 2003). These strongly modify the composition of the interstitial matrix (Fitch and Silver 2008). Altered ECM following ischemic and traumatic brain injury has been implicated as an inhibitory environment which triggers dystrophic end bulb formation on the end terminal of the regenerating axon (Steinmetz et al. 2005; Sharma, Selzer, and Li 2012). Several approaches have been tested *in vitro* and in animal models of stroke and TBI to promote axon regeneration. Some of them try to improve neuron capability in regeneration, others are aimed to remove an inhibitory environment by eliminating the astrocytes that produce them (Moon et al. 2000), blocking CSPGs synthesis, secretion and activity (Logan et al. 1994), or by degradation of chondroitin sulfate chains by chondroitinase ABC treatment (Zuo et al. 1998).

The reactive gliosis and glial scar formation is the natural environment which effectively handles sub-acute pathology and preserves tissue from the speeding injury, thus supporting spontaneous recovery that occurs to some extent (Rolls, Shechter, and Schwartz 2009). Nevertheless, it is accompanied by regeneration inhibitory effects. Proper modulatory interventions that are applied within optimal time windows can be beneficial for broad a spectrum of acute neurodegenerative disorders.

AIMS

The thesis work was undertaken to: i) determine whether there are fine morphological alterations within a living brain during and following ischemic and traumatic injury; and ii) exam novel recovery-promoting strategies aiming to overcome the inhibitory environment of the altered extracellular matrix.

The specific aims of study were:

I. To establish and validate the *in vivo* two-photon microscopy-based on:

- an animal model of acute brain injury (Study I)
- automatic analysis for neuronal mitochondrial morphology (Study IV)
- an approach for awake head-fixed recordings (Study VI)

II. To examine HB-GAM effects on dendrites plasticity/recovery following acute brain injury (Study II)

III. To study mitochondrial dysfunction in animal and *in vitro* models of acute neurodegenerative disorders as promising drug targets (Studies III and V).

METHODS

The detailed descriptions of the methods used in this thesis work are given in the "Materials and Methods" sections of the original publications. All compounds and equipment suppliers, catalog numbers and other information as well as recipes of solutions are indicated in the original publications. A brief description of the methodological approaches is given below.

1. Primary cortical astrocyte cultures (study III)

For intracellular Ca^{2+} and mitochondrial morphology and for their motility imaging experiments, primary hippocampal and cortical astrocytes were obtained from 2-4-day-old Wistar rat pups. Cells were dissociated with enzymatic treatment and plated on pre-coated glass-bottom 35 mm Petri dishes, and then cultured for 1–1.5 weeks. To visualize mitochondria, astrocytes were transfected by means of lipofectamine reagent with plasmids that encode either mitochondria-targeted yellow (mitoYFP) or red (mitoDsRed) fluorescent protein. Next day, DMEM was replaced with the standard extracellular solution, which was continuously perfused during imaging experiments. $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator Fura-2 AM.

2. Treatment and imaging (study III)

To mimic physiological and pathological increases in $[Ca^{2+}]_I$, ionomycin at either 1 µM or 5 µM concentration was applied to astrocytes over 2 minutes. For disruption of microtubules or actin filaments, astrocytes were incubated with 10 µM nocodazole or 2.5 µM Latrunculin B, respectively.

Cultured astrocytes were investigated using (i) a wide-field epifluorescence microscope that was equipped with a 175 W xenon lamp, 60X oil-immersion objective and a CCD camera, or (ii) confocal laser scanning microscope equipped with a 63x water-immersion objective, argon (488 nm) and diode-pumped solid-state (561 nm) lasers. Images were acquired every 10 s in a time-lapse mode. To achieve physiological temperatures and to improve focus stability, the microscope frame and the culture dish mounted on it were maintained at a constant temperature of 37 °C.

3. Experimental animals and surgical procedures (studies I, II, IV, V and VI)

The local authority (ELÄINKOELAUTAKUNTA-ELLA) approved the animal licenses (ESAVI/2857/04.10.03/2012 and ESAVI/11326/04.10.07/2014) that included all the procedures used in this study. The mice and rats were bred and kept in group cages in the certified animal facility of the University of Helsinki and provided with *ad libitum* food and water. Newborn (P2-P4) Wistar rat pups, C57BL/6J mice at ages ranging from 2 to 5 months were used in this study. In addition, the present study utilizes the following commercially available transgenic mouse lines that express fluorescent proteins in:

i) neuronal mitochondria; Thy1-mitoCFP mice (Misgeld et al. 2007);
ii) neurons; Thy1-YFP(H) mice (Feng, Mellor, and Bernstein 2000);
iii) neuronal mitochondria and neuronal cytoplasm; Thy1-GFP(M) × Thy1-mitoCFP mice;
iv) astrocytes; GFAP-EGFP mice (Nolte et al. 2001);
v) microglia; CX3CR1-EGFP mice (Jung et al. 2000).

During operations and all experimental procedures, the mice were allowed free access to room air and were anaesthetized (i.p.) with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). To maintain the depth of anesthesia, physiological parameters were assessed by using a pulse oximeter equipped with a mouse thigh sensor. A heating pad was used to maintain the animals' core temperature at 37 °C. Anaesthetized rodents were prepared for *in vivo* two-photon microscopy by implanting either an acute or chronic cranial window (Holtmaat, Bonhoeffer, and Chow 2009) or by skull thinning (Guang Yang et al. 2010). Two-photon imaging sessions were performed after 21-40 days recovery period following cranial window implantation or during the first week after skull thinning.

4. Immunohistochemistry and Western blotting (study II)

Animals were perfused with chilled PBS and 4% paraformaldehyde (PFA). The brains were then postfixed in 4% PFA overnight at +4 °C. After sinking in 30% sucrose, the brains were frozen at -80°C and 25µm thick coronal sections were cut. The slices were collected and stored at –20 °C in a cryoprotective solution. Double staining with anti-recHB-GAM or anti-pleiotrophin and anti-GFAP antibodies was done on floating sections. Alternatively after postfixation, brains

were embedded in paraffin and 5-7 25µm thick coronal sections were cut. To identify apoptosis, nuclear DNA fragmentation was measured by means of TUNEL staining, preformed accordingly to the supplier's protocol.

For western blotting, blood from cranial vasculature was washed by transcardiac perfusion with chilled PBS. A piece from the cortex was then cut and used for sample preparation. Proteins were separated on SDS-PAGE and blotted to nitrocellulose membranes. The membranes were used to measure the protein levels with anti-recHB-GAM and anti-GAPDH antibodies.

5. *In vivo* two-photon microscopy (study I, II, IV, V, VI)

Animals were imaged with laser-scanning two-photon microscope equipped with a 25X water immersion high NA objective and femtosecond laser. To achieve stable imaging and precise repositioning between imaging sessions, the animals were placed under the microscope by attaching the metal holder to the custom-built frame fixed to a motorized stage. The laser power required for image acquisition was adjusted to the lowest possible level for each animal. One imaging field of XYZ-stacks for large scale images comprises 500 x 500 µm (for high resolution - 165 x 165 µm) in the XY axes. Image stacks were collected with a vertical step of 0.5-3 µm. For time-lapse imaging of *in vivo* mitochondria motility, either a 100 frame movie of a single optical section or a small z-series were acquired every 0.06-2 seconds, or 6 full-depth z-stacks were imaged with a 15 minutes time interval. Cerebral vasculature was visualized by i.v. injections of 100µl 2.5% Texas Red 70kDa dextran. The velocity of blood flow was measured from repetitive longitudinal line scans along the central axis of capillaries or surface arterioles and veins (Nguyen and Nishimura 2011).

6. Traumatic and ischemic models (studies I, II, IV, V)

A new animal model of open head injury was developed and validated in Study I. This prick-injury model was then used to study dendritic damage and regeneration in the cerebral cortex (Study II). For the *in vivo* characterization of the dynamics of neuronal mitochondrial morphology during primary and secondary injury and during recovery, several animal models of stoke and TBI were adapted and accurately followed by two-photon microscopy. Three different injury types of

closed-skull TBI (namely - mild photodamage (MPD), focal laser lesion (FLL) and Rose Bengal photosensitization (RBPS)), ranging from mild to severe, were inflicted inside the somatosensory cortex through a chronic cranial window (Study V). Occlusion of CCAs and cardiac arrest were used to induce reversible (Study V) and irreversible (Study IV) global ischemia, respectively.

7. Image analysis

Images were quantified and processed using the following open-source or commercial software: ImageJ, Imaris, AutoQuant X3, AnalySIS, ImagePro 5.1 and NeuronStudio or Matlab-based BlebQuant and MicroP programs as well as custom macros and scripts.

Prior to analysis of the density of dendritic tufts and the number of apical dendrites in Study II, a surface was generated with Imaris to get rid of the blebbed dendrites and unspecific speckles. The maximal intensity z-projection of YFP-labeled dendritic tuft of cortical neurons was taken within the first 120-180 µm of cortex. Further, images were converted from grayscale to binary and automatically segmented with Otsu's thresholding method. The density of dendritic tufts was calculated as a sum of pixels belonging to dendrites per area of injury site, perilesional area or a remote region. For measurement of the number of apical dendrites, the brightness and contrast of individual z-slices was normalized to a uniform level. Next, we selected and aligned between 7 and 15 sequential sub-stacks. Apical dendrites were identified within each sub-stack using the Imaris 'surfaces' tool. The number of apical dendrites in an individual animal was calculated as an average normalized number of surfaces. The statistical unit - N values refer to the number of independent replications, each of them being performed in a different animal. In Study II, data were presented as mean ± SEM. For statistical analyses, Mann-Whitney-U test was used since the data did not demonstrate normal distribution.

To quantify the general motility and velocity of astrocytic mitochondria *in vitro* during physiological and pathological $[Ca^{2+}]_i$ rises, a sequential image subtraction protocol (Kolikova et al. 2006) and semiautomatic tracking were performed (Study III). In the same study mitochondrial morphology was analyzed with MicroP software (Peng et al. 2011) that automatically classifies mitochondria into six subtypes: small globule, large globule, straight tubule, twisted tubule, branched

tubule, and loops. The ratios of the mitochondrial subtypes were investigated to define the effects of microtubules/actin filaments disruption and different $[Ca^{2+}]_i$ elevations on mitochondria morphology. Data from individual cells were pooled and presented as mean ± SEM. Student's t-test was used for statistical analysis since the data demonstrated normal distribution.

To quantify morphological changes in neuronal mitochondria, a supervised-learning-based method was developed. This method was described in details and validated in Study IV, and it estimates the degree of fragmentation of neuronal mitochondrial populations based on large scale images of cortical tissue. The final analysis of mitochondria fragmentation score was performed only in animals with confirmed injury and without imaging- and surgery-induced artifacts. For statistical analyses, two-tail paired t-test or repeated measures one-way ANOVA followed by Bonferroni's post-tests was used to compare differences in mitochondria fragmentation score across time in each experimental group. F-tests were used to compare variance in the mitochondria fragmentation score across experimental groups at different time points.

RESULTS AND DISCUSSION

Optical methods are actively used to study the structure and function of the CNS. The combination of advanced imaging technologies and fluorescent reporters offers an opportunity to investigate the role of endogenous signaling molecules and subcellular structures in health and disease. In particular, *in vivo* two-photon microscopy (IV2PM) is a unique non-linear optical technology that allows real-time visualization of cellular and even subcellular structures in deep cortical layers of the rodent brain. Several types of cells and organelles can be imaged simultaneously by combining different fluorescent markers.

The concept of two-photon absorption was first described by Maria Goeppert-Mayer in 1931, then Winfried Denk and co-authors invented laser scanning two-photon microscopy and described its principle for imaging biological specimens (Denk, Strickler, and Webb 1990), and further for *in vivo* imaging of neuronal activity (Svoboda et al. 1997). Since then, *in vivo* two-photon microscopy has been intensively recruited in different areas of biology (Fritjof Helmchen and Denk 2005; Shih et al. 2012; Ritsma, Ponsioen, and van Rheenen 2014). These advanced optical techniques and genetic animal models provide a unique opportunity to study and manipulate mitochondria in the intact rodent brain.

1. The role of HB-GAM in dendritic recovery and plasticity following cortical injury
(study I and II)

1.1. The novel animal model of acute brain injury followed by intravital microscopy

Regeneration following injury is mainly restricted by glial scar and altered ECM. Chondroitin sulfate (CS) chains of the CSPGs is a key inhibitory element limiting regeneration and plasticity in the adult nervous system as it has been solidly demonstrated by chondroitinase ABC treatment. The initial observation by Paveliev and co-authors (Study II) revealed that HB-GAM (heparin-binding growth-associated molecule or pleiotrophin) can overcome CSPG's inhibitory effect and promote neurite outgrowth *in vitro*. HB-GAM demonstrated efficiency when coated with aggrecan as well as when added to culture media after primary cortical and hippocampal neurons were already inhibited by the aggrecan matrix.

In Study II, we hypothesized that HB-GAM can enhance CNS regeneration and plasticity since it binds GAGs with nanomolar K_d (Milev et al. 1998) and is highly expressed in the developing brain (Merenmies and Rauvala 1990). To test this hypothesis, we attempted to validate HB-GAM effects on dendritic and/or axonal regeneration and plasticity in animal models of CNS injury. It is well known that HB-GAM actively binds to ECM and it has a long matrix-bound half-life (Dreyfus et al. 1998). Taking into account these considerations, we assumed that in the living brain HB-GAM diffusion will be limited and the current well-established animal models of TBI would not allow us to reach the trauma site efficiently. To address this experimental challenge, we have developed a simple, reproducible and controlled focal model of brain injury that can be combined with chronic cranial window or skull thinning preparations and be followed by two-photon microscopy (Study I).

Our novel open head injury model produces prick-injury by means of stereotaxic targeting of a syringe needle into the cerebral cortex. The prick-injury results in parenchyma concussion, destruction of capillary networks and in elimination of dendrites at the controlled and reproducible volume of the cerebral cortex (Study I). As it was observed with *in vivo* multiphoton microscopy and Thy1-YFP mice, the prick-injury induces dendrite blebbing and formation of dendritic retraction bulbs in the peri-lesion areas. During the first two-three days the injury spreads further and the size of the lesion area increases (Study I). Although the trauma size is relatively small, it activates astrocytes in a substantial cortical volume (1-1.5 mm) that can be seen by GFAP staining (Study II). In addition, this staining also confirmed that prick-injury leads to formation of an inhibitory glial scar.

In Study I, we have estimated potential injuries induced by a thin glass pipet (outlet diameter 20-30 µm) insertion and microinjection. Dendrites expressing YFP under Thy1 promoter did not demonstrate any morphological signs of injury like blebbing or retraction bulbs 3 hours after to the intact somatosensory cortex was given a microinjection of Sulforhodamine-101 dye. Pipet insertion leaves a trace in the brain meninges, thus it can be visualized by a second harmonic generation signal. To quantify delayed cell death induced by microinjection, TUNEL staining was performed 3 days after microinjection of 1.5 µl 0.1M PBS (negative control) or 2M NaCl (positive control) at the rate of 2 to 5 nl/sec. A few cells were found to have DNA strand breaks after the negative control microinjection, in contrast more than 94% of cells were TUNEL “+” in a substantial cortical volume following

high molar salt injection (Figure 4). Further the toxicities of different HB-GAM concentrations (0.1, 1.0 and 10 mg/ml) were tested with TUNEL staining. None of the tested concentrations demonstrated significantly increased numbers of TUNEL "+" cells. Moreover, microinjection of 1.5 µl of 1.0 mg/ml HB-GAM into the injury site did not promote delayed cell death following prick-injury.

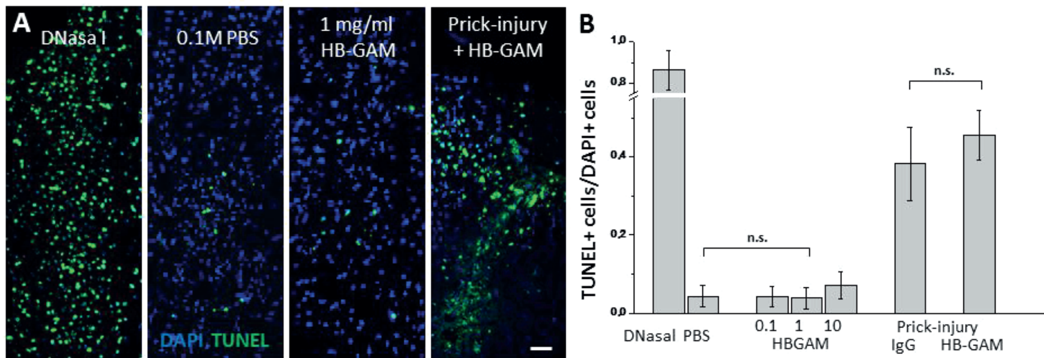

Figure 4. Histological analyses of microinjection-induced injury. A. Cortical sections from adult C57Bl mice were processed for TUNEL staining (green) to mark apoptotic cells at day 3 post-injection or 3 days after the prick-injury followed by microinjection. Brain sections were treated with DNasa I for positive control. DAPI (blue) was used to label cell nucleus. Scale bar, 30µm. B. The fraction of $TUNEL^+$ cells were calculated (mean ± SEM) to identity apoptosis following the microinjection of different HB-GAM concentrations (0.1, 1.0 and 10.0 mg/ml). There was no significant difference in number of $TUNEL^+$ cells between 0.1M PBS and HB-GAM injected animals. In addition, quantification of TUNEL staining did not show significant difference in HB-GAM injected animals after prick-injury compare to IgG injected (1.5 µl of 1.0 mg/ml either HB-GAM or IgG). This data indicates that HB-GAM at tested concentration did not induce apoptosis in cortex of intact mice and HB-GAM microinjection into prick-injury did not promote it further.

Protein delivery into the brain is one of the challenging problems in modern translational neuroscience since the BBB strictly regulates traffic from the blood (Pardridge 2012; Yu and Watts 2013). Presently, several techniques that utilize nanoparticles, exosomes, liposomes (Ramos-Cabrer and Campos 2013; Stockwell et al. 2014), chimeric peptides that promote transferrin receptor-mediated transcytosis (Niewoehner et al. 2014) or focused ultrasound-induced opening (Konofagou et al. 2012) have been developed to facilitate penetration of the proteins through the BBB. Alternatively, proteins can reach the injury site via intrathecal, intracerebroventricular or intranasal delivery since epithelial barriers permit passive transport from the cerebrospinal fluid. It has been demonstrated that proteins diffuse poorly into the brain parenchyma (Aird 1984; Yan et al. 1994) since their concentration gradually decreases in the cerebrospinal fluid due to

bulk flow (Bulat and Klarica 2011) and active clearance (Xie et al. 2013). However, it is hard to estimate how the manipulations required for these techniques will affect the activity of such a small protein like HB-GAM. Microinjection is a unique method that overcomes several disadvantages of the delivery techniques describe above. Direct delivery of substances via microinjection is a well-established way to infuse dyes (Garaschuk, Milos, and Konnerth 2006), small compounds and proteins (Davidson et al. 2010; Gonzalez-Perez, Guerrero-Cazares, and Quiñones-Hinojosa 2010), and to perform genetic manipulations of the targeted cells (Molotkov et al. 2010; Borrell 2010). Furthermore, microinjections provide opportunities to study the local effects of known concentrations of drugs.

In summary, our animal model of prick-injury assesses key features of the complex pathology following traumatic brain injury and can be combined with simultaneous local drug or protein application (Study I).

1.2. HB-GAM promotes robust dendritic recovery *in vivo* (study II)

In Study II, we examined the time course of injected HB-GAM in the brain tissue by means of western blotting. Although, two thirds of injected HB-GAM was found to be cleared within the first day after injury, it stayed as non-fragmented 18 kDa protein at least until post-injury day 7. In addition, western blotting did not show any endogenous production of HB-GAM following prick-injury in the samples from control IgG injected animals. Distribution of injected HB-GAM was detected by immunohistochemistry. The majority of the protein stays at the injury site 3 hours after injection. Notably, at post-injury day 20, injected HB-GAM was found to accumulate in the area of the astrocytic glial scar. Similarly to western blot analysis, no HB-GAM signal was detected at the site of injury after injection of IgG on days 0, 3 or 20.

Several studies indicated changes in HB-GAM expression following ischemia and traumatic CNS injury. It was found to be upregulated in astrocytes, oligodendrocytes, and neurons at days 3 and 7 after spinal cord transection in rats (Wang et al. 2004). In addition, the mRNA and protein levels of HB-GAM were found to increase in the hippocampal reactive astrocytes on day 4 following transient ischemia (Takeda et al. 1995). Another study has indicated upregulation of HB-GAM in areas of developing vasculature following focal cerebral ischemia

(Yeh et al. 1998). In contrast, in our study prick-injury did not trigger HB-GAM endogenous production (Study II).

Dendritic morphology is linked to information processing and in the adult brain it remains relatively static. It is known that brain injury dramatically changes dendritic complexity (Biernaskie and Corbett 2001), thus affecting the neuron circuitry and general brain functioning. *In vivo* two-photon microscopy allowed us to visualize neuronal morphology up to depths 800 µm below the cortical surface. We imaged dendrites in the somatosensory cortex 3 hours, 2 days, 20 days and 40 days following the prick-injury. Analysis of dendritic turf density and number of apical dendrites has revealed that HB-GAM significantly enhanced regeneration and plasticity of dendrites in the injury site as well as in the perilesional area up to 3 weeks after the prick-injury.

Until now, only a few studies have reported structural changes in the dendrites following injury. It has been shown that dendrites exhibit spontaneous regeneration following branch elimination (Sacconi et al. 2007; Allegra Mascaro et al. 2013; Yuryev, Molotkov, and Khiroug 2014) or following focal laser lesion (Study V). These small lesions did not activate astrocytes and initiate scar formation; injured tissue is thus probably effectively removed by microglia dendritic remodeling and there can be free axonal sprouting. In cases of focal ischemia, it has been shown that dendrites become highly asymmetric in the peri-infarct region and turn away from infarction (Brown et al. 2007); these changes persisted over 4 weeks (Brown, Boyd, and Murphy 2010). It was found that chondroitinase ABC treatment enhances regeneration by increasing the numbers of motor and sensory neurons that send their axons and dendrites across the injury site in different animal models of the spinal cord (Bradbury et al. 2002; García-Alías et al. 2009) and brain injury (Harris et al. 2013) as well as in ischemic stroke (Hill et al. 2012; Soleman et al. 2012; Gherardini, Gennaro, and Pizzorusso 2015). Although chondroitinase ABC treatment promotes structural plasticity, in some cases there are no functional outcomes (Harris et al. 2010) or significant effects require specific training (García-Alías et al. 2009). Anti-Nogo-A immunotherapy also enhances plasticity in the CNS by modulation of the inhibitory environment, and displays effective functional regain as a delayed treatment in animal models of CNS injury (Gonzenbach et al. 2012) and cerebral stroke (Tsai et al. 2011).

In Study II, we have demonstrated a novel strategy to enhance plasticity and regeneration, which is an alternative to chondroitinase ABC treatment and anti-Nogo-A immunotherapy. Our approach modifies the CSPG matrix through the use of HBGAM and promotes neural regeneration and functional gain.

2. Mitochondria following ischemic and traumatic injury

2.1. Ca^{2+}- dependent changes in astrocytic mitochondria *in vitro* (study III)

Astrocytes regulate actively neuronal functions via glutamate uptake and potassium clearance from synapses. Mitochondria in astrocytes not only provide ATP, but also significantly contribute to Ca^{2+}-signaling, which is well-known for its role in cell-cell communication and in the regulation of neuroactive factor release, and the glutamate-glutamine shuttle (Stephen, Gupta-Agarwal, and Kittler 2014).

In Study III, we observed mitochondria motility and morphology in primary cultivated astrocytes during physiological and pathological $[Ca^{2+}]_i$ rises. Mitochondria in astrocytes *in vitro* were found to be highly mobile in the resting conditions and to interact with microtubules and actin filaments. Mitochondria were more aligned with microtubules and their disruption strongly restricted mitochondria motility. In contrast, depolymerization of actin filaments did not affect either the morphology of mitochondria or their motility. Our results demonstrated that during physiological $[Ca^{2+}]_i$ elevation astrocytic mitochondrial motility was temporarily and partly arrested. To become “anchored”, mitochondria require intact actin filaments (Study III). Previous studies have shown that rises in $[Ca^{2+}]_i$ trigger mitochondria re-localization or trapping near a Ca^{2+} source at the plasma membrane (Rizzuto, Duchen, and Pozzan 2004; Kolikova et al. 2006; Genda et al. 2011). Recently, it has been suggested that mitochondria motility is regulated by neuronal activity, glutamate transport and Na^+/Ca^{2+} exchange. They can accumulate at glutamate transporter - enriched sites of the astrocytic membrane (Jackson et al. 2014; Ugbode, Hirst, and Rattray 2014).

We also demonstrated actin-independent arrest of mitochondria when they undergo fragmentation in a Ca^{2+}-mediated manner during pathological $[Ca^{2+}]_i$ rise, mimicked by astrocytes treatment with 5 µM ionomycin (Study III). Although mitochondria remodeling or rounding and fission have been reported in different cell types following various of stimuli (Kristal and Dubinsky 1997; Isaev et al. 1996;

Rintoul et al. 2003; Pivovarova et al. 2004; Greenwood and Connolly 2007; Brustovetsky, Li, and Brustovetsky 2009; Hom et al. 2010), the mechanism of Ca^{2+}-dependent remodeling of mitochondria is unclear. It has been suggested that Ca^{2+} can activate remodeling via calcineurin phosphatase which then dephosphorylates Drp1 – the main regulator protein of mitochondria fission (Cereghetti et al. 2008; Cereghetti, Costa, and Scorrano 2010). The role of mPTP in mitochondria Ca^{2+}-dependent remodeling is under debate since cyclosporin A does not ameliorate Ca^{2+}-mediated fragmentation (Tan et al. 2011). Recently, it has been suggested that remodeling of mitochondria morphology following $[Ca^{2+}]_i$ rise mainly happens due to fragmentation, but not via fission and regulation through the ROS-signaling mechanism (Deheshi et al. 2015).

2.2. The novel approach for quantification of neuronal mitochondria fragmentation *in vivo* (Study IV)

Presently, there are only a few *in vivo* studies of mitochondria. Several well-established methods to label mitochondria *in vitro* have been successfully transferred to *in vivo* conditions. A synthetic water-soluble dye Rhodamine 123, known as a probe of mitochondrial membrane potential, was applied to the brain surface after craniotomy by Ran R. Liu and Timothy H. Murphy (Liu and Murphy 2009). It’s loading results in ubiquitous labeling of mitochondria within the superficial cortical layers. This approach allowed them to monitor mitochondria depolarization with high temporal resolution during the onset of ischemia. There are several concerns with Rhodamine 123: the dye needs to be delivered into the brain via topical application or bulk loading through craniotomy, thus it is hard to avoid surgery-induced artifacts; the dye is rapidly (within 1 or 2 days) washed out from the cells, restricting the number and duration of imaging sessions; since the dye is not cell specific, it is impossible to distinguish between neuronal and astrocytic mitochondria.

Recently, transgenic animal lines with mitochondria-targeted fluorescent proteins have become available (Chandrasekaran et al. 2006; Misgeld et al. 2007; Shitara and Shimanuki 2010; Kim et al. 2008; Wang et al. 2011). Axonal transport of mitochondria has been described in acutely exposed peripheral axons in mice during rest and stimulation (Misgeld et al. 2007; Sajic et al. 2013) as well as in a mouse model of amyotrophic lateral sclerosis (Marinkovic et al. 2012). Nikic and

co-authors (Nikić, Merkler, and Sorbara 2011) used mice with CFP-labeled mitochondria in neurons for two-photon microscopy of single mitochondria in experimental autoimmune encephalomyelitis and multiple sclerosis. Moreover, mitochondria structure and function have been imaged in *ex vivo* mice kidney during ischemia–reperfusion injury or drug toxicity (Hall et al. 2012). Furthermore, mitochondria morphology was recorded following application of apoptosis-inducing drugs in transgenic zebrafish (Kim et al. 2008). In addition, longitudinal imaging was performed in “MitoFish” to study mitochondria lifecycle and mitochondria axonal transport in a zebrafish tauopathy model (Plucińska et al. 2012).

In Studies IV and V, we utilized the Thy1-mitoCFP transgenic mouse strain (Misgeld et al. 2007) to study the morphology and distribution of mitochondria labeled with CFP fused to the mitochondria targeting sequence. There are two major technical challenges of mitochondria *in vivo* microscopy in Thy1-mitoCFP mice. The first one is a low z-axis resolution of *in vivo* two-photon microscopy that hinders proper three dimensional (3D) neuronal mitochondria analyses. To handle this in Studies IV and V mitochondria were analyzed in individual optical sections, i.e. in two dimensions (2D). The second one arises from the properties of Thy1-mitoCFP mice. These mice are known for sparse expression of fluorescent protein in the cortex (Crowe and Ellis-Davies 2014). In addition, CFP is three times dimmer than GFP and it emits light with a lower quantum yield and extinction coefficient (Heim, Prasher, and Tsien 1994). Expression patterns and CFP properties require higher laser power and longer exposure times to reach adequate signal-to-noise ratio. Optical zooming, which provides images at reasonable resolutions for mitochondrial morphology analysis, restricts reparative acquisitions since it is accompanied by pronounced photodamage (Study V). To minimize imaging-induced artifacts, neuronal mitochondria image acquisition was adjusted to the lowest possible laser power and the exposure time was reduced by increasing the size of the image limiting time of the laser spot in the same cortical volume. The resulting large scale image contained projections from the cerebral microvasculature and tens of the cells with hundreds of neuronal mitochondria. However, existing methods for the segmentation of mitochondria, intensively used for their morphology analysis in cultured cells (Lihavainen et al. 2012; Peng et al. 2011), could not be in the analysis of mitochondria from large scale images. This was mainly due to low resolution and signal-to-noise ratio, which did not allow accurate segmentation and consequent morphometric analysis.

To overcome the limitations of *in vivo* two-photon microcopy of cortical neuronal mitochondria in Thy1-mitoCFP mice, we developed a novel approach to quantify mitochondria morphology (Study IV). This automatic method estimates the degree of fragmentation of neuronal mitochondrial populations (herein referred to as *a patch*) from large scale images of cortical tissue. To train a model to predict a *fragmentation score*, a supervised learning approach was used. A beta regression model was used (Ferrari and Cribari-Neto 2004) since mitochondrial fragmentation score is expected to be a continuous quantity in the interval (0,1), representing the range from completely intact to completely fragmented mitochondria. As explanatory variables for the regression model, we used morphometric and texture features extracted from the patch. In addition, to cope with the low resolution and signal-to-noise ratio of the large scale images, we utilized a Support Vector Machine classifier (Cortes and Vapnik 1995) to automatically exclude locations where the mitochondrial phenotype is unclear.

During the validation procedure, it was found that the method was as reliable as an expert manual scoring. In addition, synthetic mitochondria data highlighted the minimal requirements of the imaging system for mitochondria fragmentation scoring. Furthermore, the method was shown to be sensitive to changes in mitochondria morphology triggered by irreversible global ischemia in an animal model of cardiac arrest. The results (Study IV) suggested that the method can not only detect changes in neuronal mitochondria morphology, but also, it is able to quantify the degree of the changes.

Automatic quantification of *in vivo* two-photon microscopy data is an urgent problem since data manipulations are complicated and even the analysis of a single experiment can require significant human and computational resources. Previously, the quantification of subcellular organelle and neurites fragmentation has been addressed manually. Presently, automatic analysis of dendritic blebbing has been reported (Chen et al. 2011). This technique quantifies morphological changes in dendrites which is similar to some extent to our case. However, the main difference is that the method relies on comparing an image to a reference image, where the dendrites are assumed to be intact. Since it is well known that mitochondrial morphology varies even between healthy cells, this method is not applicable for mitochondria morphology analysis.

2.3. Mitochondria damage and recovery in animal models of stoke and TBI (Study IV)

In Study V, the developed method for quantification of mitochondrial fragmentation (Study IV) was applied to analyze mitochondria damage and recovery in different models of ischemic and traumatic brain injury. Three different injury types: mild photodamage (MPD), focal laser lesion (FLL) and Rose Bengal photosensitization (RBPS), ranging from mild to severe, were inflicted inside the somatosensory cortex through a chronic cranial window.

MPD was induced in a small volume (typically 165x165x100µm) by exposing the tissue to approximately 50-fold higher light energy than during imaging. MPD resulted in rapid mitochondrial fragmentation in the high-exposure region. Surprisingly, the damage did not extend to the surrounding tissue and recovery of mitochondria morphology occurred only 4 days after MPD. When MPD was applied to Thy1-YFP(H) mice, it did not induce strong morphological changes, like remodeling, blebbing or retraction of apical dendrites in the photodamaged region after 2 weeks, but there was a significant decrease of dendritic spine density two days after MPD and the number of spines regained to their initial level only after day 7. These results emphasized the important role of mitochondria in spine maintenance. Mitochondria renewal was accompanied with normalization of spine density, supporting previous *in vitro* findings suggesting that these two events are closely related (Li et al. 2004; Wilson, Slupe, and Strack 2013).

FLL was produced by targeted laser light eliminating individual branches of the apical dendrites in a controlled volume (typically 50-100 µm3) of somatosensory cortex. FLL resulted in a complete loss of the CFP fluorescence at the lesion site and induced mitochondria fragmentation at the perilesion site. Mitochondria fragmentation probably happened due to ionic stress since some dendritic branches were cut and then one segment degraded whereas others remained connected to the soma but their mitochondria were affected by the extracellular environment. Spontaneous dendritic regrowth into the lesion site occurred over 7-14 days and was accompanied by recovery in mitochondrial morphology. This type of injury has been often used to test microglia activation that rapidly responds with processes extension toward the damaged site (Davalos et al. 2005). Apparently, microglia cells effectively remove debris and promote spontaneous dendritic regeneration.

RBPS was achieved by i.v. injection of Rose Bengal followed by exposure to green light, after which mitochondrial morphology was monitored sequentially in the core and remote areas. RBPS rapidly produced a core with severe mitochondrial fragmentation that did not show any recovery over 3 weeks. Some recovery of mitochondrial morphology was observed after 3 weeks but only in relatively small parts of the remote area. Finally, a dual TG mouse line Thy1-mitoCFP×Thy1-EGFP-M was used to evaluate the temporal relationship between mitochondrial morphology and the dendritic structure during the first 3h after RBPS. Interestingly, structural changes in neurons, such as blebbing, were always observed with some delay after the mitochondrial fragmentation occurred first in response to RBPS. Mitochondria fragmentation can be thus recognized as the earliest sign of neuronal damage.

Additionally, to evaluate neuronal mitochondria morphology sensitivity to ischemia and their recovery following reperfusion, in Study V mice underwent CCAs occlusion either for 5 minutes or for 8 minutes. The occlusion duration was selected based on irreversible global ischemia data from Study IV and according to blood flow impairment. Previous reports maintained CCAs occlusion for 15-30 minutes (Eklof and Siesjo 1972; Pulsinelli, Levy, and Duffy 1982; Smith, Auer, and Siesjo 1984; Terashima et al. 1998), whereas here it was demonstrated that 8 minutes occlusion induced long-term hypoperfusion. In our hands, 8 minutes CCAs occlusion had a dramatic effect on the cortical arteries in ketamine/xylazine anesthetized mice, which led to irreversible fragmentation of neuron mitochondria. Surprisingly, neuronal mitochondria can tolerate brief impairment in blood flow and regain their initial structure within minutes. To our knowledge, there are no other experimental data demonstrating such fast reversible perturbation in mitochondria morphology within a living brain. Earlier studies that utilize the same model demonstrated that structural changes in dendritic morphology happened during propagating wave of ischemic depolarization and $[Ca^{2+}]_i$ elevation (Murphy et al. 2008). Moreover, mitochondrial membrane potential monitored by the means of *in vivo* two-photon rhodamine 123 fluorescence imaging demonstrated for the first time *in vivo* the dynamics of mitochondria depolarization with high temporal and spatial resolution. This method demonstrated the effects of CsA treatment on global ischemia (Liu and Murphy 2009). Our results amplified the picture of the ischemia onset with profound mitochondria fragmentation after the 2nd minute of ischemia induction. Taken together, when mitochondria lose their membrane potential and structural

integrity *in vivo* due to energy demand and ionic stress, there is a narrow time window when restoring energy supply can rapidly reverse mitochondria morphology and function. Notably, from a clinical view point, if emergency interventions can guarantee efficient and rapid reperfusion, neurons can be supported with internal pro-survival resources that allow normalization of the brain's circuit functioning and as a consequence improvement of the recovery processes.

3. Improvement of animal models

3.1. Anesthetic effects on injury progression

A broad spectrum of anesthetics is used in animal models for induction of ischemic and traumatic brain injury. The high variability in the size of the resulting infarct mainly arises from complicated induction procedures that require animal immobilization. Anesthetics are widely acknowledged to introduce pronounced effects in animal experiments since they are well-known to be neuroprotective in a dose-dependent manner (Matchett et al. 2009; Kawaguchi, Furuya, and Patel 2005). For example, halothane and isoflurane can reduce the size of infarction by attenuation of NMDA-mediated excitotoxicity (Miura et al. 1998; Matchett et al. 2009). Moreover, this type of anesthesia can mask the benefits of certain treatments like those recently observed following photothrombotic occlusion of cerebral vessels in fully conscious *versus* isoflurane anesthetized mice (Seto et al. 2014). Anesthesia also affects neuronal and astrocytic activity (Hentschke, Schwarz, and Antkowiak 2005; Kimura et al. 2014; Goltstein, Montijn, and Pennartz 2015; Thrane et al. 2012) which induce widespread alterations in brain metabolism (Choi, Lei, and Gruetter 2002; Mizuma et al. 2010).

A mixture of ketamine/xylazine was utilized in the present study for general anesthesia. Ketamine is a non-competitive antagonist of NMDA receptors that has a neuroprotective effect resulting in the reduction of infarction size in animal TBI and stroke models (Hudetz and Pagel 2010). In intact adult brain ketamine rapidly increases the formation of new dendritic spines and enhances synaptic function (Li et al. 2010). Xylazine is an effective alpha 2 adrenergic receptor agonist that depresses neuronal activity, relaxes skeleton musculature and constricts the arteries (Cabral et al. 1998). Ketamine/xylazine anesthetized rodents demonstrate reduced brain oxygenation that can cause hypoxia, hypercapnia and acidosis

(Wixson et al. 1987; Lei et al. 2001). These concerns of general anesthetics should be taken into account as a major disadvantage of present animal models utilized for preclinical drug research.

Anesthetics are commonly used to minimize movement artifacts during acute and chronic imaging of cells and their subcellular components within living animal. There are growing numbers of laboratories that employ *in vivo* two-photon microscopy to study a wide range of processes in translational and fundamental research (Perry, Burke, and Brown 2012; Mostany et al. 2015). Although the results are highly controlled and biologically relevant, their interpretations should always incorporate the action of anesthetics and anesthesia by itself.

3.2. *In vivo* two-photon microscopy in awake behaving mice (Study VI)

Molecular and cellular probing of neuronal circuitries during cognitive and behavioral tasks can not be performed in anesthetized animals. As cutting-edge experimental advances, *in vivo* microscopy has been implemented for recording from awake behaving rodents (Helmchen et al. 2001; Dombeck et al. 2007). Thus, this could become the most relevant approach that would overcome anesthesia drawbacks and provide high temporal and spatial resolution.

Presently, there are several approaches for two-photon microscopy imaging and patch-clamp recordings that requite head-fixed preparation or miniaturized head-mounted microscope, fiberscope or amplifiers (Fee 2000; Helmchen et al. 2001; Sawinski et al. 2009). The existing solutions for head-fixing preparation range from physical restraint (Fujiwara-Tsukamoto et al. 2012) to spherical treadmill combination with computer-generated virtual reality (Dombeck et al. 2010).

In Study VI, we developed a novel approach for *in vivo* two-photon microscopy and/or patch-clamp recording. A head-fixed mouse moved around flat-floored air-lifted platform and explored its tangible environment under stress free conditions. This approach provided firm head fixation that ensures the mechanical stability and allows the imaging of fine neuronal morphology and even mitochondrial morphology (data not shown) as well as to record single-cell electrophysiological activity in awake animals with the same stability as in anesthetized animals (Study VI). Moreover, with the air-lifted mobile homecage longitudinal studies can be

designed without limitations in duration and frequency of repeated recording/imaging episodes.

Our system provided a new relevant tool for pre-clinical drug research since experimental data obtained with this method are not compromised either by anesthesia or by constrain-induced stress. Overcoming anesthesia drawback could be especially crucial for the improvement of stroke and TBI animal models. Air-lifted mobile homecage was combined with optical imaging of intrinsic signals or fluorescent reporters of intracellular calcium, glutamate and cell membrane voltage. More importantly for translational research, clinically relevant techniques like MRI, ultrasound and high resolution microPET could be combined with our approach to study different aspects of certain diseases and to validate drug candidates' efficacy.

Finally, this approach was combined with complex behavioral tasks, namely habituation-dishabituation and novel smell recognition tasks (Study VI). Although animal movement was restricted to two-dimension, the animals exhibited a wide range of movements including horizontal locomotion, grooming, whisking, licking, nose-poking, skilled front paw movements, and wall touching with their forelimbs. All these movements could be used to report behavioral choice in a similar way as it has been done previously in sensory discrimination (Verhagen et al. 2007), detection (Houweling and Brecht 2008) and virtual navigation tasks (Dombeck et al. 2010). The air-lifted mobile homecage could be also utilized in sensorimotor system studies with a high level of control over both the stimulation conditions and the behavioral read-outs. Furthermore, studies of cognitive abilities in awake mice could be performed during conditioning, spatial navigation, decision-making tasks and social interactions.

There are several concerns with head-fixation preparation in general and with this method, particularly. First, theoretically there is sensory incongruity due to inappropriate activity of the vestibular system during locomotion, however it is hard to estimate its "artificial" contribution to neuronal circuitry activity since the brain should adapt to head-fixation following a reasonably prolonged training/habituation period. Second, the air-lifted mobile homecage provide a simple and small space to navigate, thus it could be difficult to study place cell activity in this environment. Third, visual or sensory stimulation and reward-based experiments would require the design of miniaturized devices that could be

challenging for some applications. In addition, the head-fixation preparation is a time-demanding approach since it requires sufficient time to prepare an animal for the experiment, namely 2-3 weeks of post-operation recovery and 4 days of training.

In summary, we believe that the novel awake animal models could change pre-clinical practice and increase the efficacy of transitional stroke and TBI research. It is expected that this approach will facilitate the biological relevance of studies since it allows us to combine cellular, molecular and behavioral levels of observation and manipulation within a single experiment.

CONCLUSIONS

In this thesis work three novel approaches have been describe and further used to study the fine morphology of neurons and neuronal mitochondria during and following ischemia and traumatic brain injury.

By applying our animal model of acute brain injury (Study I) we were able to demonstrate that, through the use of an HBGAM, inhibitory extracellular matrix, namely CS chains of the CSPGs, can be modulated to enhance regeneration and plasticity of dendrites following cortical prick-injury (Study II).

We studied mitochondria motility and morphology on primary cultured astrocytes and demonstrated that: i) the motility of astrocytic mitochondria is inversely related to $[Ca^{2+}]_i$; ii) mitochondria require intact microtubules for their motility; iii) elevated $[Ca^{2+}]_i$ immobilizes mitochondria by strengthening their interaction with actin filaments; and iv) an actin-independent arrest of mitochondria may also occur during pathological $[Ca^{2+}]_i$ rise when they undergo fragmentation (Study III).

By applying our automatic quantification method (Study IV) we were able to confirm that *in vivo* two-photon microscopy can be utilized for neuronal mitochondria analysis in the mice neocortex. Neuronal mitochondria were found to be extremely sensitive to laser light exposure and to blood flow interruption. Our results identify mitochondrial fragmentation as the earliest ultrastructural change in the neuronal damage cascade, which precedes the gross changes in dendritic morphology. Interestingly, our data suggest that alterations in neuronal mitochondrial morphology can be spontaneously reversible in traumatic and ischemic injuries and therefore highlight mitochondria as a potential target for therapeutic interventions (Study V).

Finally, we developed a novel approach for *in vivo* microscopy and single cell recordings in awake head-fixed rodents that can be combined with behavioral tasks (Study VI). This “awake microscopy” approach can be further combined with quantitative analysis of mitochondrial morphology in traumatic/ischemic injury models (Study V). This powerful combination can initiate a novel era of mitochondria fundamental research within the awake living brain and help to bring translational research to new horizons.

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