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ANU LIPSANEN

Secondary Neuropathology after Experimental Stroke

With Special Emphasis on Calcium, Amyloid-β and Inflammation

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ABSTRACT

One out of every three people will suffer either a stroke or develop Alzheimer's disease (AD). In a world with continuous increase of life-expectancy the burden to healthcare system caused by these two neurological disorders will increase dramatically. It has become apparent that these two diseases share common pathological features, and this knowledge can be used to our advantage in finding new treatments.

Focal cerebral ischemia in rodents is followed by delayed secondary pathology in the thalamus and this involves amyloid-β (Aβ) and calcium aggregation. This thesis has been done to further advance our understanding of the mechanisms behind this secondary pathology after cerebral ischemia and whether this pathology could be modified by the non-steroidal anti-inflammatory drug ibuprofen (study I), the non-selective calcium channel inhibitor, bepridil (II and IV) and the reverse Na^+/Ca^{2+} exchanger inhibitor, KB-R7943 (III).

In study II, bepridil treatment after middle cerebral artery occlusion decreased the amounts of $A\beta_{40}$ and $A\beta_{42}$ as well as calcium levels in the ipsilateral thalamus in rats. The sensorimotor impairment was improved in bepridil treated MCAO animals. The data indicate that bepridil treatment prevents or modifies secondary pathology in the thalamus improving functional outcome.

In study IV, transgenic AD mice were treated with bepridil after photothrombotic cortical lesion. There appeared to be less pronounced primary and secondary pathology in AD mice after ischemic cortical injury. It may be that the underlying AD pathology in the transgenic animals exerted a protective effect against cortical ischemic damage. The calcium pathology in the thalamus was effectively prevented by bepridil treatment.

In study V, common marmosets subjected to transient middle cerebral artery occlusion were followed for forty-five days. The histological evaluation conducted after the follow-up did not show Aβ or calcium aggregates in the thalamus similar to those found in rodents.

The data from studies I and III suggest that chronic ibuprofen or KB-R7943 treatment in rats does not improve behavioral outcome nor prevent secondary pathology in the thalamus after experimental focal ischemia.

Secondary pathology after stroke is an attractive drug target since it has an extended therapeutic time window. Bepridil seems to alleviate the secondary pathology via a non-inflammatory pathway and without interfering with amyloid precursor protein or Aβ cleavage and clearance. Data from these rodent studies indicates that calcium plays a more pivotal role than Aβ in the secondary pathological changes in the thalamus. The results from non-human primates after cerebral ischemia, however, show a complete lack of secondary pathology indicating that this may be a rodent specific phenomenon, which is likely to complicate translation of the data from rodents to humans.

National Library of Medicine Classification: WL 356, QV 276, WD 205.5.A6, QZ 150
Medical Subject Headings: Stroke/pathology; Brain Ischemia; Infarction, Middle Cerebral Artery; Thalamus; Calcium; Calcium Channel Blockers; Amyloid; Amyloid beta-Peptides; Inflammation; Ibuprofen; Bepridil; Sodium-Calcium Exchanger/antagonists & inhibitors; Behavior; Disease Models, Animal

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TIIVISTELMÄ

Joka kolmas meistä sairastuu elämänsä aikana aivoverenkiertohäiriöön tai Alzheimerin tautiin (AT). Keskimääräisen elinajanodotteen kasvaessa myös näiden sairauksien aiheuttama sosioekonominen taakka yksilölle ja yhteiskunnalle kasvaa merkittävästi. Näillä sairauksilla on monia yhteisiä piirteitä, mikä tarjoaa lähtökohtia uusien hoitojen kehittämiselle. Sekä Alzheimerin taudissa että aivoiskemiassa on havaittu esimerkiksi kalsiumin ja amyloidin-beeta:n (Aβ) kertymistä. Aivoiskemian jälkeen nämä muutokset ilmaantuvat viiveellä varsinaisen infarktialueen ulkopuolelle kuten talamukseen.

Tässä väitöskirjatyössä selvitettiin kortikaalisen aivoiskemian jälkeen tapahtuvia sekundaarisia muutoksia ja eri lääkeaineiden tehoa niiden estämisessä. Lääkkeinä käytettiin tulehduskipulääke ibuprofeenia, ei-selektiivistä kalsiumkanavan salpaajaa bepridiiliä tai käänteisen natrium-kalsium-ioninvaihtajan estäjää KB-R7943:a.

Osatyössä II kokeellisen aivoiskemian jälkeen rotille aloitettu bepridiili-lääkitys laski $A\beta_{40}$:n ja $A\beta_{42}$:n sekä kalsiumin määrää talamuksessa. Myös sensorimotoriset käyttäytymistestit osoittivat lääkinnän nopeuttavan rottien toipumista. Tulosten perusteella voidaan olettaa, että bepridiili-hoito joko estää tai hillitsee haitallisia muutoksia talamuksessa ja näin ollen nopeuttaa toiminnallista kuntoutumista.

Osatyössä IV AT-muuntogeenisille hiirille aloitettiin bepridiili-lääkitys valosensitiivisellä aineella aiheutetun kortikaalisen aivoiskemian jälkeen. Tulosten perusteella aivoiskemian aiheuttama kalsiumin ja Aβ:n kertyminen oli AT-hiirillä vähäisempiä kuin villityypin hiirillä. On mahdollista, että Alzheimerin taudille tyypilliset muutokset suojelevat AT-hiiriä aivoiskemian vaikutuksilta. Myös tässä tutkimuksessa bepridiili-lääkitys vähensi myös tässä tutkimusessa kalsiumin ja Aβ:n kertymistä talamukseen.

Osatöiden I ja III tulosten perusteella pitkäaikainen ibuprofeeni- tai KB-R7943- hoito aivoiskemian jälkeen ei estä muutoksia rottien talamuksessa.

Viidennessä osatyössä kortikaalinen aivoiskemia aiheutettiin apinoille. 45 vuorokautta myöhemmin tehty histologinen tutkimus ei osoittanut aivojen talamuksessa sellaisia Aβ- tai kalsiumkertymiä kuin jyrsijöillä on havaittu.

Aivoiskemian jälkeen tapahtuvat sekundaariset muutokset ovat kiinnostava kohde uusille lääkehoidoille, koska ne ilmaantuvat viiveellä ja hoidon aloittamiseen jää enemmän aikaa. Bepridiili näyttäisi vähentävän näitä haitallisia muutoksia. Se ei vaikuta tulehdusprosessiin eikä Aβ:n tai sen esiasteen käsittelyyn. Jyrsijöillä tehtyjen kokeiden perusteella kalsiumilla näyttäisi olevan Aβ:a tärkeämpi rooli talamuksen haitallisissa muutoksissa. Kuitenkin apinoilla tehty työ osoitti, etteivät aivoiskemian jälkeiset muutokset ole kädellisillä samanlaisia kuin jyrsijöillä. Näin ollen kokeelliset tutkimustulokset eivät välttämättä ole suoraan sovellettavissa ihmisiin.

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Yleinen Suomalainen asiasanasto: aivohalvaus; aivoinfarkti; iskemia; talamus; kalsium; amyloidi; tulehdus; kalsiuminestäjät; ibuprofeeni; bepridiili; koe-eläinmallit

"I have always had more dread of a pen, a bottle of ink,

and a sheet of paper than of a sword or pistol."

Alexandre Dumas, the Count of Monte Cristo

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Once there was a girl on a farm, who wanted to show that she could do at least the same things as her brothers (… she was the firstborn…). Lots have happened since, and now she is in the situation where she is obliged to thank all those marvelous people around her, who gave her the opportunity to reach the "final step" in the Finnish educational system. This is truly a miracle.

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Kuopio, March 2014

Anu Lipsanen

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List of the original publications

This dissertation is based on the following original publications:

I Lipsanen A, Hiltunen M and Jolkkonen J. Chronic ibuprofen treatment does not affect the secondary pathology in the thalamus or improve behavioral outcome in middle cerebral artery occlusion rats. Pharmacol Biochem Behav 99(3):468-74, 2011

II Sarajärvi T*, Lipsanen A*, Mäkinen P, Peräniemi S, Haapasalo A, Jolkkonen J and Hiltunen M. Bepridil decreases Aβ and calcium levels in the thalamus after middle cerebral artery occlusion in rats. J Cell Mol Med 16(11):2754-67, 2012.

III Lipsanen A, Parkkinen S, Khabbal J, Mäkinen P, Hiltunen M and Jolkkonen J. KB-R7943, an inhibitor of the reverse Na^+/Ca^{2+} exchanger, does not modify secondary pathology in the thalamus following focal cerebral ischemia in rats. Submitted

IV Lipsanen A, Flunkert S, Kuptsova K, Hiltunen M, Windisch M, Hutter-Paier B and Jolkkonen J. Non-selective calcium channel blocker bepridil decreases secondary pathology in mice after photothrombotic cortical lesion. PLoS One 8(3):e60235, 2013

V Lipsanen A, Kalesnykas G, Pro-Sistiga P, Hiltunen M, Vanninen R, Bernaudin M, Touzani O and Jolkkonen J. Lack of secondary pathology in the thalamus after focal cerebral ischemia in non-human primates. Exp Neurol 248:224-227, 2013

* Equal contribution

Throughout the thesis, these papers will be referred to their Roman numerals.

Contents

Abbreviations

AD	Alzheimer's disease
ADP	adenosine diphosphate
AICD	APP intracellular domain
AMPAR	α-amino-3-hydroxy-5 -methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
APP	amyloid precursor protein
ATP	adenosine triphosphate
Aβ	amyloid beta
BACE	β-secretase
BBB	blood-brain barrier
CBF	cerebral blood flow
cDNA	complementary DNA
CNS	central nervous system
CTF	C-terminal fragment
DNA	deoxyribonucleic acid
EMEA	European medicines agency
ER	endoplasmic reticulum
ET-1	endothelin-1
FDA	Food and Drug Administration
GA	golgi apparatus
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde-3 -phosphatase dehydrogenase
GFAP	glial fibrillary acidic protein
GGA3	golgi-localized γ-ear-containing ARF binding protein 3
GP	globus pallidus
$hAPP_{SL}$	human APP expressing mouse strain
IDE	insulin-degrading enzyme
IP_3R	inositol triphosphate receptor
LRP	low-density lipoprotein receptor-related protein
LTCC	l-type calcium channel
MCAO	middle cerebral artery occlusion
MMP	matrix metalloproteinase
mNCX	mitochondrial NCX
MRI	magnetic resonance imaging
NCS	neuronal calcium sensors
NCX	sodium/calcium exchanger
NEP	neprilysin
NMDAR	N-methyl-D-aspartate receptor
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drug
OX-42	anti-integrin αM antibody, clone OX-42
PCR	polymerase chain reaction
PMCA	plasma membrane calcium ATPase
PSEN-1	presenilin 1
PSEN-2	presenilin 2
qPCR	quantative PCR
RNA	ribonucleic acid
ROS	reactive oxygen radical
RTN	reticular thalamic nucleus
RyR	ryanodine receptor
sAPP	soluble APP
SERCA	sarco/endoplasmic reticulum calcium ATPase
SOCE	store-operated calcium entry
SPCA	secretory-pathway calcium-ATPases
STAIR	Stroke Therapy Academic Industry Roundtable
STEPS	Stem Cell Therapies as an Emerging Paradigm in Stroke
TNF-α	tumor necrosis factor alpha

tPA	tissue plasminogen activator
TRPM2	transient receptor potential cation channel, subfamily M, member 2
TRPM7	transient receptor potential cation channel, subfamily M, member 7
VDCC	voltage dependent calcium channel
VLa-VLp	ventral lateral thalamic nuclear complex
VPL	lateral ventroposterior nucleus
VPM	medial ventroposterior nucleus
VPN	ventroposterior nucleus (VPL+VPM)

1 Introduction

In global terms, in a single year, there are over 16 million strokes and almost 6 million people die because of a stroke [1]. In addition, stroke is one of the leading causes of disability. By the year 2030, it has been estimated that 10.3 % of all deaths will be caused by a stroke and the incidence rate will have increased by a three million further. Certainly, stroke is one of the most costly global wide non-communicable diseases. One can also estimate, that the cost of stroke today in the developed world (266 billion to 1038 billion USA dollars) will double by the year 2030 due to the aging of the population [2].

Alzheimer's disease (AD) is the most common type of dementia; it is characterized by neuronal loss and the typical amyloid-β (Aβ) pathology. The death or malfunction of neurons causes changes in memory, behavior, and the ability to think clearly. These neuronal changes eventually impair an individual's ability to carry out even basic functions such as walking and swallowing [3]. It is estimated that currently around from 24 to 36 million people around the world have dementia, in most cases AD, and this number is going to double every 20 years. Sixty percent of dementia patients live in developing countries; this proportion will increase to more than 70 % by 2040. The current evidence suggests that vascular factors, such as midlife hypertension and cerebrovascular disease, contribute significantly to the development of dementia and Alzheimer's disease [4,5]. Worldwide, the annual economic cost of dementia has been estimated as 315 to 604 billion USA dollars [6,7].

One out of six people will suffer a stroke during their lifetime. One in three individuals will suffer either stroke or dementia [8]. Therefore, it seems that not only can the two pathologies occur together, but they also may interact. Several studies have shown that AD and ischemic brain injury leads to altered amyloid precursor protein (APP) processing [9,10], Aβ accumulation [11–14], and increased neuroinflammation [15]. In both diseases, impaired calcium homeostasis has been demonstrated in multiple studies [16]. Neurological deficits following acute cerebral infarction are associated with not only primary injury but also with the secondary damage in remote loci linked to the infarction site [17–21].

In view of the numbers of deaths, disability and cost attributable to these neurological disorders, and the fact that these disease entities may interact, a treatment focused on the common neuropathology would be extremely beneficial. This thesis focuses on understanding the secondary pathology after experimental stroke involving pathology typical to AD.

2 *Review of the literature*

2.1 ISCHEMIC STROKE

Blood clotting is essential for survival in everyday life. Wounds will be closed because of the clotting processes activated in the blood and vessels. However, in the case of stroke, blood clots are dangerous because they block the blood flow and interrupt the supply of oxygen and nutrients in the brain. This event is called ischemia and it can occur in two ways.

In an embolic stroke, a blood clot is formed somewhere in the body, most often in the heart. This clot travels via the peripheral vascular system into the brain and eventually reaches a vessel so small that the clot cannot pass through and this will block the blood flow. This peripherally formed clot is called an embolus, which gives the name to this subgroup of strokes [22].

In a second subgroup of strokes, the clot called a thrombus develops in the arteries supplying the blood to the brain. In most cases, this plaque is a rupture from a "large artery atherosclerosis", a situation usually traceable to unhealthy living habits and increase in uptake of fatty deposits and cholesterol, which accumulate in the walls of the vessels [23]. Usually these thrombotic strokes lead to a large vessel thrombosis, which means that the combination of long-term atherosclerosis and the rapid formation and/or rupture of a clot(s) eventually prohibits the blood flow in the large brain arteries. Thrombotic stroke patients are also likely to have coronary artery disease, and ischemic heart attack is a common cause of death in patients who have suffered this type of brain attack. In addition, myocardial infarction has been shown to share causality to plaque rupture [24]. Lacunar infarction occurs when the blood flow is blocked in a smaller arterial vessel and usually the symptoms from these small strokes are milder and/or unnoticeable. The "small vessel disease" is commonly linked with hypertension [22].

Life style modifications are the primary preventive actions an individual can take to decrease his/her risk of stroke. The major risk factors of stroke have been characterized; hypertension, smoking, diabetes, atrial fibrillation and certain other cardiac related conditions, dyslipidemia, carotid artery stenosis, sickle cell disease, unhealthy diet, physical inactivity and obesity. Other risk factors are metabolic syndrome, excessive alcohol consumption, drug abuse, use of oral contraceptives, sleep-disordered breathing, migraine, hyperhomocysteinemia, elevated lipoprotein levels, hypercoagulability, inflammation and infections (see [25,26]).

Thrombolysis (tissue plasminogen activator; tPA) is at the present the only United States Food and Drug Administration (US FDA) and European Medicines Agency (EMEA) approved drug treatment for acute ischemic stroke [27–29]. It is unfortunate that tPA-treatment is possible for only patients who are in the hospital or in a special mobile stroke unit [30]. Since tPA-infusion should be started at the latest within 3 - 4.5 hours from the onset of initial signs of an ischemic event. After this time window the possible risks outweighs the possible benefits. It seems that patients under 75 years old having mild to moderate stroke symptoms are more likely to benefit from tPA treatment (see [31,32]). Nonetheless, the limitations associated with tPA-treatment leaves almost 90 - 95 % of acute stroke patients without any effective drug intervention [33–35]. In Finland, and particularily in Helsinki, the situation is much better, and almost 30 % of hospitalized stroke patients receive tPA treatment [36].

There are from 33 up to 55 million survivors living with the consequences of a stroke [1,37]. Based on statistics from the USA, at 6 months after stroke onset, 50 % of stroke survivors were experiencing some hemiparesis, 30 % were unable to walk without some assistance, and 26 % were dependent in activities of daily living, 19 % had aphasia, 35 % had suffered depressive symptoms and 26 % required institutional care [37].

Despite decades of scientific work into the pathology behind the disease, setbacks continually plague the search for an effective cure and treatment for the disease [38]. Therefore, there is a great need to develop new therapeutic approaches which can be used in patients with cerebrovascular diseases.

The events that take place after the initial stroke are schematically presented in Figure 1. After the primary blockade of the artery, many distinct processes become activated in the ischemic core and perilesional areas. Cell death and edema with energy failure develop quickly and thus these represent one possible, but challenging, target for acute neuroprotection. After the primary devastating cell death, the brains own repair pathways are activated, and these can be promoted with different activators to lead into new neuronal connections to compensate for the lost ones. Weeks after the initial stroke, cell therapy and cortical stimulations may represent novel ways to aid the brain's own plasticity and recovery [39]. It should be noted that in particular inflammatory processes remain active for months after the ischemic event which means that these secondary pathological changes may have impact on the patient's life long after the acute stroke.

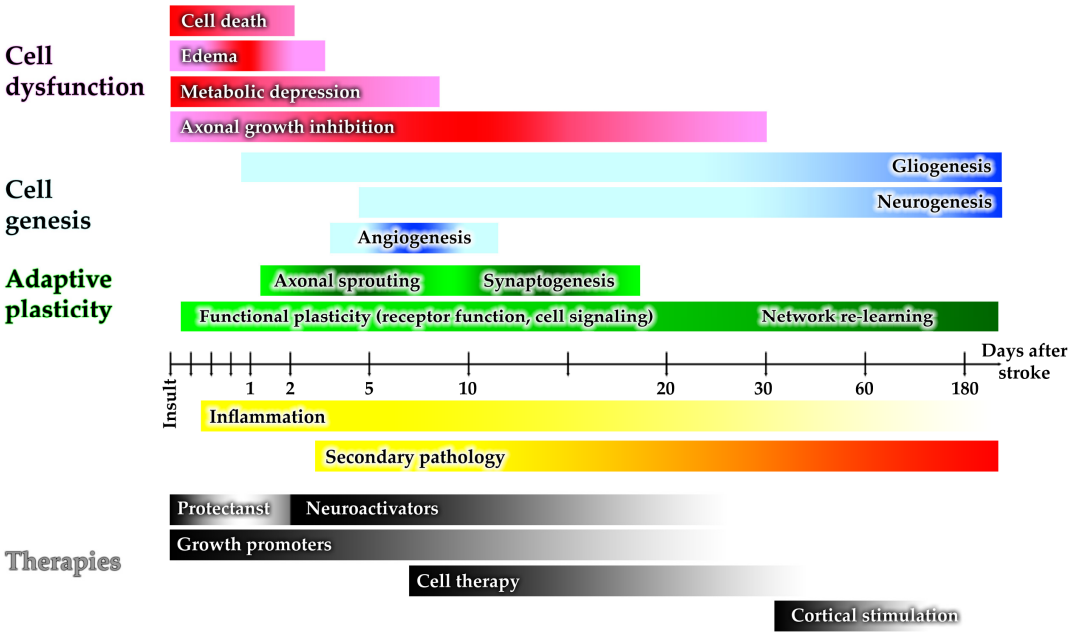


Figure 1: Timeline of events after ischemia. The intensity of color represents the level of activity in changes in the brain and the optimal time to influence these events with different therapies. The inflammation cascade begins shortly after the ischemic event and stays active for months. Secondary pathologies take place one week after the onset of stroke and also continue for months. Modified from Wieloch and Nikolich [39].

2.2 ALZHEIMER'S DISEASE

Dementia competes with stroke for first place as the leading cause of functional disability in the elderly [40,41]. In 2010, the global prevalence of dementia was estimated to be almost 36 million individuals, with an annual societal cost of 604 billion dollars [7]. In addition, another estimate is that over 115 million people will have lived with dementia before the year 2050 [5]. It is well known that AD represents the majority (60-70 %) of all dementia cases [6]. Although it is difficult to assess the cause of death in AD patients, the general estimation is that the majority (61 %) of individuals with AD at age 70 will die before the age of 80, as compared with only 30 % of correspondingly aged people not suffering from AD [42].

The key protein aggregates that are considered as hallmarks for AD are the extracellular Aβ-plagues which are mainly comprised of $A\beta_{40}$ and $A\beta_{42}$ monomers derived from APP, and intraneuronal neurofibrillary tangles (NFTs) containing microtubular hyperphosphorylated tau (see [43]). It is noteworthy that there is a vigourous debate about whether these two hallmarks should be considered as "damage response proteins" rather than being actual causes of the disease [44].

Furthermore, epidemiological studies have shown that the risk factors for stroke are associated with AD and that malign cerebrovascular changes can be detected in a high proportion of autopsy samples of AD patients [45,46]. In addition, stroke intensifies the presence and worsens the severity of the clinical symptoms of AD as well as increasing AD-specific pathology in the brain [47,48].

However, AD is not simply a secondary disease caused by a brain injury. In addition, genetic factors can be a major contributor to early-onset AD. In these relatively rare early-onset ADs, the neurological symptoms are diagnosed before the age of 65 (34% from all dementias under the age 65) [49]. In both the early-onset and late-onset forms of AD, the 4-kDa Aβ peptide cleavage products from β- and γ- secretases have been considered as causal factors in AD [50–52]. In early-onset AD, there is an increase in Aβ cleavage due to an inherited mutation in APP or presenilin (*PSEN*) [53]. In late-onset AD, the elevated enzymatic cleavage occurs in some patients who have a high level of β-secretase (BACE) [54]. In addition, a decrease in the Aβ degrading enzymes may lead to late-onset AD [55].

At the moment, there is no treatment for reversing the progress of AD. During the last decade, over 100 compounds for the treatment of AD have been tested in clinical trials but all have failed [56]. The treatments for AD at the moment are merely palliative for the symptoms rather than curing the disease itself. The main target for these compounds are based on two hypothesis; first that in AD there is a cholinergic deficit (e.g., treatment with a cholinesterase inhibitor) or second, there is an excessive NMDA receptor tone (e.g., treatment with an NMDA receptor antagonist such as memantine). Both of these treatment approaches have achieved at best only mild to moderate treatment effects in patients [57].

Similar to stroke, the only way to reduce the risk of developing AD can be found in life-style modifications. By preventing any additional brain injury such as stroke or traumatic brain injury, the risk of premature development of AD can be also decreased.

2.3 PRIMARY NEUROPATHOLOGY AFTER STROKE

Acute ischemic damage is a consequence of a cascade starting from energy depletion in the brain. Thus the fast restitution of blood flow into the ischemic areas is crucial in preventing further energy and sodium/potassium (Na^+/K^+) pump failure, increase in ionic influx, increase in intracellular calcium (Ca^{2+}), depolarization, cell swelling, generation of free radicals, blood-brain barrier (BBB) damage, inflammation and apoptosis (see Figure 2 and [58–60]).

The ischemic event is described as a situation when cerebral blood flow (CBF) is reduced to 75-80 % below normal level or is less than 10 ml/100g/min [58,61]. Since nutrients, like the levels of glucose and oxygen are too low in target brain areas, adenosine triphosphate (ATP) production and cellular metabolism in the brain becomes dysfunctional. This rapidly leads to an efflux of K^+ ions, which in turn causes an influx of Na^+ and Ca^{2+} into the cells (see [62]) and release of excitatory neurotransmitters such as glutamate (see [63]). The accumulation of the calcium in the cells leads to a rapid and extensive breakdown of phospholipids, proteins and nucleic acids by activation of calcium-dependent phospholipases, proteases and endonucleases [58]. Due to the K^+ synergized neurotransmitter release, N-methyl-D-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) are activated, thus elevating the intracellular Ca^{2+} concentration. In addition, a high intracellular Ca^{2+} concentration activates cytosolic proteases, which can directly affect the intracellular microtubule-trafficking and proteolytic degradation of structural and functional proteins (see [58]).

Subsequently, free fatty acids are transformed via lipid peroxidase into toxic substances, which in turn endanger cellular functions. Free radicals inflict damage to lipids, deoxyribonucleic acid (DNA) and proteins, thus accelerating neuronal death. Highly reactive oxygen species also contribute to the BBB breakdown and brain edema. Plasminogen activators and matrix metalloproteinase (MMP) are the two major protease systems that modulate the matrix in the brain thus having a critical impact on functioning of the BBB. Inhibition of MMPs after ischemia has been shown to reduce infarct size, the extend of brain edema and hemorrhage [64]. The disruption of the matrix leads to dysfunctional neurovascular signaling between cells [65]. In conjunction with endothelial hypoxic damage, inflammatory molecules and free radicals, MMPs further aggravate the damage done to the BBB by the stroke [58,66]. Other studies have shown that MMPs are intimately involved in angiogenesis via vascular endothelial growth factor [67].

Within minutes of blood deprivation in the brain, the endothelial cells start to express adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 that help leukocytes adhere to the endothelium in the arterial wall, thus enabling these molecules to migrate into the brain parenchyma [68–70]. At the same time, activated leukocytes (e.g., granulocytes, monocytes/macrophages, lymphocytes) produce proinflammatory cytokines, like tumor necrosis factor alpha (TNF-α) as well as interleukins, and chemokines [58,65,71]. Microglial cells participate not only in producing proinflammatory cytokines, but also in the production of free oxygen radicals and the enzyme cathepsin during the initial inflammatory response.

In addition, nitric oxide (NO) has an important role in multiple physiological processes like neuronal communication, host defense and regulation of vascular tone (see [71]). Inflammation causes excessive NO release in the brain and is also one of the culprit factors in axonal degeneration [72]. Pharmacological studies have shown that inhibition of the inducible calcium independent isoform of NO synthesis can reduce the infarct size by about 30 % after cerebral ischemia [73].

DNA can be damaged if it becomes exposed to severe oxidative stress such as occurs in a stroke. The DNA damage can be divided in two distinct types; active and passive DNA damage (see [74]). In active DNA damage the endonucleases orchestrate apoptotic DNA fragmentation, in which the two main endonucleases are caspase-activated deoxynuclease and apoptosis-inducing factor. In passive DNA damage, the main players are reactive oxygen radicals (ROSs). During the postischemic DNA accumulation, the DNA synthesis process is dysfunctional and gene transcription is disrupted thus activating apoptotic cell death [75]. Apoptotic cell death, mainly observed in the penumbra, begins immediately after the onset of the stroke and it remains highly active for days [76,77].

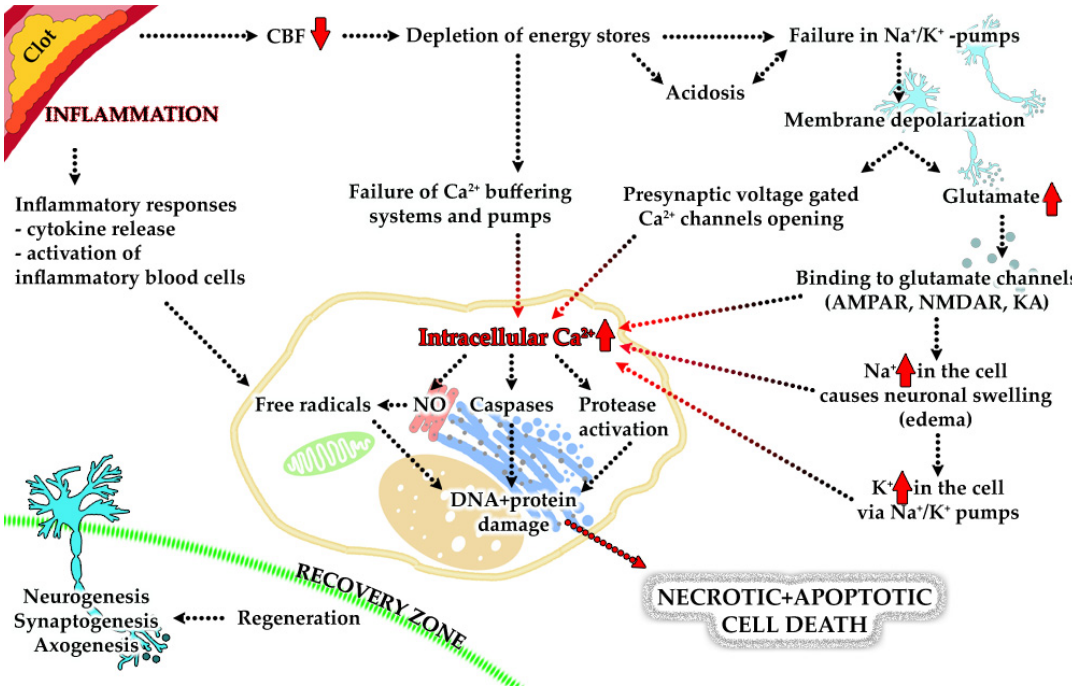


Figure 2: Schematic picture of ischemic cascade in cellular level. Ischemic cascade starts from the blockage of blood flow (CBF) which inhibits oxygen and nutrition transport to the brain eventually leading to intracellular calcium overload. AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, NMDAR: N-methyl-D-aspartate receptor, KA: kainate receptor, NO: nitric oxide. Endothelial inflammation activates cytokine release and inflammatory blood cells, which in turn promotes intracellular free radicals and thus accelerates cell death.

2.4 SECONDARY NEUROPATHOLOGY AFTER STROKE

After cerebral infarction, neuronal death, gliosis, and axonal degeneration have been found in the ipsilateral thalamus, substantia nigra and distal pyramidal tract, all of which are located outside the regions supplied by blood from the middle cerebral artery (MCA) [17,78–82]. In addition to axotomy and neuronal loss, secondary pathology in the remote areas seems to be far more complex, including Aβ and calcium deposition.

2.4.1 Degeneration of corticothalamic and thalamocortical connections

It is well established that in mammals the ascending information reaching the cerebral cortex derives primarily from thalamic relay nuclei. The prominent thalamocortical projection is associated with an even denser reciprocal corticothalamic projections, allowing the cerebral cortex to exert what is generally considered as a descending feed-back control on the thalamus [83].

In general, the thalamus can be divided into two regions based on the origin of the input. The so called "first-order" thalamic nuclei receive connections from subcortical centers (e.g.,, retina, spinal cord and cerebellum) and thus transmit visual, somatosensory and motor information. "Higher order" thalamic nuclei, such as the lateral dorsal nucleus (LD) and the lateral posterior nucleus (LP), receive inputs from cortical layer 5 pyramidal cells and participate in cortico-cortical information transfer via the thalamus (see [83–85]). Rodent studies have revealed that corticothalamic connections from the primary somatosensory cortex terminate in the reticular nucleus of the thalamus (RTN), ventroposterior medial nucleus

(VPM) and ventroposterior lateral nucleus (VPL), as well as the nuclei of the posterior complex [86]. A recent study revealed that the ventral lateral thalamic nuclear complex (VLa–VLp) is more connected to the motor cortical areas, also receiving cerebellar and basal ganglia afferents and sending projections to the motor-associated cortical areas [87]. Corticothalamic relays are mostly connected via gamma-aminobutyric acid (GABA)ergic connections between globus pallidus (GP), RTN and ventroposterior nucleus complex (VPN), which in turn projects with glutamatergic signals to cortical layers (Figure 3, mouse sagittal images from [88]).

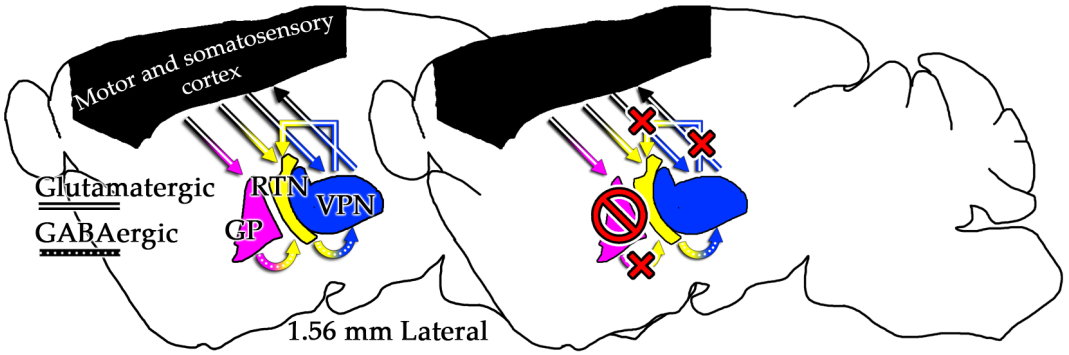


Figure 3: Schematic figure shows corticothalamic and thalamocortical connections (modified from Dihné et al. 2002 [108] and Paxinos 2001 [88]). After MCAO, thalamocortical connections undergo retrograde degeneration. In addition, damage to globus pallidus (GP) decreases inhibitory control on the reticular thalamic nucleus (RTN) leading to overexcitation of ventroposterior thalamic nucleus (VPN) and excitotoxic cell damage.

Since it known that thalamus has an important role as a signal controller between cortical and subcortical areas, one may ask what would happen were the thalamus to be damaged? In experimental middle cerebral artery occlusion (MCAO) studies, the thalamus is spared from acute ischemic damage, but because of its synaptic connections to the cortex, it can suffer delayed retrograde degeneration of thalamocortical neurons. In addition, the Wallerian (anterograde) degeneration of corticothalamic connections takes place soon after the cortical injury. At the ultrastructural level, retrograde degeneration involves degeneration of corticothalamic terminals, swelling of thalamic astrocytes and dissolution of endoplasmic reticulum (ER) within perikarya of affected neurons. As the degenerative process continues, thalamic neurons die [89].

APP is one of the markers for axonal injury; this form accumulates because of the disruption of fast anterograde axonal transport after various brain insults [90]. After MCAO, APP immunoreactivity is acutely localized within axonal swellings, dystrophic neurites, and neuronal perikarya all along the periphery of the infarct [91]. Later, there is an increase in APP staining in the corpus callosum in crossing axons, in descending axons leaving the lesioned area, and in the terminal zone of these axons in the thalamus [92].

Understandably, impaired axonal transport has been described also in AD. Recent studies have shown axonal deficits in AD through swellings that have accumulated abnormal amounts of microtubule-associated and molecular motor proteins, organelles and vesicles [93]. It has been postulated, that this is largely mediated by the kinesin-I-related fast anterograde transportation pathway within the axon [94,95]. The same mechanism also seems to be relevant in the accumulation process of $A\beta_{42}$ and increase of the $A\beta_{42}/A\beta_{40}$ ratio in the axons [93]. Poon et al [96] also showed that soluble Aβ assemblies cause synaptic dysfunction by disrupting both neurotransmitter and neurotrophin signaling, thus again blocking the axonal trafficking.

Furthermore, evidence from mouse models of AD have revealed a correlation between white matter diminish and APP deficiency [97], and this was also detected in the magnetic resonance imaging (MRI) imaging studies [98]. Interestingly, it has been shown that axonal swelling occurs long before detectable Aβ deposits can be observed in the AD mouse [93,99] and in humans [100]. Several studies have demonstrated that the presence of Aβ can accelerate the accumulation of microtubule-associated tau-protein, which deposits in AD brain as hyperphosphorylated aggregates or neurofibrillary tangles [101–106]. Similar changes have also been seen after a traumatic brain injury in transgenic AD mice; in these animals the intra-axonal Aβ accumulation and increased phosphorylated tau immunoreactivity was detectable for seven days. Treatment with a γ-secretase inhibitor was able to prevent the axonal Aβ accumulation without effecting the tau pathologies [107], suggesting that Aβ and tau are independently affected by trauma. More recent studies have shown that also hyperphosphorylated tau tends to aggregate in the ipsilateral thalamus after MCAO [108].

2.4.2 Neuronal loss and shrinkage of thalamus

Acute ischemic cell death is mediated mainly by necrotic and apoptotic pathways. The primary ischemic insult can be considered mainly as a necrotic event, in which a cell and its organelles swell and lose membrane integrity and ultimately release inflammation inducing factors which can damage the neighboring cells [109].

In addition, MCAO results in delayed neuronal loss in both the VPN and RTN. Glial activation occurs in both nuclei beginning after 24 hours [110]. The RTN, like other thalamic nuclei, is supplied by the posterior cerebral artery [111]. Thus, occlusion of the MCA alone does not induce any significant reduction in regional cerebral blood flow within the thalamus [112,113]. Vasogenic fluid spreading from the area suffering the infarction may be a contributor to the neuronal damage in the VPN after transient MCAO [114,115] but not after photothrombotic cortical lesion [116] possibly because of a smaller size of edema or because of the intact GABAergic projection from the GP into the RTN [110].

Due to anterograde and retrograde axonal degeneration and neuronal damage, severe atrophy takes place in the thalamus [117]. Within weeks, the the atrophy can be seen in imaging studies from stroke patients [80] as well as in rodents [117].

2.4.3 Inflammation

The majority of stroke patients have a co-morbid systemic inflammatory disease like diabetes, atherosclerosis, obesity, hypertension or peripheral infection [118,119], which are all known to be risk factors for stroke. A chronic increase in the numbers of inflammatory cells in the blood circulation has been proposed to be one of the causal factors also in AD, since the factors released by these cells can increase the permeability of the BBB [120]. In addition, dysfunctional Aβ clearance through BBB via RAGE-mediated pathway may be affected by inflammation and thus accelerate the onset of the disease [121].

In acute stroke, there seems to be BBB leakage into the ischemic core, thus enabling blood derived cells to gain access into the brain, which in normal situations has a very different immune system compared to that present in peripheral tissues (see [122]). In addition, upregulation of TNFα expression after stroke by neurons and astrocytes has been shown to increase the BBB permeability [123]. The brain has a tendency to quickly restore the cerebral blood flow after stroke, thus BBB permeability is at its highest during this restoring process [124].

In the brain, the immediate response to injury is the activation of astrocytes, which in turn facilitates microglia activation (see [125]). Astrocytes maintain the homeostasis in the brain, thus when they are dysfunctional, this may lead to multiple other neurological diseases. There are studies of experimental models of stroke revealing a connection between inhibition of astrocytic activation and improved functional recovery [126]. In addition, microglia cells have

multiple ways to facilitate rapid sequestration and destruction of invading microorganisms as they strive to limit the effect of trauma and cell necrosis (see [127]). The activation of microglia cells has a contribution to neurodegenerative diseases, but the question "how" is still a matter of debate. Migration and phagocytosis are promoted via activated microglia cells; there is also the release of many inflammatory mediators e.g., superoxide anions and nitric oxide (NO). Recent studies have shown that microglia could be an additional source of inflammatory cytokines, which makes these cells an interesting target for acute stroke treatment (see [128]). Since the activation of microglia is a slow process (hours to days), it offers a wider time window for drug intervention [129].

Thus, if one inhibits the inflammatory response, then the infarct size may be decreased and neurological deficits improved in experimental stroke [130]. There is a report that TNF-α was upregulated in the ipsilateral thalamus one day after experimental MCA occlusion, and the microglia/astrocyte reaction which became activated after three days persisted for up to 6 months [131,132]. In contrast, neuronal degeneration was initiated only four days after MCAO, and it became evident after 14 days [131,133].

In addition, prevention of peripheral infections with antibiotic treatments has been shown to improve the outcome after stroke [134–136]. The rationale for this might be that by preventing the excess activation of immune cells in the peripheral tissues, also the amount of peripheral immune cells penetrating through the BBB would be reduced and thus this would lessen the burden within the ischemic area.

2.4.4 Autophagy

Autophagy is a major intracellular degeneration cascade which acts in contact with lysosomal elimination and recycling of damaged organelles and aged proteins (see [137]). Effective clearance of autophagosomal structures needs retrograde transportation into the soma, where the majority of lysosomes are situated. Beclin 1 and LC3-II are two of the best known autophagosomal protein markers [138] which have been shown to be upregulated after focal ischemia [139]. Gabryel et al [140] hypothesized that by keeping autophagy as an active process and removing damaged organelles, then secondary damage could be prevented. In contrast Ginet et al [141] claimed that autophagy exhibited regional specificity and this exerted an impact on the worsening of the secondary damage after hypoxia in young rats [142].

Inhibition of Beclin 1 has been shown to prevent autophagy orchestrated secondary damage in the ipsilateral thalamus after MCAO [143], which is in line with the findings that autophagosomes can accumulate within thalamic cells after a cerebral cortical infarction. This in turn is associated with thalamic Aβ deposition and secondary neuronal degeneration via elevation of β-site APP-cleaving enzymes [144].

In AD autophagy has been more intensively studied. It seems that the retrograde trafficking system and lysosomal activity is dysfunctional in AD, thus causing a massive accumulation of autophagic elements along degenerating neurites and blocking the vital trafficking, and additionally releasing toxic peptides (see [145,146]). In *in vitro* and *in vivo* models of AD, even before the appearance of plaques in the brain, the presence of APP and BACE cleavaged fragments of Aβ_{42} have been shown to trigger autophagosomes to convert into as endogenous source of Aβ [147].

2.4.5 Aβ pathology

The cleavage and processing of APP can be divided into two pathways; a non-amyloidogenic and an amyloidogenic pathway (see Figure 4). In the non-amyloidogenic pathway, a large proportion of APP is cleaved on the cell surface by α-secretase producing an extracellular soluble N-terminal fragment (sAPPα) and a non-amyloidogenic carboxy-terminal fragment of APP (APP-CTFα/C83) [148]. This in turn is cleavaged by presenilin-containing γ-secretase (see [149]) to yield a soluble N-terminal fragment p3 and a APP intracellular domain (AICD [150]). In the amyloidogenic plaque formation, the pathway follows a similar set of cleavages, but instead of α-secretase, the BACE cleaved fragment of APP, APP-CTFβ/C99 is further processed in the endosomal compartments within the cell (see [151,152]). Subsequently, after the γ-secretase cleavage, the fragments of Aβ_{40}/Aβ_{42} and AICD are produced. There is some evidence indicating that the delayed AICD and Aβ peptide accumulation may contribute to the secondary changes in brain and worsen the post-ischemic outcome by increasing neuronal death [153–158].

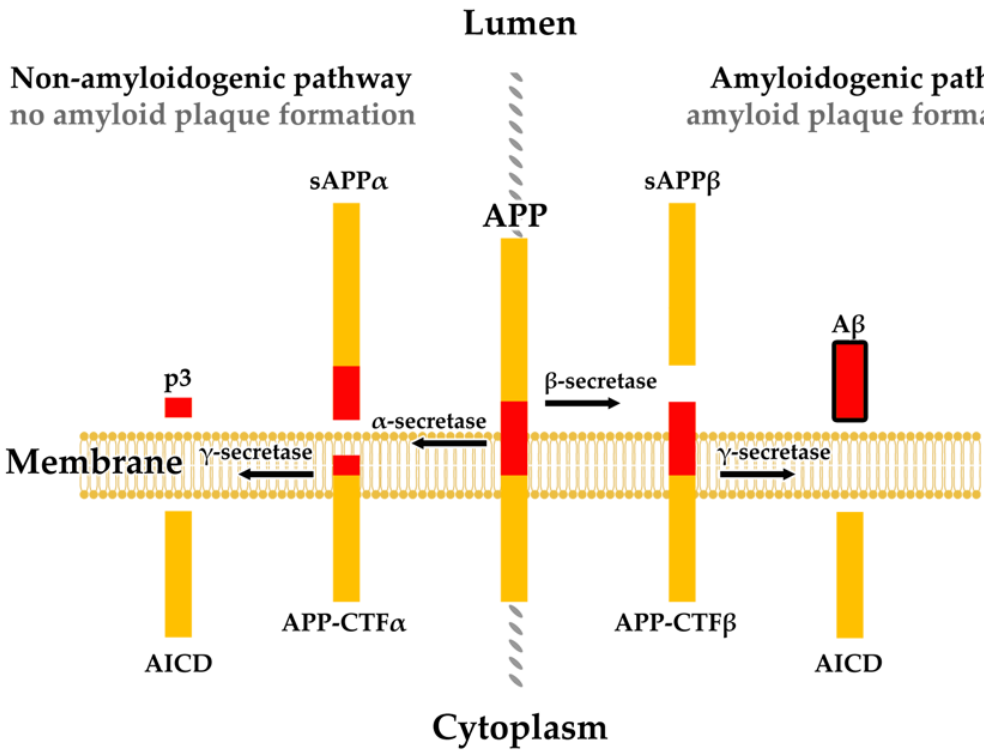


Figure 4: Amyloidogenic and non-amyloidogenic APP pathways in a schematic picture.

After the initial amyloidogenic cleavage of APP into Aβ, the peptide is eventually cleared from the central nervous system (CNS) via low-density lipoprotein receptor-related protein 1 (LRP1) mediated pathway (see [159]). In the periphery, the cleavage products of LRP1, sLRP1s, sequesters the Aβ in the plasma and transports it to liver for further degradation. The reverse transportation from periphery to CNS can occur with the help of transporters such as receptor of advanced glycation end products (RAGE; see [160]).

In both the early-onset and late-onset forms of AD, the 4-kDa Aβ peptide cleavage product of two key enzymes reactions produced by β- and γ- secretases has been considered as a causal factor of AD [50–52]. Previous studies with mice models of traumatic brain injury have demonstrated that blockade of either β- or γ-secretases can ameliorate motor and

cognitive deficits and reduce cell loss [161]. In early-onset AD, there is an increase in Aβ cleavage due to an inherited mutation in *APP* or presenilin (*PSEN*) [53]. In late-onset AD, the elevated enzymatic cleavage occurs in some patients who have a high level of BACE caused by a deficiency of microribonucleic acids (microRNAs) that control its expression [54]. In addition, a decrease in the Aβ degrading enzymes may lead to late-onset AD [55]. Different fragments of APP were noted in astrocytes, neurons, oligodendrocytes, and microglia [12,91,162–166], and in addition to brain, APP have been found all over the peripheral organs, e.g., heart, kidneys, lungs, spleen and intestine (see [167]). The fragment size of Aβ can vary between 39 and 43 amino acid residues, with $A\beta_{40}$ being the most common species and $A\beta_{42}$, a longer, hydrophobic species with a tendency to be more fibrillogenic. Recent studies have demonstrated that a soluble form of oligomeric Aβ is more toxic than the monomeric form, and consequently a conscious effort has been made to reconstitute oligomers from monomers in the laboratory setting to demonstrate their toxicity in neuronal cells [96,168–171].

There is growing evidence to suggest that brain ischemia may play a role in the etiology of late-onset AD. The calcium hypothesis postulates that there is an increase in the intracellular calcium content as a response to Aβ oligomer formation and this may lead to neuronal cell dysfunction and death in AD [172]. *In vitro* studies have further demonstrated with human neuroblastoma (MC65) cells that the neurotoxicity of soluble Aβ oligomers is accompanied by a marked increase in the intracellular Ca^{2+} content [173]. In addition, memantine, an antagonist of the glutamate activated calcium channel NMDAR, improves cognition and reduces AD-like neuropathology in mouse model of AD and has beneficial effects in AD patients [174,175]. Thus, treatments that interrupt aberrant Ca^{2+} influx may represent promising therapeutic strategies for AD.

Additional research has demonstrated an interesting phenomenon, i.e., that human Aβ peptide removal/treatment is more effective in experimental ischemic brain injury in rats [176,177175,176] and less effective in mice with overexpressed Aβ pathology [178]. Furthermore, when administered immediately after global ischemia, the secreted forms of APP proteins have been shown to exert a protective effect on neuronal injury after an ischemic insult [179]. In particular, sAPPα has been shown to possess neuroprotective effect, rather than the amyloidogenic pathways sAPPβ fragment [180,181]. By activating K^+-channels [182–184] and down-regulating NMDAR [185] sAPPα could prevent calcium overload in the neuronal soma via reducing the calcium load in the cells. Other possible cascades in which APP may have a protective role are by moderating hippocampal calcium responses to glutamate [186] and inhibiting proapoptotic *PSEN-1* mutant expression [187].

Altered APP processing indeed seems to play an important role in calcium homeostasis and this may lead to degeneration in AD [188]. Thus it has been speculated that altered processing of APP could contribute to neuronal injury by compromising this normal function of soluble APPs. There is growing evidence that APP also plays a role in other crucial processes like cell adhesion [189], cell proliferation [190] and neurite extension [191]. Nevertheless, the role of soluble APPs and the neuroprotective effects have recently suffered a setback due to the fact, that excessive increase in sAPPα may also shift proliferating cells toward tumorigenesis and the activation of microglia may cause neurotoxicity. Another fact is that at normal concentrations, Aβ is indeed cleaved during brain embryogenesis and it seems to be essential for normal brain development [192,193]. Immunodepletion of the monomeric form of Aβ has been shown to cause neuronal cell death without any effect on a variety of non-neuronal cells [194]. There are other important beneficial effects of monomeric Aβ, e.g., an increase in the survival hippocampal neurons and developing neurons, and protection of neurons against excitotoxic cell death [195]. Furthermore, Aβ fragments have been reported to play a major role as a guide in the transformation of the neural progenitor cells into neurons ($A\beta_{40}$) or astrocytes ($A\beta_{42}$) [196].

Recently, it was noted that neuropathological processes in ischemic neurons continue well beyond the acute stage of insult [153,197–199]. In these situations, the presence of a leaky BBB [200–203] probably leads to a risk that neurons in the ischemic area are made vulnerable to Aβ peptide [204]. In addition, Aβ is known to causes vasoconstriction and endothelium damage [205204], which in turn may reflect to the outcome in recirculation injuries occuring in the brain.

2.4.6 Dysregulation of cellular calcium homeostasis

Calcium is the major trigger of neurotransmitter release, a process that has been thoroughly investigated during the past decades (see [206]). Moreover, it has also become clear that calcium is essential for a variety of other neuronal functions, including neuronal excitability [207], integration of electrical signals [207,208], synaptic plasticity [209], gene expression [210], metabolism [211], and programmed cell death [212]. In the thalamus, calcium has been shown to progress from a diffuse distribution forming more dense and larger deposits with an overlapping pattern with phosphorus after MCAO [213].

Free Ca^{2+} concentrations in the neuron. Under physiological conditions, intracellular Ca^{2+} is tightly regulated, not only in the cytosol, but also within the organelles. Calcium transporters, pumps and exchangers control the intracellular free Ca^{2+} concentrations against a huge extracellular calcium gradient difference (Figure 5). The resting free Ca^{2+} concentration in the cytosolic milieu is approximately 0.1-0.2 μM, whereas it is 10 000-20 000-fold higher in the extracellular space [214,215]. The majority of the intracellular Ca^{2+} (< 99%) is bound to cytosolic proteins or stored in ER. The ER holds significant amounts of free Ca^{2+} in its lumen, and even in the resting state, the concentration of free Ca^{2+} in ER is 500-5 000-fold higher than that in the cytosolic space [215–217]. Similar gradients of Ca^{2+} can be also stored in the GA, which functions in close collaboration with ER [215,216]. Under normal conditions, mitochondria do not act as calcium storage sites, but however, after stimulation, they are able to rapidly accumulate large amounts (up to 80 μM) of calcium (see [218,219]).

Plasma membrane, influx. Calcium influx into dendrites and the soma is largely dependent on presynaptic neurotransmitter release and the membrane potential, with the latter being mainly controlled by Na^+ and K^+-channels [220]. Channels in the plasma membrane allow passive Ca^{2+} influx down an electrochemical gradient. There are two main channel types which have been categorized based on the movement-control mechanism; voltage-dependent calcium channels (VDCC) and ligand binding gated calcium channels (see [221]). In particular, glutamate receptor family members are known to mediate exitotoxicity in the cells. The activation of ionotropic glutamate receptors NMDA, AMPA and kainate leads to an enhanced permeability to Na^+, K^+ and/or Ca^{2+} in the associated ion channels [222,223], and thus plays a crucial role in the Ca^{2+} influx.

Plasma membrane, efflux. Two major plasma membrane mechanisms are responsible for the extrusion of calcium from cells. One is the ATP-driven plasma membrane calcium-pump (PMCA), the other is the Na^+/Ca^{2+} exchanger (NCX), a complex similar to that responsible for the removal of Ca^{2+} from the mitochondrial matrix into the cytoplasm [224–226]. Unlike mitochondria, plasma membrane NCX has the inherent ability to move Ca^{2+} into or out of the cell depending on the prevailing conditions. There are reports that the loss of PMCA function (for example dysfunction of the calcium binding protein calmodulin [227]) causes an increase in the intracellular Ca^{2+} levels and this can trigger apoptotic death of cerebellar and spinal cord neurons [228]. Transient receptor potential cation channels, subfamily M, members 2 and 7 (TRPM2 and TRPM7) are non-selective ions channels, which have been shown to become activated in ischemic conditions and mediate oxidative stress-dependent cell death [223,229]. Interestingly, TRPM7 is believed to regulate cell adhesion via calpain mediated influx of Ca^{2+} [230].

Neuronal Ca^{2+} homeostasis, intracellular. The ER is a complex system of endomembranes and it is present in all neurons extending from the nucleus to the soma, dendrites, and dendritic spines, and down the axons to the presynaptic terminals. The ER can act as a dynamic calcium store, accumulating calcium and releasing it in response to physiological stimulation. Within the ER, Ca^{2+} binds to molecular chaperones such as calreticulin and calnexin (see [231]). In neurons, Ca^{2+} exits from the ER mainly through the isoforms of inositol triphosphate receptors (IP_3Rs) and the Ca^{2+} activated isoforms of ryanodine receptor (RyR), both of which are large tetrameric channel proteins [221]. Store-operated calcium entry (SOCE) refers to an ER operated influx of extracellular calcium across the plasma membrane. Recently, this calcium entry mechanism has been demonstrated to occur following cerebral ischemia and contribute to neuronal death [232]. In addition to its known functions in processing and sorting of lipids and proteins [233], the Golgi Apparatus (GA) is also known to play a role as an intracellular calcium store, in conjunction with the ER [234–236]. Ca^{2+} homeostasis in the intracellular golgi is regulated by two groups of pumps: sarco/endoplasmic reticulum calcium ATPases (SERCAs) and secretory-pathway calcium ATPases (SPCAs) [237]. The relative contribution of SERCAs and SPCAs to the total uptake of Ca^{2+} into the GA seems to be cell-type-dependent, for example, the highest dependence of SPCAs occurs in human keratinocytes [238]. One of the main functions of mitochondria is to produce nicotinamide adenine dinucleotide and ATP, but it is also now well accepted that this organelle also acts as a Ca^{2+}-buffer. Mitochondrial Ca^{2+} buffering is more efficient than can be achieved by expelling Ca^{2+} through the plasma membrane or into the ER, and thus this mechanism is considered to be very relevant for neurons [239]. In a crisis situation such as during ischemia there are high demands for mitochondria-derived ATP. Because of the low ATP/adenosine diphosphate (ADP) ratio in the ischemic core, additional Ca^{2+} is released, which in turn, activates enzymes in the Krebs cycle, thus causing a boost in ATP production [240]. In mitochondria, the majority of Ca^{2+} is expelled via mitochondrial NCX (mNCX). A secondary route to Ca^{2+} to traverse to cytosol occurs via the proton/Ca^{2+} exchanger (MPT pore) [241]. In addition, lysosomes have been shown to act also as a component of the Ca^{2+} buffering system in the cell by storing any excess calcium [242].

Ca^{2+} buffers and sensors. In addition to the cell organelles mentioned above, also a large set of proteins can act as buffers and sensors needed in the maintenance of Ca^{2+} homeostasis. For example, EF-hand Ca^{2+}-binding domains (for more information see [243]) can possess both buffering and sensoring functions. Calmodulin serves as a Ca^{2+} sensor, instead calretinin, calbindin and parvalbumin seem to function as buffers. Another group of Ca^{2+}-binding proteins are called intracellular neuronal Ca^{2+} sensors (NCS; see [244]). When considering about ischemia and AD linkage, the most interesting NCS subfamily is the potassium channel interacting protein 3, coding calsenilin, which has been shown to interact with presenilin 1 (*PSEN-1*) and 2 (*PSEN-2*; see [245]).

Intracellular Ca^{2+} removal. Unlike calcium influx, calcium clearance requires ATP, which may originate from either glycolysis or mitochondria. PMCA, mMCA and SERCA are especially dedicated to removing calcium ions against an electrochemical gradient, and these processes require hydrolysis of ATP in order to work. Recent studies have shown that excess calcium within neurons is removed especially via PMCA and it is powered by glycolysis [246].

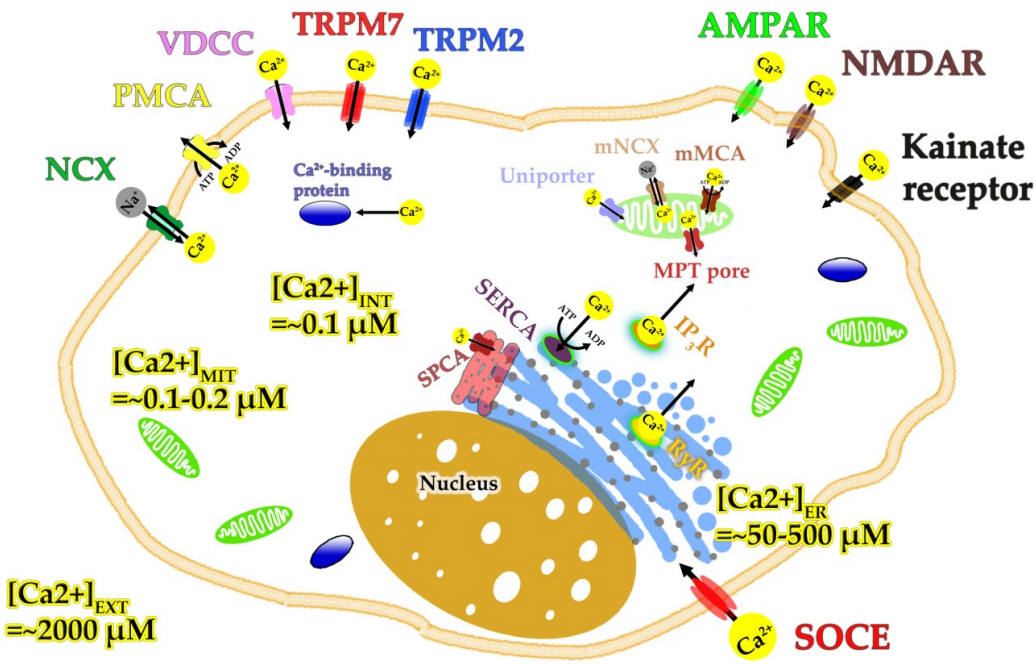


Figure 5: Representative picture of calcium channels and concentration in a cell. The intracellular calcium concentration may be over 10 000 to 20 000 times smaller than in extracellular space. Especially endoplasmic reticulum and mitochondrias store excess calcium in the intraorganelle spaces. **Transmembrane transporters and channels;** transient receptor potential cation channel, subfamily M, member 2 (TRPM2), transient receptor potential cation channel, subfamily M, member 7 (TRPM7), voltage dependent Ca^{2+} channel (VDCC), plasma membrane Ca^{2+} ATPase (PMCA), Na^{+}/Ca^{2+} exchanger (NCX), store-operated calcium entry (SOCE). **Excitatory amino acid receptors;** 2-amino-3-3(3-hydroxy-5-methylisox-azol-4-yl) propionate receptor (AMPAR), N-methyl-D-aspartate receptor (NMDAR), Kainate receptor. **Endoplasmic reticulum (ER)**; sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), inositol triphosphate receptor (IP_3R), ryanodine receptor (RyR). **Mitochondria (MIT);** mitochondrial Na^{+}/Ca^{2+} exchanger (mNCX), mitochondrial membrane Ca^{2+} ATPase (mMCA), uniporter, mitochondrial permeability transition (MPT) pore. $[Ca^{2+}]_{EXT}$: extracellular, $[Ca^{2+}]_{INT}$: intracellular, $[Ca^{2+}]_{ER}$: endoplasmic reticulum and $[Ca^{2+}]_{MIT}$: mitochondrial calcium concentrations.

2.4.7 Dysregulation of axonal calcium

Focal cerebral ischemia in the cortex evokes inflammatory responses in perilesional areas. The thalamus is spared from acute ischemic damage because it receives a blood supply through the posterior cerebral artery. Nevertheless, due to its synaptic connections, signs of delayed damage in various thalamic nucleus are obvious as neuronal loss and atrophy are seen weeks after the onset of the stroke [80,117]. This is thought to be due to the loss of GP and the effect of this loss in the glutamatergic and GABAergic pathways innervating the cerebral cortex and thalamus [110]. The microglia activation and both anterograde and retrograde degeneration lead to severe thalamic atrophy after ischemia [247] (see Figure 6 and [248]).

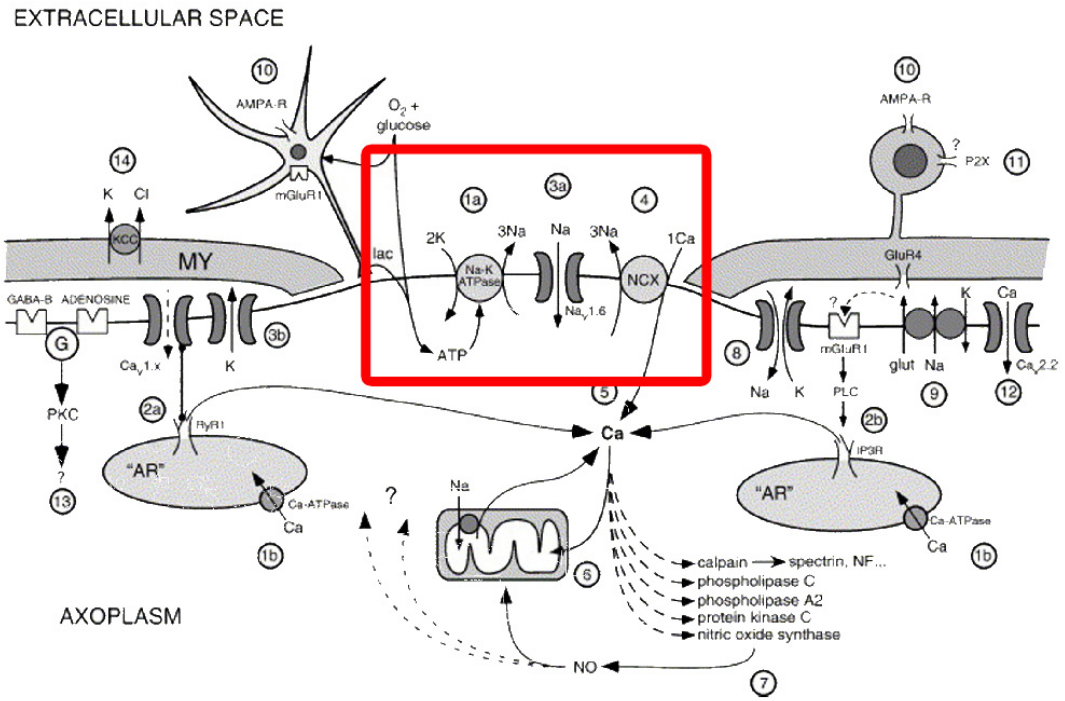


Figure 6: Representative picture of acute axonal injury modified from Stys 2005 [247]. Area presented in white background illustrates the pathways described at the cellular level in this thesis. Energy deficit causes impairment in ATP-dependent Na^+/K^+–pumps in cell membrane (1a). The rise of intracellular sodium (Na^+) (3a) and potassium (K^+) (3b), stimulates the Na^+/Ca^{2+} exchanger (NCX) to operate in the reverse Ca^{2+} import mode (4). This Ca^{2+} accumulation (5) promotes destructive events including mitochondrial Ca^{2+} overload (6), and over-activation of several Ca^{2+}-dependent enzyme systems (7).

Special emphasis on Na^+/Ca^{2+} exchanger. NCX is the main calcium export mechanism across the plasma membrane and it is responsible for restoring the resting level of Ca^{2+} in neurons after stimulation. Two families of NCX have been found, of which one requires the co-transport of K^+ (NCKX). NCKX is rather less understood, but it does seem to be a less important factor in the acute calcium overload in the cells (see [249,250])

NCX is a nine transmembrane segment protein which is widely distributed in the brain [251250]. It functions in a bidirectional way, coupling the movement of Ca^{2+} and Na^+ ions across the cell membrane in the CNS. NCX has also been found in the inner membrane of the nuclear envelope and has been proposed to mediate Ca^{2+} flux between the nucleoplasm and the nuclear envelope [252] and then to the ER [253].

Under normal conditions, NCX is the main pathway for calcium efflux, but it can also contribute to Ca^{2+} influx (reverse mode exchange), especially during intense depolarization, and with increased intracellular sodium (see [62]). The affinity for calcium and its high capacity make NCX suitable for the rapid clearance of large amounts of calcium without the need of ATP hydrolysis [254]. Thus, NCX may act as a protective mechanism against Ca^{2+} overflow [255].

NCXs are presented in three different forms (NCX1, NCX2 and NCX3), with NCX3 being mostly presented in subpopulations of neurons, where it is localized predominantly in dendrites, and distal astrocyte processes that contact excitatory synapses [256]. In the cerebral cortical areas, the NCX1 isoforms are highly expressed in pyramidal neurons of layers III-V. In addition, NCX1 protein is mainly detected in the hippocampus, in the hypothalamus, in the substantia nigra, in the ventral tegmental area, and in the granular layer of cerebellum. In the same study, NCX2 was expressed mainly in somatosensory cortical areas, but also in hippocampus, thalamus and hypothalamus. NCX3 protein was found particularly in the hippocampal subregions and in ventral striatum and cerebellar molecular layer [251]. Interestingly, transcription of the NCX1 gene is controlled by three separate promoters and it is independent of calcium levels, but in contrast, NCX2 and NCX3 genes are strictly dependent on calcium levels, although in different and reverse manners (see [250]).

Studies with transgenic and knockout animal models have revealed that animals lacking the NCX2 isoform exhibits an improvement in several hippocampal-dependent learning and memory tasks [257]. In addition, animals lacking the NCX3 gene display reduced motor activity and weakness in their forelimb muscles [258]. The homozygous NCX1 knockout experiment lead to non-viable embryos, possibly due to failure of the foetal heart to beat [259]. The NCX3 dysfunction has been connected also to overproduction of Aβ and oxidative stress activated calpain [260] and caspase [261,262]. It has also been shown that aggregated Aβ peptides could interact with the hydrophobic intracellular loop of the exchanger inhibiting its calcium-extruding functions [263]. A great many other, some partly conflicting, reports have been published about the effect of NCX modulation on cell damage [264–268]. For example, NCX blockade worsens infarction volumes in rodent models of focal cerebral ischemia [269]. In contrast, there are also reports in which a NCX inhibitor has been able to reduce infarct volume in animal stroke models [270,271].

One possible explanation for these controversial results may be traced to the fact that after ischemic hypoxia/anoxia, the expression of these three genes is differentially regulated. As explained by Gomez-Villafuertes et al [250], there is a massive downregulation of NCX2 after cerebral artery occlusion and at the same time there is an increase in accumulation of messenger RNAs of NCX1 and NCX3 in the peri-infarct brain area, as well as in non-ischemic surrounding areas, leading to the conclusion that the putative neuroprotective effect may be attributable to NCX1 and NCX3 gene products rather than emanating from NCX2. Another explanation for some of these variations in outcome could be due to the differential NCX responses in the ischemic area; in mild injury where Na^+/K^+ ATPase is preserved, blockade of NCX prevents normal Ca^{2+} extrusion from the cell. In contrast, in areas where ATPase activity is severely reduced, the elevated intracellular Na^+ load evokes NCX to operate in its reverse mode as a Ca^{2+} -influx pathway, and under these conditions a blocking of NCX channel would be protective. Earlier studies have already provided some insights into the different expression of the three subtypes of NCX after focal cerebral ischemia [272] laying the foundations for future interventions with NCX inhibitors. For a more detailed review about NCXs, see Annuziato et al [273].

2.4.8 Aβ and calcium interaction

One interesting phenomenon which can be observed days after the ischemic insult in rodents has been the overlapping accumulation of Aβ and calcium in the thalamus [92,213,274]. It is difficult to conduct studies evaluating the temporal pattern, e.g., which one is the first to be activated, since it entrails gathering data separately from both stroke and AD studies.

In a study done in our laboratory, APP/Aβ activation in the thalamus was seen one week after ischemia and it persisted for up to one month in rats, whereas in the same study also calcium and increased activity of BACE, insulin-degrading enzyme (IDE) and NEP were observed in the thalamus [9]. These results are in line with concept that increased BACE activity may result from caspase-3 mediated cleavage of Golgi-localized γ-ear-containing ARF

binding protein 3 (GGA3), which under normal conditions inhibits the excessive activity of BACE in the APP-cleavage pathway [275]. On the other hand, Ca^{2+} released from ER has been shown to be crucial in the activation of caspase-3 [276], which would suggest that an increase in Ca^{2+} -levels in the cell is the primary factor underpinning the BACE overexpression.

Additional support for the hypothesis that Ca^{2+} is the initial trigger for dysfunction in APP/Aβ clearance is, that already three days after the ischemic insult, calcium accumulates in the VPN thalamus [132]. Related to the dysfunction of thalamo-cortical signaling after stroke, a study from Zhang et al., showed that axonal swelling is detectable already 6 hours after ischemia in rats and this persisted for up to 4 months, but neither Aβ plaques nor overexpression of $Aβ_{42}$ were seen within and/or close to the swollen axons [277]. In addition, activation of tau phosphorylation was observed at 6 hours after ischemia in the same brain areas, but this declined at the later time points [277]. Similar changes have been reported in the thalamus two days after ischemia [108].

In short, the primary changes encountered after ischemia at the cellular level are cell swelling, which in turn with the increase of Na^{+} allows Ca^{2+} to flood into the cell. This may interact with other overlapping pathologies such as the increase in the NO levels increases caspase-3 activities, leading to a vast supply of BACE and this would bias the APP processing into the amyloidogenic direction.

In contrast to the primary changes occurring in the brain after ischemia, long follow-up studies have shown that the diffuse N- and C- terminal APP and Aβ deposits have disappeared in the cortical areas at one month after an experimental stroke, but instead the deposits in the thalamus start to accumulate [92]. At the timepoint 9 months after ischemia, neither APP nor Aβ was seen in the cortical areas, but in the thalamus, the deposits had been transformed into plaque-like deposits containing only N-terminal APP and Aβ. In the same study, acute activation of astrocytes and microglia was detected in the border of ischemic area. After one month the number of activated glial cells had decreased and totally disappeared after nine months. However, in the VNP, the time course of glial activity was quite different; one week after MCAO very few microglia cells and astrocytes were present. In contrast, after one month a large number of microglial cells and astrocytes were present in the same area. After 9 months, the Aβ deposits were surrounded by an astroglial scar, and there were no activated microglia present [92]. An even longer follow-up from Iwata et al [278] revealed that one year after a traumatic brain injury there was still accumulation of Aβ.

Dysregulation of Ca^{2+} in AD. In AD, the accumulation of oligomeric species of Aβ protein and neurofibrillary tangles in the brain are considered to be key factors that cause the disease. It has been postulated that excessive release of Aβ incorporates into neuronal membranes, forming calcium-permeable channels, thus increasing the intracellular calcium concentration [279–281].

Studies with young and old rodents have shown that neuronal calcium-regulating systems undergo many age-dependent changes. These include elevated intracellular calcium levels, increased calcium influx through VDCCs, enhanced calcium release from intracellular stores through IP_3R and RyR, reduced contribution of NMDAR-mediated calcium influx, impaired ability of mitochondria to buffer or cycle calcium, reduced cytosolic calcium buffering capacity and activation of calcineurin and calpains (see [282]).

There is evidence from both *in vitro* and *in vivo* studies indicating that Aβ can trigger Ca^{2+} influx through endogenous membrane channels, usually via the NMDAR [283]. In addition, other data suggests that calcium influx through synaptic NMDAR can promote non-amyloidogenic APP processing, leading to the formation of the beneficial sAPPα [284]. Another hypothesis is that VDCCs can be activated by Aβ in the early phase of AD pathogenesis (see [285]) thus elevating the intracellular calcium levels. Furthermore, presenilin proteins which are involved in AD pathogenesis, are located in the ER and if they are dysfunctional, there can be leakage of Ca^{2+} into the cytoplasm or they may interact with RyR,

IP_3R, and SERCA to increase their activity [286–290]. Interestingly, in the study of Kuchibhotla et al., Aβ deposits were required in order that they could induce calcium overload in transgenic mouse model of AD [291], which emphasized that the presence of oligomeric Aβ fragments would lead to dysfunction in calcium homeostasis in the neuron, not vice versa.

A more recent study demonstrated that especially the NCX subtype NCX3 functioning through calpain activity has a major impact on $A\beta_{42}$ toxicity in the AD brain [292]. The review of Yu et al [282] evaluates several possible mechanism through which Aβ could stimulate calcium uptake. In general, most of the Aβ fragment targets are ER and mitochondria-related channels and receptors, which in turn could well evoke an increase in the intracellular Ca^{2+} level.

2.5 ANIMAL MODELS TO STUDY STROKE

2.5.1 STAIR guidelines for stroke research

The Stroke Therapy Academic Industry Roundtable (STAIR) recommendations were first time published in 1999 to improve the quality of preclinical stroke studies [293,294]. Although over a decade has elapsed, the implementation of these guidelines into practice has been a slow process.

The main concerns highlighted in the STAIR recommendations are the appropriate selection of animal models and conducting good quality research including randomization, study inclusion/exclusion criteria and power- and sample-size calculations. In addition, it is recommended that studies should be conducted also in old animals, in both sexes, and in animals with co-morbidities like hypertension and diabetes. If one wishes to adequately assess the treatment efficacy, then clinically relevant biomarker-data should be collected from multiple time points and also from longer follow-up studies.

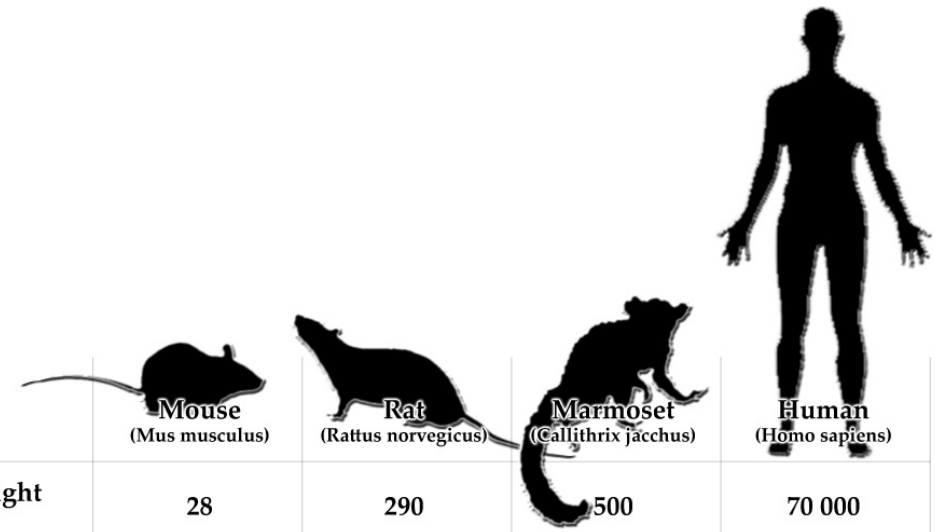
The use of primates in stroke research is included in STAIR recommendations. After initial testing of treatment efficacy in rodents, non-human primates or large animals should be included in the studies before embarking on clinical studies. The guidelines also recognize the cost, availability and ethical problems related to use of primates [293,294].

2.5.2 Brain – comparison from mouse to human

Before new drug compounds can be tested in humans, large amounts of preclinical studies have to be conducted. Most of the preclinical in vivo studies are conducted with mice (Mus musculus; over 7 million mice were used in EU in 2008) and rats (Rattus norvegicus; over 2 million). For comparison, only 904 non-human New World monkeys, such as common marmosets (Callithrix jacchus) were used in preclinical studies [295].

However, quantity is not synonymous with better impact, in terms of translation of the data. For comparison, the mouse brain weighs approximately 0.4 grams, the rat brain is 2 grams, the common marmoset's brain is 8 grams whereas the human brain weighs approximately 1500 grams [111,296–300]. Thus the difference between mouse and human brain weight is 3750-fold. Furthermore the number of neurons differs, from mouse's 75 million to a human's 86 billion [301–304]. It is noteworthy that the common marmoset has a similar distribution of neuronal and glial cells in the brain compared to the human brain [304]. There are also the gray/white matter ratio differences between the species [296,305]. See Table 1 for summary.

Table 1: Comparison between the laboratory animals and human. Estimate means are collected from the references mentioned in the text 2.4.2.



	Mouse (Mus musculus)	Rat (Rattus norvegicus)	Marmoset (Callithrix jacchus)	Human (Homo sapiens)
Body weight (g)	28	290	500	70 000
Brain weight (g)	0.4	2	8	1500
Neuronal cells (*10^6)	75	200	635	86 000
Nonneuronal cells (*10^6)	23	131	600	84 000
Neurons (*10^6) /Brain weight (g)	187,5	100	79,375	57,333
Gray/White matter ratio	12,2	6,7	2,2	1,5

2.5.3 Different ischemia models

One needs valid experimental models if one wishes to understand the basic mechanisms of ischemic damage and functional recovery. Relevant animal models are essential in the development of new therapies. Rodent models for stroke are most commonly used due to the similar vasculature to humans, low costs, vast supply of different behavioral tests and imaging opportunities.

The majority of all the human strokes are ischemic, and the MCA is the most commonly affected vessel in stroke. Spontaneous reperfusion occurs in the majority of untreated stroke patients during the first week after the onset of symptoms [306]. One of the most widely used reperfusion models in rats is transient middle cerebral artery occlusion using a filament [307]. In this model, a intraluminal thread is inserted into the internal carotid artery through the external carotid artery in order to block blood flow to the MCA territory. The filament is removed after 60 to 120 min thus allowing reperfusion. The model is invasive and the animals require post-operative care.

Permanent ischemia can be produced by either the proximal (Tamura model) or the distal MCAO. In the Tamura model, the proximal regions of the MCA are first exposed through a craniotomical window next to the olfactory tract, and then the artery is occluded by electrocoagulation. As a result, an extensive neocortical and striatal infarct is produced, and this is associated with persistent sensorimotor and cognitive deficits [268,308,309]. In the distal MCAO model, the artery is occluded through a small burr hole made between the eye and external auditory canal [310]. In order to produce a consistent lesion in normotensive rats, the ipsilateral common carotid artery has to be occluded permanently or both common carotid arteries transiently from 45 to 60 minutes. This model causes a frontoparietal cortical lesion, while the subcortical structures are spared. In contrast to the proximal MCAO occlusion model, partial recovery in sensorimotor deficits can be seen over time [308–312]. The weight loss and feeding difficulties encountered after the operation are less severe compared to those found with the filament model.

Endothelin-1 (ET-1) is a potent vasoconstrictor that has been used to produce ischemic injury. In this method, the animal is placed into a stereotaxic frame and endothelin-1 is injected adjacent to the MCA resulting in a rapid blood flow with the artery only reopening hours later [313]. In order to achieve an irreversible injury in the brain, after the ET-1 injection, the CBF has to decline significantly (~50 ml/100 g/min) and to stay below 100 ml/100 g/min for at least 10 hours [314]. In this stroke model, a typical injury occurs in the lateral cortex and dorsolateral striatum, while the forelimb motor cortex is spared. Missing hyperthermic response and less vascular invasive method are the major advantages of this model compared to the intraluminal methods. The correct placement of the ET-1 is the most common technical difficulty encountered with in this model, and this may account for much of the variability in the success rate.

Photothrombotic models are one of the less invasive models to produce stroke in rats and mice. Photothrombosis requires the use of photosensitive dyes like Rose Bengal or erythrosine B to cause intra-arterial endothelial irritation and eventually a formation of the thrombus and occlusion [315–317]. In this model, the animal is placed into a stereotaxic frame and the skull is exposed. After the intravenous injection of the photosensitive dye, a cold white light or a laser beam is targeted to the cortical area of interest. Skull thickness, time of the illumination and concentration of the dye will determine the severity of the cortical lesion. Photothrombotic lesions are characterized by occlusion of small cortical vessels, small penumbral area and diminished collateral blood flow. Infarcts are consistent with a precise location and size. Other advantages of photothrombotical models are the low mortality of animals and high success rate.

Embolization is an attempt to mimic the formation of the thromboembolic stroke in animals. The embolus is achieved after an injection of microspheres, macrospheres, clot or purified thrombin into the internal carotid artery [318–321]. The major problem is high variability limiting a more general use of these models.

In larger animals which have larger vascular volumes, the stroke models are more limited. For example in rabbits, a transient model occluding the MCA was developed for testing the tPA efficacy already in the early 1980s [322]. Different embolic methods have also been used in awake animals, enabling real-time behavioral testing while occlusion is developing [323]. There are some stroke models which better resemble the human brain structure and vasculature using gyrenecephalic animals, like pigs, sheep, cats, dogs and non-human primates. The primary difficulty with using these animals are the cost and ethical issues. In addition, their large size usually requires a more mechanical approach to produce the occlusion. Similar to the rabbit studies, also in gyrenecephalic animals, the most common way to produce the clot is to utilize an embolic method (see [324]).

Due to their analogous vascular system, one could hypothesize that the non-human primates would be by far the most appropriate subjects in which to study the pathology of stroke. Embolic models have been used also in non-human primates [325–327], although open surgery occlusion models have greater precise in location and size (see [328]). Recently also a transient filament model has been described for the stroke studies using common marmoset [329,330].

2.5.4 Behavioral tests to study stroke

In humans, a large MCA territory infarct causes neurological deficits, such as contralateral hemiplegia affecting the face, arm and the leg. This is also associated with a loss of superficial and deep sensations. In addition, one can encounter major higher function disturbances, including aphasia, visuopatial impairment, and hemineglect [331].

Rats having a focal cerebral ischemia after MCAO have been shown to exhibit deficits in several tests assessing sensorimotor and cognitive functions [332]. In contrast to memory and learning tasks, brain ischemia does not seem to result in long-lasting neurological deficits in alertness nor in sensimotor capacities [333]. However, with more demanding behavioral outcome measures, then functional deficits can be revealed even a long time after the ischemic insult [334–336]. Depending on the model selected to mimic the ischemic conditions, different behavioral tests can be used to follow the recovery.

The ischemic rats are impaired in tests which assess coordination and integration of motor movement, such as beam walking [337,338] and the grid-walking test [339]. Similar foot-fault measurements can be attained with a rotating wheel [340]. Montoya's staircase test measures skilled coordination of the forelimb in the ability to reach and grasp food pellets from different distances [341,342]. The limb placing test can be used to measure the hindlimb and forelimb responses to tactile and proprioceptive stimulation [343–345]. The cylinder test can be used to assess asymmetry in the use of forelimbs in the lateral movements against the cylinder wall [346,347].

In addition to sensorimotor dysfunction, cognitive deficits following focal cerebral ischemia have been found in tests measuring associative memory such as passive avoidance [348–351] or spatial memory such as water maze [352,353] and radial arm maze [354,355].

If one wishes to assess true neurological recovery instead of compensation (such as the use of the tail as an aid when the animal is performing the beam walking and Rotarod test) or adaptation (as can occur in cylinder test when the test is repeated multiple times within a short time period), then a battery of different behavioral tests should be selected for preclinical testing. A well designed test battery with cross validation of the observations is more powerful than any single test. In addition, high speed video recording for frame-by-frame analysis can be useful in differentiating between compensatory mechanisms and true recovery [356].

It is challenging to distinguish between primary neurological deficits and those induced by secondary changes. The extend of thalamic atrophy in MCAO rats has been demonstrated to correlate with the late sensory deficits revealed through the adhesive-removal test and in other tests measuring skilled forelimb function [336,357]. Studies with human APP (hAPP) transgenic animals with an excessive Aβ load in the thalamus have also shown sensorimotor deficits compared to wildtype littermates [358].

2.6 TREATMENTS TO PREVENT THE SECONDARY PATHOLOGY

As previously mentioned, only a subpopulation of patients benefit from the US FDA and EMEA approved tPA treatment for stroke because of the strict therapeutic time window (<4.5 hours). Surgical interventions and manual removal of the intravenous clot require highly specialized hospitals and surgical staff. In contrast, the secondary pathology in the brain offers a completely different time window for treatment. However, insufficient attention has been paid to the secondary pathological changes.

Pharmacological studies have revealed that delayed administration of various drugs prevents ischemia-induced pathology in thalamus, which in turn is reflected in improved sensorimotor or cognitive functions [142,359–362]. Different pharmacological approaches have been attempted to target this secondary neurodegeneration. For example, ciliary neurotrophic factor (CNTF) treatment after distal permanent MCAO has reduced the neuronal loss and improved cognitive water-maze performance [360]. The inhibitor for protein synthesis (CHX) after a permanent MCAO was also found to decrease neuronal death [132]. Treatment after transient MCAO with the iron chelator deferoxamine was claimed to decrease infarct size, to prevent thalamic shrinkage and to improve the behavioral recovery [363]. Similarly carbamylerythropoetin lessened inflammation markers and improved behavioral outcome [21]. An antagonist for axonal outgrowth inhibitor receptor, Nogo-66 (NEP1-40) has been shown to lessen the axonal injury and to prevent axonal degeneration after permanent MCAO [364]. In addition, treatment with a γ-secretase inhibitor (DAPT) has been shown to prevent secondary damage after permanent MCAO by decreasing levels of Aβ, gliosis and neuronal loss in the thalamus leading to improvements in behavioral tests assessing sensory functions [362].

Another striking feature of secondary pathology after stroke is its similarity to AD. This raises the possibility that one can use experimental stroke as a model with which to study AD pathology and therapies. Current treatment for AD includes symptomatic drugs, which have only modest efficacies, and none of them have shown any ability to reverse the underlying progression of the disease. Nevertheless, several lines of evidence have indicated that calcium dysregulation plays a major role in both in the pathogeneses of AD and stroke [62,172,365–367].

2.7 RATIONALE

There are many studies which have been conducted ranging from preclinical to clinical studies in stroke and AD. However, only a few studies have examined calcium and Aβ accumulation in the thalamus and even fewer studies have investigated whether preventing this type of secondary pathological changes in the brain could be reflected in a better functional outcome. Therefore, in the present thesis, the author has focused on the secondary pathology in the thalamus affected by inflammation and calcium dysregulation after stroke.

Inflammation is known to be a major contributor to the pathological changes in the brain during acute and chronic phase of stroke. In addition, excessive calcium accumulation into cells after stroke has been shown to be lethal to nerve cells and cause axonal dysfunction. Synaptic dysfunction and transportation deficits may lead to additional Aβ accumulation in the brain and again increase the neuronal loss occurring after stroke.

Given this background, this thesis examined whether the inflammatory effect in the thalamus could be ameliorated by chronic ibuprofen treatment, and whether inhibiting calcium accumulation in the cells with bepridil or KB-R7943 could improve the functional outcome after experimental stroke.

In addition, much less is known about the stroke-related thalamic pathological changes present in species genetically more close to humans. In accordance with the STAIRs recommendations about the use of multiple species, this thesis investigated the changes present in the thalamus after experimental stroke in marmosets.

3 *Aims of the study*

The aim of the study was to understand secondary pathology in the thalamus following stroke and to test pharmacotherapies which would target these pathological mechanisms. The specific aims included:

1. To test the effect of the non-steroidal anti-inflammatory drug, ibuprofen, in chronic stroke in rats and to measure the outcome via behavioral tests and histological analyses.

2. To prevent calcium accumulation after stroke in the rat brain by chronic administration of the non-selective calcium channel inhibitor bepridil, or an inhibitor of the reverse Na^+/Ca^{2+} exchanger, KB-R7943, and to follow the behavioral, histological and molecular outcomes.

3. To investigate the interaction between calcium and Aβ accumulation in the thalamus by inducing a cortical lesion in AD mice and treating the animals with the non-selective calcium channel inhibitor, bepridil.

4. To study whether there is similar pathology occuring in non-human primates after an experimental stroke.

4 Materials and methods

4.1 ANIMALS AND HOUSING CONDITIONS

4.1.1 Rats (studies I, II and III)

A total of 116 (study I: 40, study II: 31, study III: 45) male Wistar rats (age 2-3 months) from the Laboratory Animal Center, Kuopio, Finland, weighing 251-384 g were subjected to either MCAO or a sham-operation. The rats were housed individually under 12:12 hour light/dark cycle in a temperature controlled environment (20 ± 1 °C), with food (2016, Teklad) and water available *ab libitum.* All animal studies were approved by the Animal Ethics Committee (Hämeenlinna, Finland) and conducted in accordance with the guidelines set by the European Community Council Directives 86/609/EEC. All efforts were made to minimize the number of animals used and to ensure their welfare throughout the studies.

4.1.2 Mice (study IV)

A total of 63 male transgenic mice that overexpress human APP ($hAPP_{SL}$) within a C57BL/6xDBA background, and their non-transgenic littermates, were used in this study at an age of 4-5 months (QPS Austria, former JSW Lifesciences, weight 25.9-46.7 g). There was no human Aβ-positive staining in the wildtype mice, thus confirming the phenotype. The mice were housed in ventilated cages on standardized rodent bedding material (Rettenmaier®). Each cage contained a maximum of five mice. The mice had free access to food (Altromin®) and water throughout the experiment and were housed on a 12-hour light cycle in a temperature-controlled environment (24 °C, humidity 40-70 %). All animal procedures were approved by the Styrian Government, Austria, and conducted in accordance with the guidelines set by the European Community Council Directives 86/609/EEC. All efforts were made to minimize the number of animals used and to ensure their welfare.

4.1.3 Marmosets (study V)

A total of six male or female common marmosets from the breeding center at the University of Caen were used (age 22-30 months, weight 280-360 g). All procedures were performed according to the European Directive (86/609/EEC) and approved by the Regional Ethics Committee.

4.2 STUDY DESIGNS

Figure 7 is an overall picture of the study designs. The behavioral tests are described in detail in section 4.4. The animals were habituated to the laboratory environment and handling well before the projects began.

Drug administration. In studies I, II, III and IV, *per os* -drug administration was started on post-operative day 2 and continued once a day until the euthanasia of the animals.

Imaging. In study V, the marmosets went through MRI on post-operative day 7.

Euthanasia. All animals were perfused for histological analysis. In studies I, II and III euthanasia was performed on day 29. Mice in study IV were perfused on day 30. Marmosets were perfused on day 45 after the MCAO.

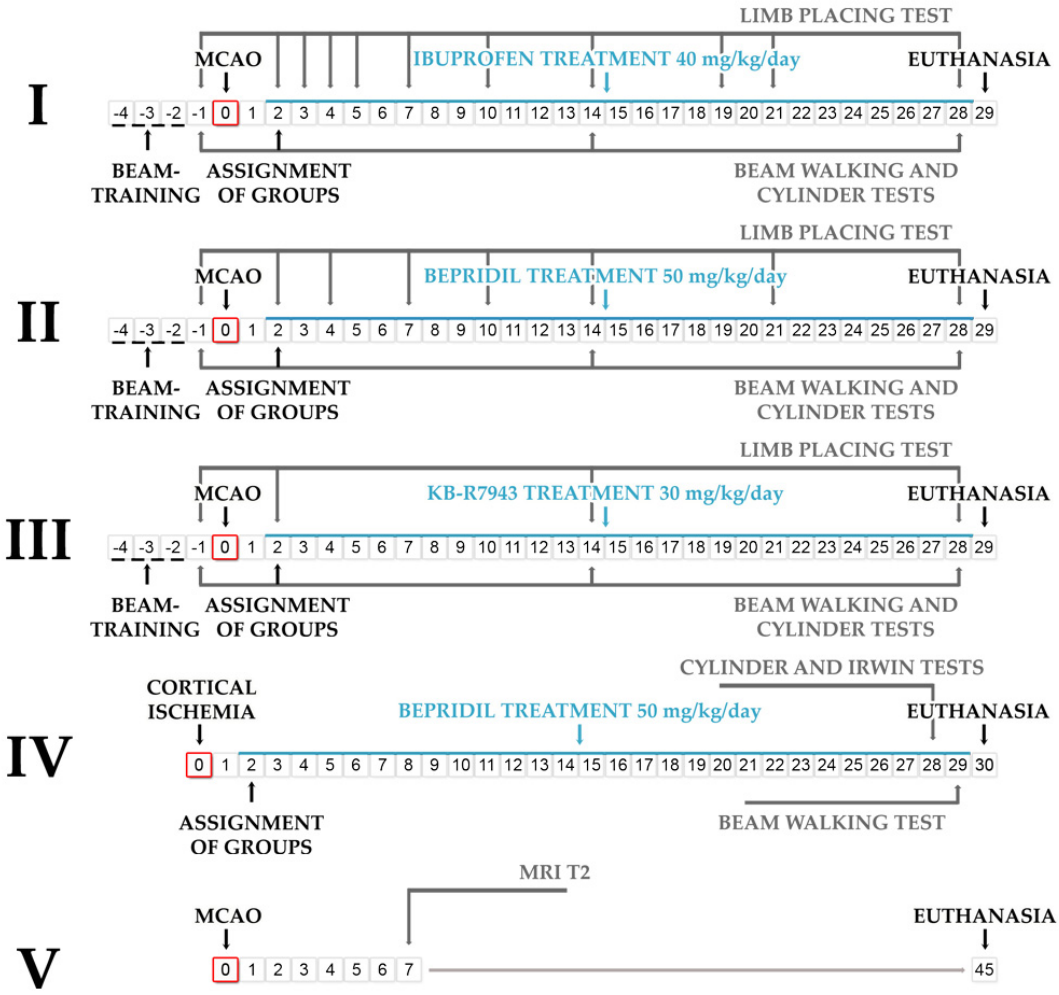


Figure 7. Study designs.

4.3 EXPERIMENTAL STROKE MODELS

4.3.1 Filament model (studies I, II, III and V)

Rats: Focal cerebral ischemia was induced by the intraluminal filament technique [307]. Anesthesia was induced in a chamber with 5 % halothane in an O_2/N_2O mixture (30 %/70 %). A surgical depth of anesthesia was maintained throughout the operation with 0.9-1.3 % halothane delivered through a nose mask. Body temperature was monitored and maintained at 37 °C using a heating pad connected to a rectal probe (Harvard Homeothermic Blanket Control Unit, Harvard Apparatus).

The right common carotid artery was exposed through a midline cervical incision under a surgical microscope and gently separated from the nerves. The external common carotid artery was closed with a suture and cut with microscissors. A heparinized nylon filament (diameter 0.25 mm, rounded tip) was inserted into the stump of the external common carotid artery. The filament was advanced 1.8-2.1 cm into the internal common carotid artery until

resistance was felt. The filament was held in place by tightening the suture around the internal common carotid artery and placing a microvascular clip around the artery.

After 120 min (studies I and II) or 90 min (study III) of MCA occlusion, the filament was removed and the external carotid artery was permanently closed by electrocoagulation. The sham-operated rats were treated in a similar manner, except that the filament was not placed into the internal carotid artery. Buprenorphine (0.03 mg/kg, subcutaneous injection, *s.c.*) was used to relieve postoperative pain. In addition, postoperative care of MCAO rats included supplemental 0.9% NaCl (intraperitoneal injection, *i.p.*) and softened food pellets to prevent weight loss.

Marmosets: Focal cerebral ischemia was induced using the intraluminal filament technique as described previously [329,330,368]. Anesthesia was induced with isoflurane (5%) and maintained during the entire experiment with isoflurane (1% to 1.5%) in an O_2/N_2O mixture (30%/70%). The animals were orally intubated and mechanically ventilated (Harvard Apparatus).

Briefly, a nylon thread, with a distal cylinder of 3 mm length and 0.54 mm diameter, was inserted into the external carotid artery and gently advanced up to the origin of the MCA (approximately 27 mm from the bifurication between internal carotid artery and external carotid artery. The thread was removed after 3 h in MCAO animals (n=4, 3 male/1 female) or immediately in the sham-operated animals (n=2, 2 female).

After reperfusion and suturing the skin, each animal received an injection of an analgesic (tolfenamic acid, 4 mg/kg, intramuscularly, Tolfedine 4%, Vétoquinol, Lure, France) and an antibiotic (cefamandole 15 mg/kg, intramuscularly; Kefandol, France, for the next 3 days). After recovery from anesthesia, the marmosets were returned to their cages and given access to water and soft food, and then observed for 1 to 2 h and daily afterwards.

4.3.2 Photothrombotic cortical lesion (study IV)

Animals were anesthetized with a mixture of Ketasol (100 mg/kg) and Xylasol (0.8 mg/kg) in 0.9 % NaCl. Anesthetized animals were mounted to a stereotactic frame (Stoelting, Wood Dale, IL) and the skull was exposed with an approximate 1 cm incision along the midline. Then the cold light source was placed on the skull over the right sensorimotor cortex (-2.4 mm from bregma). Before illumination, Rose Bengal (50 mg/kg, Sigma, St. Louis, USA) in saline was given via the tail vein as a bolus. Subsequently, the cold light (diameter 1 mm) was switched on for 10 minutes. After the operation, the skin was glued with VetGlue and the animals were treated for postoperative pain with 10 ml/kg body weight of Rimadyl (0.05 mg/ml, Pfizer Animal Health, The Netherlands) and the local painkiller Xylocain gel (2 %, AstraZeneca, Södertälje, Sweden).

4.4 BEHAVIORAL TESTS

All behavioral tests were carried out in a blind manner, meaning that the behavioral tests and analysis were conducted by person unaware of the treatment groups.

4.4.1 Tapered/ledged beam walking test (studies I, II and III)

Sensimotor functions of hindlimbs were tested using a tapered/ledged beam walking test [337336]. The rats were pretrained on the beam for 3 days before ischemia induction. The animals were tested before surgery and on post-operative days 14 and 28.

The beam-walking apparatus consisted of a tapered beam with underhanging ledges on each side to permit foot faults without falling. Rats with ischemic damage are able to use the ledge as a “crutch” and thus are less prone to learn compensatory postural strategies. The beam has a starting point with a platform at the other end, and a black goal box

(20.5 cm × 25 cm × 25 cm) at the other end. A bright light was placed above the start point to motivate the rats to traverse the beam.

All trials were videotaped and later analyzed by calculating the slip ratio of the impaired (contralateral to lesion) forelimb and hindlimb. Steps onto the ledge were scored as a full slip, and a half slip was given if the limb touched the side of the beam. The used formula for calculation was; (number of full slips + ½ *half slips/total steps)*100%. The mean of three trials was used in the statistical analyses.

4.4.2 Cylinder test (studies I, II and III)

The cylinder test was used to assess spontaneous forelimb use and imbalance between impaired and non-impaired forelimbs [369]. In the test, the rat was placed in a transparent cylinder (diameter 20 cm) and videotaped during the light part of the light/dark cycle before, and 14 and 28 days after surgery.

Rats were transported to the videotaping room 20 minutes before the test. After the habituation phase and tapered/ledged beam walking test, one animal at a time was placed in the cylinder, and videotaped for 5 minutes. A mirror was placed under the cylinder so that behavior could be viewed. Exploratory activity for 1–5 min was analyzed in a blind manner to the treatment groups using a program with slow-motion capabilities.

Forelimb use was observed during the first contact against the wall after rearing and during lateral exploration of the wall. Independent use of impaired and non-impaired forelimbs as well as simultaneous use of both forelimbs were calculated. After the start of the trial, the first forelimb to contact the wall with weight support was scored as an independent wall placement for that limb. A simultaneous (both forelimbs) movement was scored if the first limb maintained its position and the other forelimb was placed on the wall. If the animal placed both forelimbs simultaneously on the wall, that was scored as a simultaneous movement. To score again, the rat had to remove both forelimbs from the surface of the cylinder. After the rat removed both forelimbs from the wall, stood with its hindlimbs, and then contacted the wall with the other forelimb, that was scored as an independent wall placement for that limb. When the rat explored the wall laterally, alternating both limbs, simultaneous movement was scored. If one forelimb was stationary and the other made several movements, it was scored as one simultaneous movement. Forelimb use during the ascent and landing was not analyzed [370]. Cylinder score for impaired forelimb was calculated as [contralateral contacts + ½ *bilateral contacts/total contacts]*100%.

4.4.3 Limb placing test (studies I, II and III)

The modified version of the limb-placing test [343] was used to assess hindlimb and forelimb responses to tactile and proprioceptive stimulation [344,345]. The limb-placing test was also used to assign ischemic animals to equivalent groups on the day after ischemia induction.

The test had seven limb-placing tasks that assess the forelimb and hindlimb responses. The tasks were scored in the following manner: the rat performed normally, 2 points; the rat performed with a delay (2 s) and/or incompletely, 1 point; the rat did not perform normally, 0 points. Both sides of the body were tested.

In the first task, the rat was positioned towards the table and its forelimbs were placed on the table. Each forelimb was gently pulled down and retrieval and placement were checked. The second task was the same as the first one except that the rat's head was kept upward at a 45° angle. Next, the rats were placed along the table edge to check for lateral placement of the forelimb (third task) and the hindlimb (fourth task). In the fifth task, the rat was again positioned towards the table with the hindlimbs just over the table edge. Each hindlimb was pulled down and gently stimulated by pushing towards the side of the table. In the sixth task, the forelimbs of the rat were on the edge of the table and the rat was gently pushed from behind toward the edge to assess its ability to maintain its grip. In the final,

seventh task, the rat was suspended 10 cm over a table and the stretching of its forelimbs towards the table was observed. The rats were habituated for handling and tested before ischemia induction and then again on postoperative days 2, 3, 4, 5, 7, 10, 14, 19, 21 and 28 (study I); on postoperative days 2, 4, 7, 10, 14, 21 and 28 (study II) or on postoperative days 2, 14 and 28 (study III).

4.4.4 Behavioral tests in mice (study IV)

Beam. This test was performed on day 29. The analysis focused on the total traverse time, the active time during the test and the number of foot slips.

Cylinder test. The spontaneous activity of the animals was determined by using the cylinder test modified for mice [369,371]. Therefore, spontaneous movements were videotaped for 1 minute in a small transparent cylinder (height: 15.5 cm, diameter: 12.7 cm) placed on a piece of glass. The number of rearings, forelimb and hindlimb steps, and the time spent grooming were measured. The test was performed on day 28 as the first test before the Irwin test.

Irwin test. To test and survey the general health, each mouse underwent an Irwin test [372]. The measured physical characteristics were body weight, body temperature, existence of whiskers, constitution of the fur and the eyes as well as individual remarks.

The wire suspension test. Neuromuscular abnormalities can be detected with a simple measure of motor strength. In the wire suspension test, a standard wire cage lid was used. The test was performed by placing the mouse on top of the lid and gradually turning the lid upside down. A cut-off time of 60 second was used. The latency to fall down was measured.

4.5 HISTOLOGY

All histological procedures and analyses are also presented in Figure 8.

4.5.1 Perfusion

Animals were perfused transcardically on postoperative day 29 (study I), day 30 (study III) or day 45 (study V) with 0.9 % NaCl followed by 4 % paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4). The brains were removed from the skulls, postfixed and cryoprotected. Frozen sections (40 μm, study I; 35 μm, study III; 50 μm, study V) were cut with a sliding microtome and stored in a cryoprotectant tissue collection solution at – 20 °C.

4.5.2 Inflammatory markers (study I)

Glial fibrillary acidic protein (GFAP) was stained as a marker for astrocytes (mouse anti-GFAP, Sigma, St. Louis, USA) and OX-42 as a marker of microglia (mouse anti-rat CD11b, Serotec, Oxford, UK). Then sections were transferred to a solution containing the primary antibody (GFAP at 1:1,000, OX-42 at 1:4,000) and Tris-buffered saline with 0.5 % Triton X-100 (TBS-T). After incubation in this solution overnight on a shaker table at room temperature (20 °C) in the dark, the sections were rinsed three times in TBS-T and transferred to a solution containing the secondary antibody (sheep anti-mouse Ig*biotin; Serotec, Oxford, UK or goat anti-mouse*biotin; Sigma, St. Louis, USA). After 2 h, the sections were rinsed three times with TBS-T and transferred to a solution containing mouse ExtrAvidin (Sigma, St. Louis, USA), and then incubated for approximately 3 min with diaminobenzidine (DAB) [92].

4.5.3 Aβ staining in rats and mice (studies I and IV)

The accumulation of Aβ in the thalamus was examined using a rodent-specific primary antibody (rabbit anti-rodent Aβ3-16, #9151; Covance, USA) and human-specific primary antibody (mouse monoclonal anti-Aβ, clone W0-2, Millipore, USA). Adjacent sections were pretreated for 30 minutes with hot (85 °C) citrate buffer. After incubation in citrate buffer, the sections were rinsed three times with TBS-T (rodent Aβ staining TBS-T pH 7.6 and W0-2 pH 8.6) and transferred to a solution containing the primary antibody (rodent specific Aβ at 1:5,000, human specific at 1:20,000) and Tris buffered saline with TBS-T. After incubation in this solution overnight on a shaker table at room temperature (20 °C) in the dark, the sections were rinsed three times in TBS-T and transferred to a solution containing the secondary antibody (goat anti-rabbit Ig*biotin 1:500, Chemicon/Millipore USA; goat anti-mouse*biotin 1:500, Vector, USA). After 2 h, the sections were rinsed three times with TBS-T and transferred to a solution containing mouse ExtrAvidin (Sigma, St. Louis, USA), and then incubated for approximately 3 min with DAB [92].

4.5.4 Double immunofluorescence staining (study IV)

For assessing the cellular localization of Aβ deposits, a double immunofluorecent staining for rodent Aβ, neuronal nuclei (NeuN) and GFAP in the thalamus was carried out. Pretreated sections (see 4.5.3) were incubated overnight with a mixture of primary antibodies (Aβ: rabbit anti-rodent Aβ3-16, 1:2,000, Covance, USA; NeuN: mouse anti-NeuN, 1:2,000, Millipore, Temecula, CA, USA; GFAP: anti-mouse anti-GFAP, 1:1,000, Sigma, St. Louis, USA). After rinsing in TBS-T, the sections were incubated with secondary antibodies (Aβ: Alexa 488-conjugated goat anti-rabbit IgG, 1:250, Invitrogen, Carlsbad, CA, USA; GFAP and NeuN: Alexa 594-conjugated goat anti-mouse IgG, 1:250, Invitrogen, Carlsbad, CA, USA).

4.5.5 Aβ and GFAP stainings in marmosets (study V)

Sections from the four anterioposterior levels (A4, A5.5 for the MD, A8 – for the VPL/VPM and P3) were used for staining [373]. A human-specific antibody (mouse monoclonal anti-hAPP, 6E10, Covance, Emeryville, USA) was used for Aβ staining. Citrate buffer pretreated sections (30 min, 85 °C) were incubated with the primary antibody (1:1,000) and Tris buffered saline with 0.5 % TBS-T overnight at room temperature (20 °C). The sections were then rinsed three times with TBS-T and transferred to a solution containing the secondary antibody (goat anti-mouse*biotin 1:400; Sigma St. Louis, USA). After 2 h, sections were rinsed and transferred to a solution containing mouse ExtrAvidin (Sigma, St. Louis, USA) and then incubated for approximately 3 minutes with DAB [92]. Adjacent sections were stained for GFAP (rabbit anti-GFAP, 1:5,000, Dako, Trappes, France) [329]. The used antibody showed Aβ deposits in brain sections from transgenic Alzheimer's disease mice and patients, which were used as a positive staining control.

4.5.6 Calcium staining (studies I, IV and V)

Calcium was stained with the Alizarin red method [213]. In brief, sections were mounted on gelatinized glass and immersed in 2 % Alizarin red (w/v, distilled water, pH 4.1 to 4.3; Merck, Darmstad, Germany) for 30 s followed by rinsing in distilled water. The sections were quickly dehydrated with acetone and xylene and coverslipped in Depex.

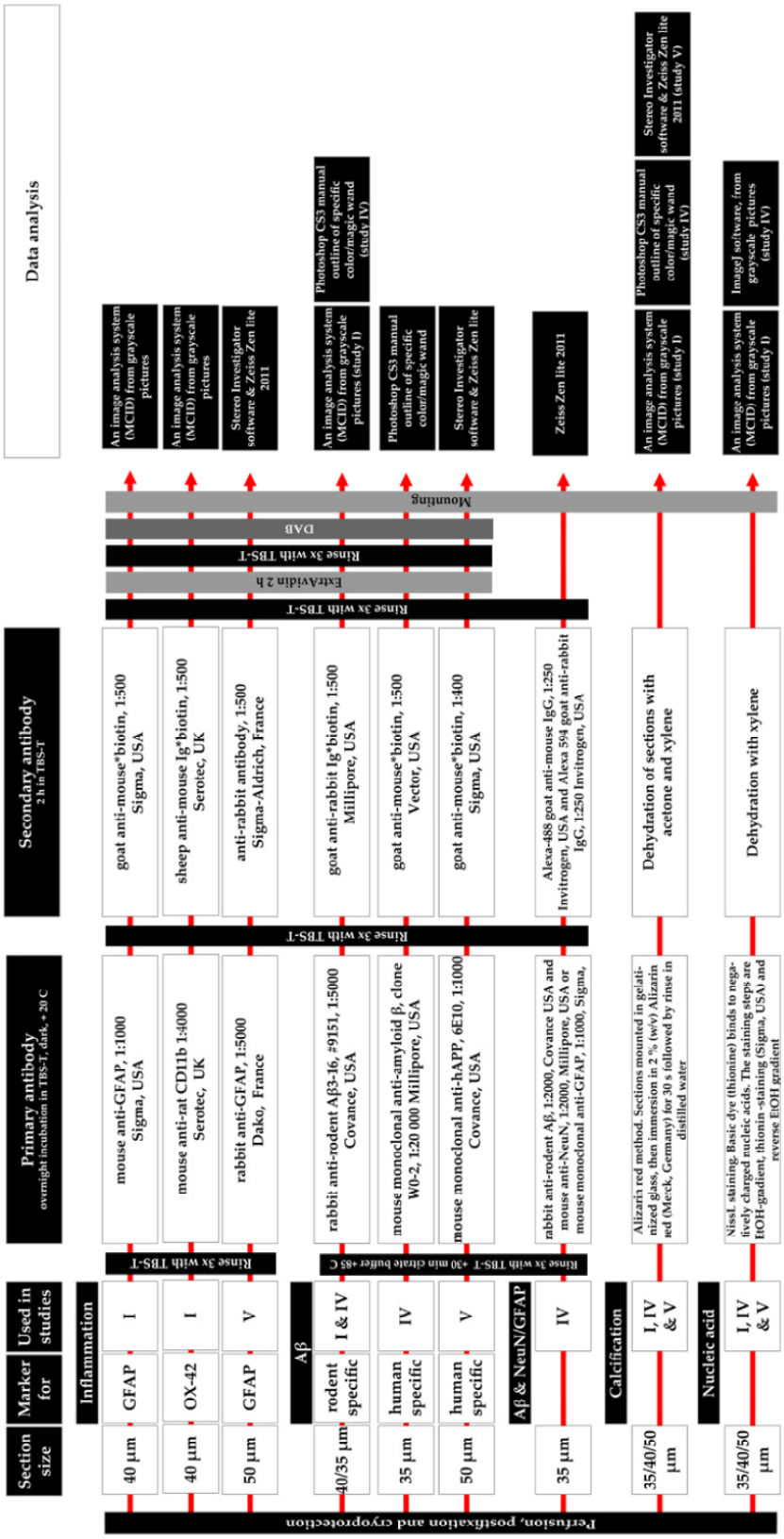


Figure 8: The histological methods and analysis of the stainings used in this thesis

4.6 HISTOLOGICAL ANALYSES

4.6.1 Assessment of infarct volumes (studies I, IV and V)

Infarct volumes were measured in study I by using an image analysis system (MCID, Imaging Research Inc., Canada) from Nissl-stained sections collected at 0.4 mm intervals. The image of each section was taken as a 1280x1024 matrix of calibrated pixel units. The digital image was displayed on a video screen and areas of surviving grey matter in the cortex and striatum were outlined separately from each hemisphere. The difference between the size of an outlined area in the contralateral hemisphere and the respective residual area in the ipsilateral hemisphere were taken as the infarcted area. The total infarct volume was calculated by multiplying the infarct area by the distance between the sections and summing together the volumes for each brain.

Infarct volumes in study IV were measured by using ImageJ from Nissl-stained sections collected at 0.175 – 0.245 mm intervals. The mean area of tissue damage between two sequential sections was multiplied by the distance between the two sections, and these values were summed up to obtain total infarct volume (mm^3).

Lesion volumes in MCAO marmosets were measured from MRI-pictures taken on post-operative day 7.

4.6.2 Calcium, Aβ, GFAP and OX-42 analysis (studies I and IV)

An image analysis system (MCID) and a DAGE MTI CCD-72 series camera were used to quantify activated astrocyte (GFAP) and microglia (OX-42) and Aβ and calcium load in the thalamus (study I). The digitized image was displayed on a video screen and converted to gray scale, after which the thalamus was manually outlined and optical densities above the threshold level taken from the contralateral thalamus were recognized automatically by the image analysis system. The areal value was taken for statistical analysis.

Images from calcium and Aβ stained sections (study IV) were taken with an Olympus BX40 microscope and an Olympus digital camera DP50-CU (Japan). From each image, the thalamus was manually outlined (Photoshop CS3) and the areas of interest were automatically analyzed by using the 'magic wand' tool to outline the specific color of staining from the background. Measurements were performed from two replicates and the mean values were used for statistical analysis.

4.6.3 Mapping of Aβ positive cells and calcium in marmoset brains (study V)

6E10-positive cells and calcium positive areas were manually mapped using Stereo Investigator software (MicroBrightField, Inc., VT, USA). A motorized stage with a microcator (Heidenhain EXE 610C) attachment (providing a 0.1 μm resolution in the *Z* axis) was mounted on the microscope. The brain sections were first outlined using a CFI Plan Achro 2× objective (N.A. 0.06, W.D. 7.5). Thereafter, a CFI Plan Achro 10× objective (N.A. 0.45, W.D. 4.5) was used to map cells and areas of interest. The shrinkage of thalamus was assessed from stained sections by outlining the ipsilateral and contralateral thalamus and calculating the % change.

4.7 BIOCHEMICAL ANALYSIS

Experimental stroke related changes have been previously demonstrated in biochemical analyses [99] and thus these kinds of results represent a good supplement to the studies with only histological and behavioral data. In the present studies (II and III), the changes in Aβ and calcium levels, as well as in a set of other Aβ and APP processing proteins, were assessed from thalamic brain lysates.

4.7.1 Thalamic tissue samples (studies II and III)

Tissue samples from the ipsilateral and contralateral thalamus of MCAO or sham-operated rats were weighed and mechanically homogenized in 400 µl of Dulbecco's phosphate buffered saline (DPBS, Lonza, Basel, Switzerland) in an ice bath. Approximately 10-fold excess of PBS/mg tissue was used. The tissue homogenates were subdivided into two (study III) or three fractions (study II) as follows; (study II; 10 % RNA extraction, 45 % total protein I, 45 % total protein II; study III: 50 % total protein I and 50 % total protein II). For the RNA extraction, the homogenant was mixed with 500 µl of TRIzol® reagent (Invitrogen, Carlsbad, CA, USA); for total protein I, the homogenant was suspended in DPBS containing EDTA-free protease and phosphatase inhibitors (1:100; Thermo Scientific, Rockford, IL, USA); and for total protein II the homogenant was suspended in DPBS without inhibitors. All aliquots were stored at -70 °C. Total protein I fraction was subjected to Western blotting and Aβ analyses. Total protein II fraction was used for measurement of calcium and α-, β- and γ-secretase enzyme activity.

4.7.2 RNA extraction and qPCR analysis (study II)

Total RNA was extracted from homogenized thalamic tissues using TRIzol® reagent according to the manufacturer's instructions. Equal quantities of total RNA samples were subjected to complementary DNA (cDNA) synthesis using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Subsequently, SYBR Green Master PCR Mix (Applied Biosystems, Foster City, CA, USA) and target-specific PCR primers for seladin-1 (5′-CAAGCCGTGGTTCTTTAAGC-3′ and 5′-CATCCAGCCAAAGAGGTAGC-3′), TNF-α (5′-CGAGTGACAAGCCTGTAGCC-3′ and 5′-GTGGGTGAGGAGCACGTAGT-3′), L-type calcium channel (LTCC) (5′-TTCGATGTGAAGGCACTGAG-3′ and 5′-TATGCCCTCCTGGTTGTAGC-3′), and glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) (5′-CGTCCCGTAGACAAAATGGT-3′ and 5′-TCTCAATGGTGGTGAAGACA-3′) were used for amplification of cDNA samples by using a real time quantitative PCR machine (7500 Fast Real Time PCR System, Applied Biosystems, Foster City, CA, USA). PCR primers were designed to amplify a region extending across at least two different exons. A standard curve method was used to obtain seladin-1, TNF-α, LTCC, and GAPDH levels. The mRNA levels of each gene were normalized to those of GAPDH from the same samples.

4.7.3 Western blotting (study II)

Total proteins were extracted by using TPER protein extraction buffer (Pierce/Thermo Scientific, Rockford, lL, USA) containing protease and phosphatase inhibitors (Pierce/Thermo Scientific, Rockford, lL, USA) from an aliquot of total protein I fraction. After protein quantification with the bicinchoninic acid (BCA) assay (Pierce/Thermo Scientific, Rockford, lL, USA), the proteins (30–50 µg/lane) were separated by 4–12% Bis-Tris-polyacrylamide gel electrophoresis (PAGE; Invitrogen) and blotted onto Immun-Blot polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). Primary antibodies against APP C-terminus (A8717, Sigma-Aldrich, Saint Louis, MO, USA), GGA3 (BD Biosciences, Dako, Glostrup, Denmark), GFAP (BD Biosciences, Dako, Glostrup, Denmark), GAPDH (ab8245, Abcam, Cambridge, MA, USA), IDE (ab32216; Abcam, Cambridge, MA, USA), neprilysin (NEP, AF1126; R&D Systems, Minneapolis, MN, USA) and low-density lipoprotein receptor-related protein (LRP, ab92544; Abcam, Cambridge, MA, USA) were used for immunoblotting. After incubation with appropriate secondary horseradish peroxidase-conjugated antibodies (GE Healthcare, Piscataway, NJ, USA), the membranes were exposed to a chemiluminescent substrate (ECL™ Advance Western Blotting Detection Kit, Amersham Biosciences/ GE Healthcare, Piscataway, NJ, USA), and protein bands were detected with ImageQuant RT ECL Imager (GE Healthcare, Piscataway, NJ, USA). Western blot images were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA). SH-SY5Y human neuroblastoma cells over-expressing APP751 isoform were used as a control for size comparison of the bands [374].

4.7.4 Aβ and soluble APP and Aβ measurements (studies II and III)

Soluble or insoluble Aβ x-40 and Aβ x-42 ($A\beta_{40}$ and $A\beta_{42}$) levels were measured from the total protein I fraction. Protein aliquots were first ultracentrifuged (100,000 x g, 50.4 Ti rotor; Beckman Coulter, Palo Alto, CA, USA) for two hours at 4 °C, and the supernatant (= soluble fraction) was collected. Subsequently, the remaining pellet was resuspended in guanidine buffer (5 M guanidine-HCl / 50 mM Tris-HCl, pH 8.0), incubated for 2 hours at room temperature on a shaker, and diluted 1:50 in BSAT-DPBS (5% BSA/0.03% Tween-20 in DPBS, pH 9.0) containing protease and phosphatase inhibitors. Finally, the suspension was centrifuged for 20 minutes at 15700 x g and the supernatant (= insoluble fraction) was collected for Aβ measurements. Both insoluble and soluble $A\beta_{40}$ and $A\beta_{42}$ levels were determined using monoclonal and HRP-conjugated antibody-based Human/Rat Aβ 40 (294-62501) and Human/Rat Aβ 42 (High-Sensitive; 290-62601) ELISA Kit (Wako, Osaka, Japan). After 30-minute incubation at room temperature, the reaction was terminated and the absorbance was measured at 450 nm with an ELISA microplate reader (Wallac/Perkin Elmer, Waltham, MA, USA). Aβ concentrations were expressed per tissue weight, and in study III per amount of soluble total protein. Soluble sAPPα and sAPP total (= sAPPα + sAPPβ) levels were detected from the same protein fraction as soluble Aβ using Western blot analysis with 6E10 (Signet Laboratories, Dedham, MA, USA)) and 22C11 (Mab348, Millipore, Darmstadt, Germany)) antibodies, respectively.

4.7.5 Calcium measurements (studies II and III)

Tissue homogenate samples (20 µl) were digested (CEM MDS-2000 microwave digestor) in 100 µl suprapure nitric acid (Merck, Merck, Whitehouse Station, NJ, USA). After digestion, 100 µl 2% lanthanum solution (Riedel de Haen, Honeywell Riedel-deHaën, Hanover, Germany) was added and the samples were diluted to a volume of 1 ml with Milli-Q water. Calcium measurements were carried out with a ZEEnit 700 atomic absorption spectrometer (Analytik Jena AG, Jena, Germany) with a calcium hollow cathode lamp at wavelength of 422.7 nm using an air-acetylene flame and SFS6 injection module. Calcium concentrations were expressed per tissue weight.

4.8 STATISTICAL ANALYSIS

All statistical analyses were performed with SPSS software. The non-parametric Mann-Whitney U-test (equal variances not assumed) was used for statistical analysis of limb placing test. Beam walk and cylinder test were analyzed using repeated measures analysis of variance (ANOVA). Then comparisons between groups were made using one-way ANOVA followed by a post hoc test (LSD). The effects of genotype and treatment on infarct volumes, Aβ and calcium staining were analyzed using two-way ANOVA followed by Student t-test, if needed. Differences in the infarct volumes, calcium staining and immunohistochemistry between vehicle controls and drug treated rats and mice were analyzed using one-way ANOVA followed by LSD. Non-parametric Mann-Whitney U-test (equal variances not assumed) and one-way ANOVA with a post-hoc test (LSD) was used for statistical analysis of biochemical data. Correlations were determined using Pearson's correlation coefficient. GraphPad Prism5 software was used to finalize the figures.

5 Results

5.1 STUDY I

5.1.1 Experimental groups

From forty animals 10, were sham-operated. One of these died during the follow-up. From the thirty MCAO-operated animals, seven died <48 hours after the surgery and three died during the follow-up. One animal was excluded from the study since its limb placing test score was 10 or higher. Thus, the experimental groups were: sham+vehicle (n=4), sham+ibuprofen (40 mg/kg; n=5), MCAO+vehicle (n=11) and MCAO+ ibuprofen (40 mg/kg; n=8). Sham-operated animals did not differ in their behavioral or histological results and thus were pooled into one group.

During the course of the study, the animals were observed daily and their weight was monitored. MCAO rats lost weight (20%) during the first postoperative days. In addition, at later time points, ibuprofen treatment seemed to decrease body weight in both sham-operated and in MCAO rats (4-8%) as compared to vehicle-treated rats.

5.1.2 Ibuprofen treatment does not change infarct volume nor mitigate inflammation

The infarct size in the cortex and striatum was measured from Nissl-stained sections in MCAO rats after the 28-day follow-up. There was no significant difference in infarct size between vehicle and ibuprofen-treated rats. Note that the thalamus was spared from acute ischemic damage because of its blood supply through the posterior cerebral artery.

Activation of astrocytes and microglia in the thalamus was measured from GFAP and OX-42-stained sections, respectively. A robust increase in both GFAP and OX-42 (Figure 9) staining was observed in the ipsilateral thalamus and in the areas adjacent to the infarct. Ibuprofen treatment increased OX-42 staining ($P<0.05$) as compared to vehicle treatment.

Rodent specific Aβ antibody was used to reveal the Aβ deposits in the thalamus. Calcium accumulation was measured from Alizarin red -stained sections. Both Aβ and calcium stainings were observed only in the thalamus of MCAO rats (Figure 9). With both stainings, statistical differences were observed only between sham-operated animals and MCAO animals. Ibuprofen treatment did not alter the extent of accumulation of Aβ or calcium in the thalamus.

5.1.3 Behavioral tests revealed no differences between the groups

All behavioral tests indicated that the MCAO rats were severely impaired shortly after the operation. A partial recovery was evident in all the behavioral tests, particularly in the limb placing test. Ibuprofen treatment did not have any significant effect on hindlimb function as measured in the tapered/ledged beam or in the cylinder test, which measures spontaneous forelimb use. The significant overall group effect was attributable to the difference between sham-operated and MCAO rats during the follow-up.

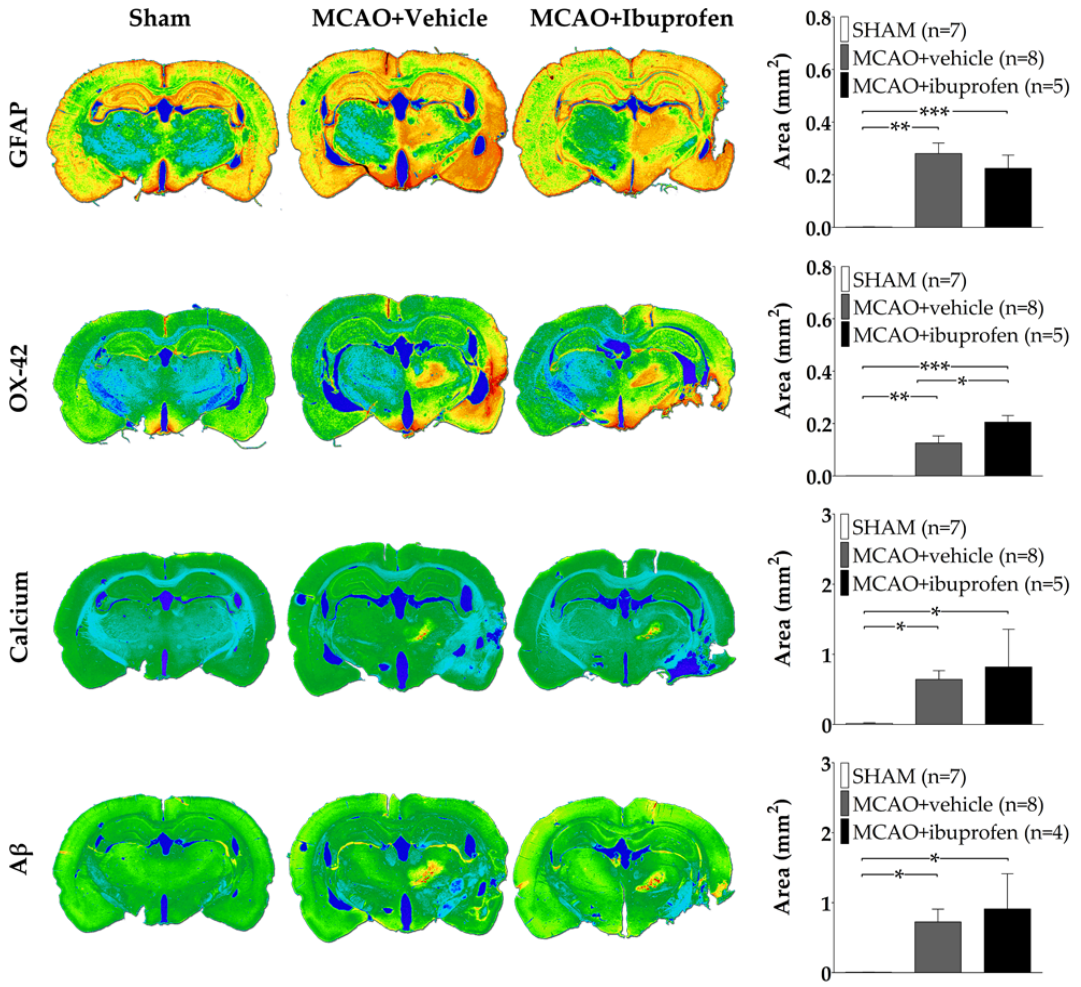


Figure 9: GFAP, OX-42, calcium and Aβ staining 29 days after MCAO in rats. The area of staining in the thalamus was measured by image analysis software (MCID; values are mean±SEM; **p<0.01; ***p<0.001). Robust GFAP and OX-42 stainings in the ipsilateral thalamus and cortex adjacent to the infarct area were observed in MCAO rats. Ibuprofen treatment (40 mg/kg/day) effect was seen in the OX-42 microglia staining between MCAO groups. Calcium and Aβ stainings were observed in the same areas in the ipsilateral thalamus in both MCAO groups.

5.2 STUDY II

5.2.1 Experimental groups

Eight out of a total of 31 animals were sham-operated. Two of sham-operated animals died during the follow-up due to unsuccessful *per os* administration. Twenty-three animals went through the MCAO-operation; of these 8 died within 48 h of surgery. One animal died during the follow-up. A total of two animals were excluded from the study because they had a limb placing test score 10 or higher. Thus the experimental groups were: sham+vehicle (n=6), MCAO+vehicle (n=7) and MCAO+bepridil (50 mg/kg; n=5). Bepridil treatment did not influence the body weight during the follow-up.

5.2.2 Calcium, Aβ40 and Aβ42 levels decline in the ipsilateral thalamus after bepridil treatment

After the 29-day follow-up, the thalamus samples were analyzed for levels of calcium, Aβ40 and Aβ42. Bepridil treatment decreased the measured levels as compared to vehicle treated animals (Figure 10). Correlation analyses revealed a positive correlation between the levels of Aβ42 and calcium (r=0-85, P<0.01) and between Aβ40 and calcium (r=0.74, P<0.01; data not shown) in the ipsilateral thalamus.

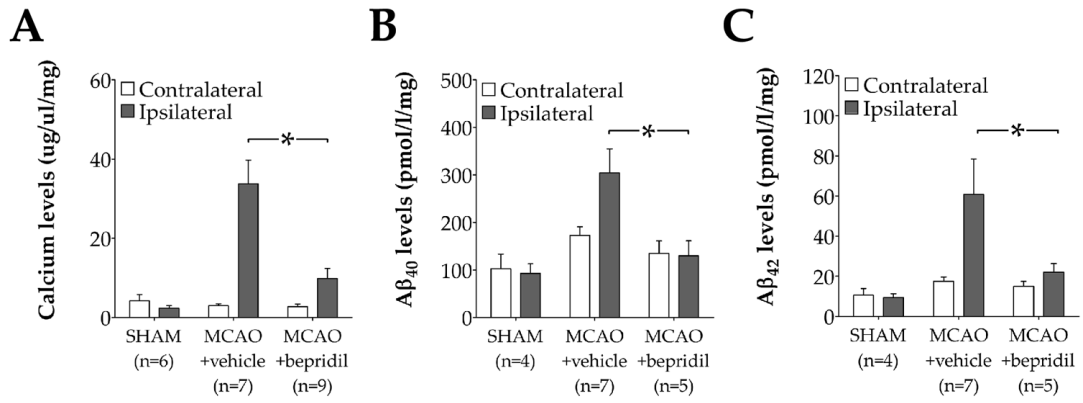


Figure 10: **A)** Calcium levels in the thalamus after 29-day follow-up. **B)** Aβ40 and **(C)** Aβ42 levels in the thalamus after 29-day follow-up. There was a statistical difference between bepridil- and vehicle-treated MCAO rats. Statistical analysis revealed a significant difference between bepridil- and vehicle-treated MCAO rats in the ipsilateral thalamus. Values are mean±SEM, *P<0.05.

5.2.3 Bepridil treatment does not influence the APP cleavage enzymes

Consistent with the previous studies [9], the levels of CTF were increased in the ipsilateral thalamus after MCAO (~1.4-fold). In addition, the levels of total APP were significantly decreased in the ipsilateral thalamus. Bepridil treatment did not change the GGA3 levels as compared to vehicle-treated animals, explaining also the increase (~1.8-fold) of BACE-activity in the ipsilateral thalamus in both MCAO groups. The activity of α- and γ-secretases did not differ between the MCAO groups. Sham-operated animals did not display any alterations in the GGA3 or BACE levels.

5.2.4 Bepridil treatment has no effect on levels of IDE, NEP, LRP, LTCC or on the inflammation response

In agreement with previous findings [9], IDE levels were significantly increased in the ipsilateral thalamus in both MCAO groups, whereas NEP and LRP levels remained unchanged in MCAO- and sham-operated animals. LTCC levels were significantly decreased after MCAO, without any differences between vehicle- and bepridil-treated MCAO animals. The levels of two inflammation markers, GFAP and TNF-α, were increased in the ipsilateral thalamus in both MCAO groups.

5.2.5 Bepridil restores seladin-1 mRNA and protein levels

Previous studies have shown that the mRNA levels of seladin-1, which in the normal situation protects cells from Aβ toxicity and oxidative stress, are down-regulated in brain regions vulnerable to AD [375,376]. Quantitative PCR analysis revealed a significant 30-40% decrease in GAPDH-normalized seladin-1 mRNA and protein levels in the ipsilateral thalamus after MCAO in rats treated with vehicle. In contrast, a similar change was not observed in the bepridil-treated MCAO rats.

5.2.6 Bepridil treatment improves the behavioral recovery of MCAO rats in the cylinder test

Behavioral measurements detected the presence of a severe motor deficit initially after MCAO operation, which was followed by a partial recovery. The significant group effect in all behavioral test was explained by the difference between sham-operated and MCAO-operated animals. Limb placing test or beam walking test did not show any statistical differences between the treatment groups. Spontaneous forelimb use, as measured by the cylinder test, revealed a statistical improvement in favor of bepridil treatment on post-operative day 28.

5.3 STUDY III

5.3.1 Experimental groups

In study III, the occlusion time was 90 minutes instead of the 120 minutes used in studies I and II. Twelve animals had to be excluded from the study since they had a score 10 or more in the limb placing test. Three animals died >48 h after the surgery and three animals died during the follow-up period. Weight loss was observed in the KB-R7943-treated MCAO rats.

5.3.2 Calcium, $A\beta_{40}$ or $A\beta_{42}$ levels in the thalamus does not differ between KB-R7943 and vehicle treated MCAO animals

Statistical analysis of calcium and Aβ in the thalamus did not reveal differences between the treatment groups. The mean ipsilateral thalamic levels for calcium was 24 ug/ul/g and for $A\beta_{40}$ 35 pmol/l/µg and for $A\beta_{42}$ 3.3 pmol/l/µg in the KB-R9743 treated rats and 20.6 ug/ul/g / 38.7 pmol/l/µg / 3.7 pmol/l/µg in the vehicle-treated groups (Figure 11).

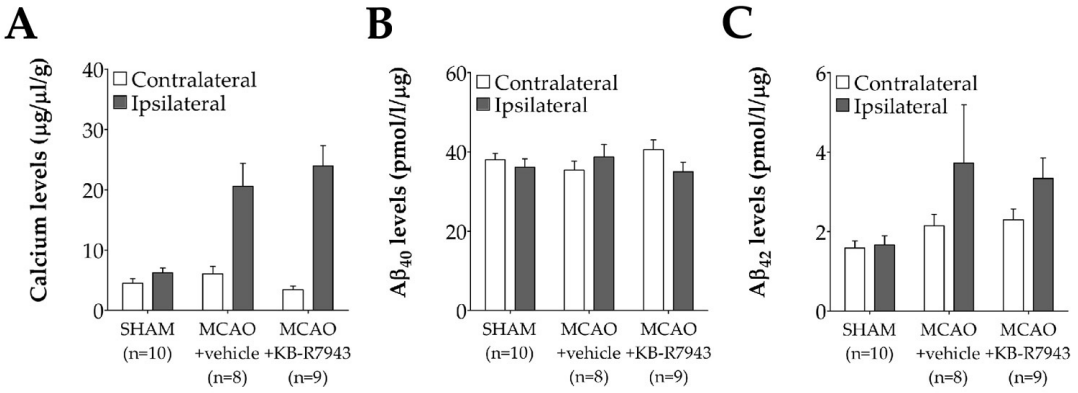


Figure 11: Analysis of **(A)** calcium, **(B)** $A\beta_{40}$ and **(C)** $A\beta_{42}$ in the thalamus after the 28-day follow-up. Statistical analysis did not detect any difference between MCAO groups. Values are mean±SEM.

5.3.3 Behavioral tests do not reveal any beneficial effect after KB-R7943 treatment

The limb placing test was used to analyze the sensorimotor, tapered/ledged beam for hindlimb, and cylinder for forelimb deficits. None of these behavioral tests detected any differences between the MCAO groups. An overall group effect was explained by the difference between sham-operated and MCAO animals.

5.4 STUDY IV

5.4.1 Experimental groups

In study IV, the total of 63 animals were used; 33 transgenic mice and 30 non-transgenic wildtype mice. The animals killed during the operations were euthanized due to an unsuccessful intravenous injection. Six animals died during the follow-up and a total of six animals had to be excluded from the study since they did not have any lesions according to the histological analysis. The groups were: Tg (transgenic)+bepridil 50 mg/kg (n=10), Tg+vehicle (n=12), nTg (non-transgenic)+bepridil 50 mg/kg (n=13) and nTg+vehicle (n=10). The cortical ischemic model caused an approximately 1 mm^3 lesion. A small but not statistically significant increase in the infarct volume was detected in the non-transgenic bepridil-treated animals after the 29-day follow-up.

From four to five months old $hAPP_{SL}$ transgenic and non-transgenic animals were used in this study. Already at the beginning of the study, wildtype mice were heavier than their transgenic littermates. The photothrombotic cortical lesion model caused a slight increase in the body weight shortly after the operation.

5.4.2 Behavioral tests do not reveal any differences between the groups

Behavioral tests for mice did not show statistical differences between the groups one month after the operation. Only in the beam test was there a trend that non-transgenic animals treated with bepridil made fewer slips in the most demanding beam (round and diameter 0.5 cm). The 60 s wire test or cylinder test did not reveal any differences between the groups.

5.4.3 Rodent Aβ and calcium accumulation decreases in bepridil-treated non-transgenic mice, but not in $hAPP_{SL}$ mice

A rodent specific Aβ antibody was used to reveal endogenous Aβ deposits in the thalamus after cortical photothrombosis. The genotype had a significant effect on Aβ load in the thalamus ($P<0.01$) and the accumulation of Aβ was more pronounced in non-transgenic animals ($P<0.01$) (Figure 12A).

A significant treatment-effect was seen in the Alizarin red –stained sections ($P<0.01$). However, there was no treatment and genotype interaction, indicating that bepridil-treatment affected similarly the transgenic hAPPSL and wildtype mice (Figure 12B).

5.4.4 Aβ deposits are extracellular when examined 30 days after the cortical lesion

Double immunofluorescence staining for rodent Aβ, neuronal nuclei (NeuN) and GFAP showed that Aβ-positive deposits were not localized in neurons or in astrocytes, evidence that Aβ deposits are extracellular for at least the first 30 days after the cortical lesion.

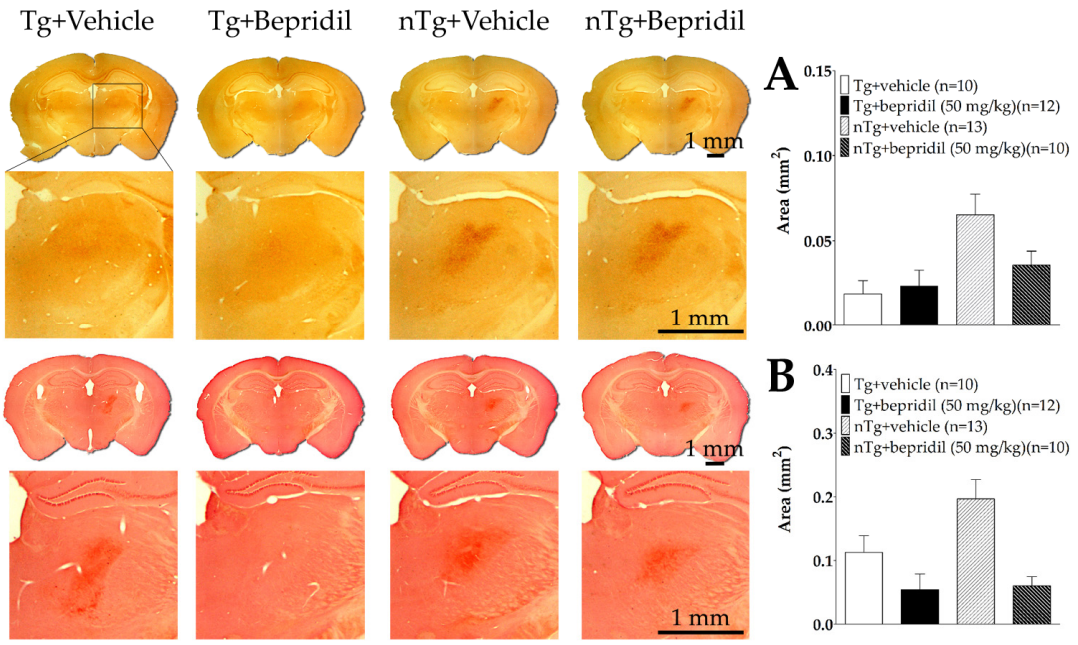


Figure 12: Representative images from rodent Aβ (upper panel) and Alizarin red –stained (lower panel) sections 30 days after photothrombotic cortical lesion. **A)** Quantitative analysis of Aβ staining. **B)** Quantitative analysis of Alizarin red –staining. Values are mean±SEM.

5.5 STUDY V

5.5.1 Experimental groups

In study V, six common marmosets (three males and three females) were used, from which four (3 males, 1 female) were subjected to MCAO and two (2 females) to the sham-operation. During the surgical procedure, arterial blood pressure, heart rate and body temperature remained stable within physiological ranges (mean arterial blood pressure: 67±6 mmHg; heart rate: 198±2 min-1; temperature 37.1±0.1 °C). Thalamus atrophy was seen in all the animals in the histological analysis (-21.8 ±7.4 %, mean ± SEM) but MRI imaging did not reveal any signs of neuronal degeneration in the thalamus at seven days after the MCAO. In addition, a hyperintense signal was found in substantia nigra in all of the animals with subcortical infarcts.

The histological analysis demonstrated patchy areas of calcium and Aβ-positive neurons in the ipsilateral cortex next to the infarct area. In one animal, calcium staining was observed also in the contralateral hemisphere (#9142). Remote regions like substantia nigra and hippocampus were occasionally stained for calcium. Sham-operated animals did not exhibit any calcium or Aβ-positive staining. On the contrary, GFAP immunostaining displayed a marked increase in the astroglial marker in the ipsilateral cortex and thalamus (see Figure 13).

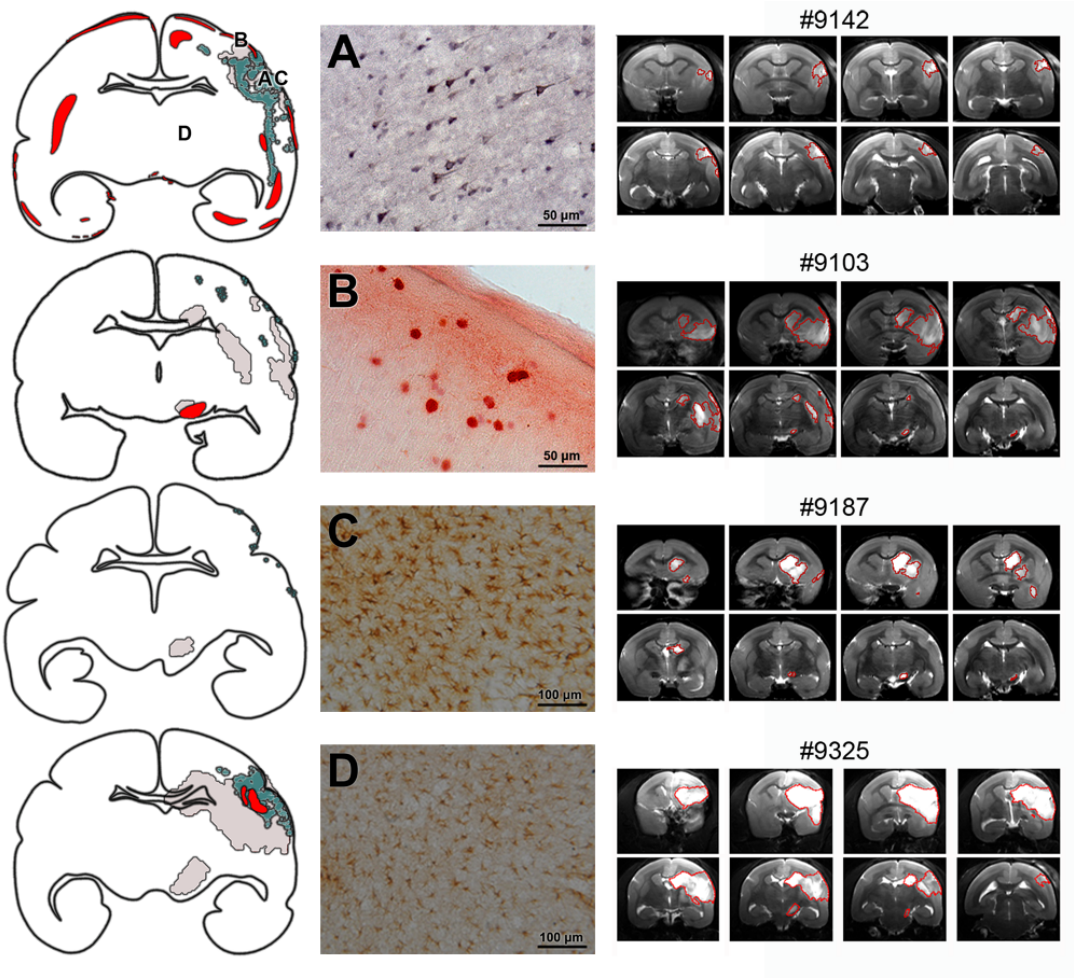


Figure 13: Representative illustration of pathological changes in marmoset brains 7 days (MRI images) and 45 days (immunohistochemical stainings) after MCAO. Grey areas in the schematic brain pictures on the left side images display the ischemic area corresponding to the MRI image from the same coronal level in the right column. Red areas represent the stainings for calcium, and the green areas are for Aβ. High magnifications images from patchy groups of Aβ-positive neurons **(A)** and calcium deposits **(B)** in the peri-infarct regions in the marmoset subjected to MCAO (#9142). Staining for glial fibrillary acid protein (GFAP) showed a greater extent of astrogliosis in the ipsilateral cortex **(C)** and thalamus **(D)** after MCAO.

6 Discussion

Stroke is one of the leading causes of death and disability worldwide [2,377]. Thrombolytic therapy is currently the only available drug treatment in the acute phases of stroke; its mode of action is to restore the blood flow to the ischemic brain before there is any permanent damage. However, thrombolytic therapy is only available for a small proportion of stroke patients due to the narrow therapeutic time window, contraindications to therapy, and limited access for the individuals living in remote regions (see [33,378]). An alternative strategy is to target secondary damage which would have a wider therapeutic time window.

The secondary pathology in stroke resembles in several respects that are seen in the AD [92,282,285,291], making it an interesting phenomenon for use as a stepping stone towards novel drug discoveries. There is ample evidence that ischemic stroke may contribute to the pathogenesis of AD. First, there is epidemiological studies indicating that risk factors for stroke are associated with AD [45]. Second, 60-90 % of AD patients exhibit cerebrovascular pathology at autopsy, and the coexistence of stroke and AD occurs more than can be accounted by chance alone [46] Third, stroke intensifies the presence and severity of the clinical symptoms of AD and increases AD pathology in the brain [47,48]. The following discussion starts with the methological issues, since these have to be taken into considerations in all stroke experiments. Most importantly, an attempt is made to find reasons why translation of stroke therapies from *in vitro* and *in vivo* studies to clinical trials has been so challenging [38]. The second part of discussion delves more into the results achieved from the five studies included in the thesis.

6.1 METHOLOGICAL CONSIDERATIONS

International recommendation such as STEPS (Stem Cell Therapies as an Emerging Paradigm in Stroke; [379]) and STAIR (Stroke Therapy Academic Industry Roundtable; [294]) provide guidelines and recommendations to improve the quality of research, which are also relevant to the studies conducted in this thesis.

When one assesses the possible pitfalls in the preclinical studies, the first point to be considered is anesthesia. It is well known that many anesthetic gases, like halothane used here, are neuroprotective [380]. It is important to note that halothane has also been shown to inhibit the activity of NCX [381]. Lidocaine and buprenorphine, which are used for postoperative care, have also putative neuroprotective properties. Taken together, this possible "second" treatment may counteract acute neuronal loss. However, it is unlikely that the anesthesia used during the operation would have any effect on delayed secondary pathology occurring after cerebral ischemia.

A relevant issue in drug research and *in vivo* testing is valid drug dose. In the present studies, maximal drug doses were selected based on values in the literature. The reasons why it was decided not to undertake a proper dose-response evaluation were the preliminary nature of the studies and complicated and laborious study design with additional experimental groups. Similar to dose, the route of dosing will need to be investigated more carefully in the future studies.

The time window for drug treatment is one of the major questions in preclinical stroke research. In the present studies the drug administration was started on postoperative day two since it was decided to avoid interference with the maturation of ischemic damage. Whether a more delayed start of treatment would have been more effective in targeting

secondary pathology, remains to be determined. As mentioned in the STAIR guidelines [294], also co-morbidities should be studied in greater detail, i.e., examining old animals with pre-existing hypertension, diabetes and hyperlipidemia (see [382]) or daily treatment with non-steroidal anti-inflammatory drugs (NSAIDs) [383]. For example, aged neurons are likely to display enhanced release of Ca^{2+} from the ER [384,385], diminished Ca^{2+} extrusion through the plasma membrane ATPase [386], reduced cellular Ca^{2+} buffering capacity due to impairment of the SERCA pumps [387], and a diminished mitochondrial Ca^{2+} sink capability [388,389]. It remains to be seen whether industry and academia would be willing to fund these complex studies mimicking real life scenarios after so many failures in the past.

The white and gray matter proportion difference between species may be one of the most important issues preventing preclinical data transfer to clinical practice. As seen in Table 1, the ratio between the grey and white matter differs from 12:1 (in mice) to 1.5:1 in humans, clearly emphasizing the evident physiological difference. This difference has been seen also in preclinical studies, where relatively a large lesion in rodent brain causes significantly smaller deficits in functionality as compared to humans. On the other hand, atrophy of the brain is an important biomarker for AD [390]; it seems that the early changes prior to the symptomatic onset of disease occurs in the white matter, which eventually lead to the pathological changes in the grey matter [391]. In addition to physiological differences between species, neuroprotective therapies that are effective in the gray matter do not necessary work in the white matter. MK-801 is good example of this phenomenon i.e., it was claimed to be neuroprotective against selective neuronal necrosis [392,393], but it was not able to attenuate white matter damage in cats [394]. However, the AMPA antagonist, SDB502, has been shown to reduce both grey and white matter damage in cats [395]. Indeed, blockade of sodium channels, calcium or AMPA receptors has been hypothesized to be important for white matter protection (see [396,397]) which could mean that targeting the similar channels in pre- and postsynaptic areas might eventually lead to a better secondary damage protection and recovery. A recent study with mice has also shown that a white matter injury differs depending on the age of the animal, e.g., a white matter injury in older mice is more related to glutamate activated AMPA/kainate receptors rather than to removal of calcium or inhibition of NCXs [398].

Functional behavioral tests have been developed to assess neurological outcome following stroke in rats, but there has been only modest success in determining a correlation between the size of ischemic injury and the extend of the behavioral impairment [340,369,399,400]. This may be partly related to the rapid spontaneous recovery of rodents or to the compensatory strategies that they develop. The battery of behavioral tests selected in the present studies are relatively easy for rats to perform. More demanding behavioral tests might have revealed differences between treatment groups, but on the other hand, the number of tests would have been lower due to time and personnel related issues. At the same time it has to be remembered, that all the extra handling and change in the environment may stress the animals and thus may worsen the behavioral outcome. Cognitive abnormalities are also common after ischemic brain injury [153,333], but these were not studied in this project.

It is noteworthy that ischemic brain injury does not result in long-lasting neurological deficits in ischemic animals [153,333], meaning that ischemic animals eventually seem to function normally in the behavioral tests, even though the ischemic lesion is still evident in the brain. After an ischemic brain injury in gerbils, impaired behavior lasted up to 6 months as revealed by longer exploration time in open-field test [401,402]. However, it must be acknowledged that any treatment-induced behavioral improvement may be due to enhanced compensatory learning (see [357,403]). To illustrate this possibility in a stroke-like behavior, if one hindlimb of a neurologically intact animal is peripherally incapacitated, tapered/ledged beam and treadmill walking and rotarod performance are compromised severely at first but gradually improve because the animal may learn new ways to rely on the other limbs or to use a tail-

based strategy for balance and movement [404]. Analysis of high speed video recording frame-by-frame is a powerful tool with which to differentiate true recovery and compensation.

One of the most important issues is blind assessment of all behavioral studies and randomization. In the present studies, the persons who tested the animals and later analyzed video recording were unaware of the treatment. Thus, one can be sure that these data were not biased in this respect. Allocation of animals to different experimental groups (randomization), may also bias the data. It was decided to assess the behavioral impairment after the operation, in order to exclude those rats with incomplete lesion and then the rest of the animals were allocated to behaviorally equal groups. The criteria for inclusion/exclusion were determined before the study.

Another aspect as important as the blind assessment and randomization, is to conduct power calculations prior to the studies. This is a mathematical method used to evaluate the number of animals needed in the study to achieve statistical differences in behavioral analysis.

6.2 INFLAMMATION IN STROKE AND AD

Proinflammatory pathways are triggered both in stroke [58,60,405,406] and AD [407,408]. In addition, it has long been known that individuals who have suffered severe or chronic periods of hypoxia are more likely to develop AD later in life [409]. Up-regulation of APP mRNA and protein after ischemia has been shown in various studies [14,410,411] to lead consequently to an increase in Aβ levels. Interestingly, many of the cell functions related to Aβ production seem to involve calcium signalling. Previous studies have shown that ibuprofen treatment decreases the formation of Aβ plaques in transgenic AD mice and improves functional outcome in association with a reduction of inflammatory mediators [412–418]. However, the preclinical studies have not been unambiguous [419]. For example, Morihara and co-workers have proposed that ibuprofen may interact via interleukin pathways rather than via impairing the production of Aβ to prevent AD in mice models [420]. In ischemia studies, the putative pathways/targets are ibuprofen suppression of NMDAR induced cell death [421], reduced striatal damage via suppression of ICAM-1 [422], and/or cyclooxygenase inhibition during reperfusion [423]. In addition, systemic inflammation may be one of the common causes leading to a worse outcome in stroke [118,119] and to accelerating the onset of AD [120,121]. The importance of BBB breakdown in both diseases is evident, the main difference is in the robustness of blood-derived cells penetrating into the brain [123,124,136]. Logically, one could speculate that this knowledge forms the basis for the hypothesis of using NSAIDs as an acute treatment for stroke and as a preventive treatment for AD. However, randomized clinical trials summarized in a large meta-analysis have not proven the preventive hypothesis of these drugs in AD [424]. In addition, the chronic use of NSAIDs in clinical trials has been claimed to increase the risk of stroke [425].

In the present study, it was decided to evaluate the anti-inflammatory hypothesis in MCAO rats with ibuprofen treatment starting on day two after ischemia and to follow the functional recovery with a battery of behavioral tests.

Ibuprofen treatment (40 mg/kg/day, *per os*) did not exert any beneficial effects during the 28-day follow-up. The ibuprofen-treated animals lost more weight during the first days of recovery after stroke than the vehicle-treated animals. However, the behavioral tests did not reveal any differences between the treatment groups. Histological staining for astrocytes in the ipsilateral thalamus did not detect difference between the MCAO groups. Instead, microglia staining did exhibit an increase in the ipsilateral thalamus of the ibuprofen-treated animals, which is quite opposite to the original hypothesis.

In conclusion, ibuprofen treatment did not prevent or modify secondary thalamic changes in the thalamus. This may have been because the dose was too low or due to inadequate transportation into the thalamus through the BBB. However, the delayed recovery (slow weight gain) indicates that with *per os* administration this is the maximum dose of ibuprofen which can be given in chronic administration. It seems that the underlying mechanisms of secondary pathology in the thalamus in stroke cannot be reversed by treatment with a non-steroidal anti-inflammatory drug.

6.3 CALCIUM CHANNELS AS A TARGET FOR STROKE AND AD

The influence of calcium dysregulation on the metabolism and production of Aβ has not been thoroughly studied as the role of Aβ in calcium signaling. Nevertheless, recent studies have revealed changes in calcium levels and dynamics sufficient to alter the metabolism and production of Aβ, leading to the proposal that of calcium may be one of the causative factors in late-onset AD [426].

During the past decade drug discovery for AD has focused on inhibitors of γ-secretase and BACE responsible for the cleavage of Aβ peptide from APP [427–429]. Studies with positive behavioral outcomes have been conducted with anti-BACE treatment in transgenic model of Aβ pathology, even though that the burden of Aβ in the brain did not change [430]. This indicates that other cascades, rather than the formation of Aβ plaques, have a major impact on the development of the disease. One suggestion is that there is a pre-existing calcium dysfunction in the brain. This raises the possibility of determining whether prevention of calcium accumulation by treatment with calcium channel blocking drugs could also decrease the Aβ burden.

The major sources of intracellular Ca^{2+} are influx through NMDARs or VDCC and release of Ca^{2+} from intracellular Ca^{2+} stores. Calcium channel inhibitors have been designed to target VDCC and thus prevent intracellular calcium overload. Earlier studies have shown that Aβ causes an increase in the intracellular calcium content via VDCC and calpain related functions [282], which in turn leads to further increases in Aβ production. Epidemiological studies have revealed that calcium channel inhibitors prevent [431] or slow the rate of progression of AD [432,433].

Bepridil is a diarylaminopropylamine derivate having both antianginal and antiarrhythmic effects [434]. It has multiple inhibitory effects on ionic currents, including the L-type [435] and T-type Ca^{2+} currents [436], as well as on K^+ channels [437–440] and Na^+ channels [441]. In addition, bepridil is known to inhibit BACE mediated cleavage of APP both *in vitro* and *in vivo* by increasing the pH in the endosomal compartments [442]. Furthermore, bepridil modulates γ-secretase-mediated APP cleavage independently of endosomal alkalization.

Previous studies have shown that NCX, operating in the forward mode, is more strongly inhibited by bepridil rather than in the reverse manner of action [443,444]. The site of action of bepridil may be located on the cytoplasmic side of the exchanger [434], thus raising the question of whether other calcium channel inhibitors would be more effective against the NCX by targeting the extracellular compartments of the exchanger. In *in vitro* studies, bepridil has been shown also to bind to albumin [434], thus decreasing the amount of free bepridil available to cross the BBB. Another protein compromising the free amount of bepridil is alpha-1-acid glycoprotein, which has high affinity for bepridil [445,446]. These previous results together with the hypothesis that calcium may be the primary trigger for caspase-3 mediated GGA3 cleavage leading to overexpression of BACE and thus increase of Aβ production [9,275,276] are the reasons why it was decided to test whether bepridil treatment could prevent calcium and APP/Aβ dysfunction in the thalamus after experimental ischemia.

Studies II and IV evaluated the effects of treatment with a non-selective calcium channel inhibitor, bepridil, after experimental focal ischemia in rats and in AD transgenic mice. The ischemic methods selected are described in more detail described in the materials and methods. The main difference between studies being the intra-arterial filament model was used in rats, whereas photothrombotic occlusion was used in mice.

In study II the transient filament model induced a large cortical infarction in the ipsilateral hemisphere and caused a severe initial sensorimotor impairment. Bepridil (50 mg/kg/day, *per os*) treatment decreased calcium and soluble $A\beta_{40}$ and $A\beta_{42}$ levels in the ipsilateral thalamus of MCAO rats when it was assessed at the 29-day follow-up. In Pearson correlation analysis, calcium levels strongly correlated with the soluble $A\beta_{42}$ levels evidence for an association between these two factors. In addition, there was also a significant improvement in the rat's performance in the forelimb function measuring cylinder test in the bepridil-treated MCAO rats 28 days after the ischemic onset as compared to the vehicle treated MCAO rats. However, bepridil treatment did not change the expression of the inflammation markers (GFAP and TNF-α) in the ipsilateral thalamus in the western blot analysis. In addition, expressions of IDE, NEP and LRP did not differ between the bepridil and vehicle treated MCAO, suggesting that some other factor(s) must play a more important role in Aβ clearance after stroke.

Bepridil treatment (50 mg/kg/day, *per os*) in study IV was started two days after the photothrombotic cortical lesion. At the follow-up assessment, bepridil treatment seemed to have increased infarct volumes in the transgenic AD animals, but not in the wildtype littermates. Increases in infarct volumes have been observed in previous transgenic AD-mice studies, when compared with wildtype mice [204,447,448], supporting the concept of increased susceptibility to ischemia as a result of neurotoxic effects of Aβ in the post-ischemic brain. On the contrast, in this present study the presence of the transgene did not effect the infarct size in vehicle treated animals. One possible explanation is the fact that quite young animals (4-5 months) were used at a timepoint, at which the early signs of Aβ plaque formation have been seen [449,450]. In addition, Aβ and calcium analyses were performed in the thalamic areas, and it was observed that cortical lesion increased Aβ and calcium burden in the non-transgenic animals, but not as much as in the transgenic animals. Indeed, bepridil treatment seemed to decrease the Aβ burden in the non-transgenic animals, but no effect was seen in the transgenic AD animals. Statistical analysis revealed that bepridil treatment decreased the calcium burden in both genotypes.

The behavioral tests in study IV did not detect statistical differences between the groups. This might be due to the two different neurological disorders, which should be tested so that common symptomatic impairments in both diseases could be distinguished, e.g., cognitive tests that would to measure AD symptoms and sensorimotor tests that would assess stroke-related impairment. The small size of mice will make it necessary that more sophisticated behavioral tests are used to avoid any possible compensatory movements.

Based on the histological results, one might say that in the early phase of AD development, the underlying APP/Aβ cascades may even be protective against cortical stroke.

6.4 A REVERSE SODIUM/CALCIUM CHANNEL INHIBITOR DOES NOT HINDER SECONDARY DAMAGE AFTER STROKE

Similar to bepridil (used in studies II and III), originally KB-R7943 [2-[2-[4-(4-nitrobenzyloxy)phenyl]-ethyl]isothiourea methanesulfonate] was developed for treatment of cardiac arrest, a condition where calcium overload triggers arrhythmias in the heart muscle. Recent reports have shown that KB-R7943 exerts an inhibitory effect on several other ionic

transport mechanisms, such as L-type VDCC. Moreover, KB-R7943 has been reported to inhibit NMDAR [270], a process already described important in ensuring intracellular Ca^{2+} homeostasis [223]. In addition, KB-R7943 can inhibit the reverse mode of NCX more potently than the forward mode [451,452]. Interestingly, KB-R7943 seems to have a different mechanism of action to block NCX activity depending on the gene product involved; NCX3 inhibition requires concentrations that are 3-fold lower than those needed to inhibit NCX1 and NCX2 [453]. In contrast to bepridil, KB-R7943 the site of action is located in the extracellular membrane [454]. Previous studies have also indicated that KB-R7943 would be a more neuroprotective compound than bepridil [269].

It has been speculated that KB-R7943, with its property to work as an inhibitor for Ca^{2+} influx without effecting the normal Ca^{2+} efflux [455], could be a better brain homeostasis stabilizer in ischemic conditions. Study IV tested whether chronic administration of KB-R7943 (30 mg/kg/day, *per os*) would prevent secondary neuropathological changes after focal ischemia in rats. KB-R7943 treated MCAO rats seemed to recover from the post-operative phase more slowly than vehicle treated MCAO rats based on the weight gain. Behavioral tests did not reveal any differences between the treatment groups (beam data not shown). Biochemical analyses from the thalamic samples also found no differences between the treated MCAO groups.

In conclusion, 30 mg/kg/day KB-R7943 treatment starting two days after the initial ischemic insult did not prevent calcium and Aβ accumulation in the thalamus nor was any behavioral recovery seen.

6.5 RODENT AND MARMOSET STUDIES – LOST IN TRANSLATION

Although decades of research in the field of stroke have produced tremendous amount of new knowledge about the pathology of the disease, it has to be stated that we only partially understand the exact chains of events that begin with the occlusion and end with the delayed stroke-related pathological changes.

One of the solutions may be the use of non-human primates as a model of stroke [330]. The translational barriers between rodents and humans are poorly defined and the anatomical and physiological differences between the rodent and human brain have been frequently cited as potential reasons for pitfalls encountered in extrapolating experimental work to clinical success [456–458]. For example, multiple studies have detected APP/Aβ accumulation in the thalamus after experimental stroke in rats [92,213,274], but this is not seen in *post mortem* samples from patients with cerebrovascular lesions [459].

The study of Tagaya and colleagues showed also that the neuronal response to acute ischemia is significantly different between rodents and baboons [460]. Notably also studies cross-checking the calcium related NMDAR inhibitor and sodium channel inhibitor effect on primates and rodents have revealed distinctly different outcome results between the species [461]. In addition, several reports have described Aβ plaques in aged marmosets, suggesting that this species may represent a novel animal model of Alzheimer's disease [462,463]. The use of common marmosets in the stroke-research could easily combine all the important factors needed in the preclinical generation of new treatments, i.e., behavioral testing [464], imaging and histology [465,466].

The ethical challenges inherent in primate research must be carefully considered in embarking on experiments with primates. The argument often made to justify primate stroke research tries to weight the discomfort experienced by the primate subjects against risk avoidance in subsequent human studies and potential benefits to humans through new discoveries [467,468].

In study V, common marmosets underwent 3 hour transient MCAO. This filament model is identical to the model used in rat studies I, II and III. Although the number of animals was low and areas of infarct varied from animal to animal, one common finding was observed in all the subcortically infarcted marmosets; a hyperintense MRI signal originating from the substantia nigra. Alizarin red staining for calcium was also occasionally observed in the substantia nigra. The inflammation marker, GFAP, showed evidence of an increase in astroglia reaction in the ipsilateral cortex and thalamus. Most important, there was absolutely no evidence of calcium or Aβ pathology in the thalamus.

These results suggests that the secondary changes occurring in common marmosets after a stroke are different to those observed in rodents. Due to the low number of animals, further studies will be required to verify this conclusion.

6.6 FUTURE PERSPECTIVES

Drug development for acute stroke has proven to be a challenging and difficult road. More than 1000 neuroprotective compounds have been shown to be effective in experimental models, but although 114 of these compounds have been tested in stroke patients, it is disappointing that not one has been clinically effective [38]. Although there was a broad definition of what was meant by the term neuroprotection (e.g., intention to prevent stroke-related neuronal death, restoring blood flow or merely investigating the mechanism of stroke), the result emphasizes the importance of critical preclinical research and for taking common directions towards additional phase III preclinical trials. The reasons for failures have been discussed [293,294,379,457], but may be partly related to the extremely narrow therapeutic time window. Thus, alternative strategies are urgently needed. One of the most plausible strategies to overcome the roadblocks in the translation from bench to bedside is international collaboration between basic scientists and clinicians. Appeals have been made for more large-scale collaboration and harmonized ways to undertake stroke-research need to be established [469,470]. As highlighted in the report from Howells et al. [471], multicentral preclinical collaboration with additional computational and in vitro-studies might be a way to reduce drug development costs by as much as 31 %.

However, less attention has yet been paid to the secondary degeneration in remote regions after cerebral ischemia. This has been detected in rodents [89,110,117] and imaging data from stroke patients suggest that there is some degree of shrinkage in the thalamus [80]. The secondary pathology typically develops in delayed manner, thus offering a target for stroke management since it would have a much wider therapeutic time window. There are preliminary data, including work done in this thesis, showing that this secondary pathology is amenable to treatment [132,142,359–364]. However, the question remains whether these effects can be translated into clinical success. The present data from marmoset revealed a lack of Aβ and calcium pathology, suggesting that results obtained from rodents may not be relevant to stroke patients. On the other hand, understanding the reason for the difference may provide a hint of the direction that should be taken by future research.

Another striking feature in the secondary pathology is the similarity to AD pathology. One immediate question arises: can we use this as a "fast" model for AD pathology? AD is a slowly developing neurodegenerative diseases and its pathology is usually seen in old animals. On the other hand, it is not clear whether removing, dissolving or dissociating Aβ aggregates, the type of neuropathology often seen towards the end stage of disease, will be helpful. Often such treatments have secondary effects that are more devastating than the disease itself. In addition, regrettably few studies have investigated the axonal changes affecting outcomes in stroke and AD.

As a conclusion, there is an obvious association between stroke and AD. Changes in human vascular pathology have been revealed to increase the risk of AD [45], similarly to the presence of AD pathology in the human brain has been shown to increase the risk of stroke [45,48]. As seen in experimental rodent studies, since calcium and Aβ exhibit two distinct, but also overlapping pathologies, one could speculate that targeting their crossroads could lead to a common therapy for both diseases.

7 Conclusions

The inflammatory process related to the secondary pathology in the thalamus was not affected by treatment with the anti-inflammatory drug, ibuprofen, or by the non-specific calcium channel inhibitor, bepridil.

Bepridil alleviates the accumulations of calcium and Aβ observed in the thalamus after cerebral ischemia, and this was to some extent reflected into the behavioral improvement of the rats. A specific inhibitor of the reverse Na^+/Ca^{2+} exchanger KB-R7943 did not lessen the pathology or sensorimotor impairment in MCAO rats indicating that excessive Ca^{2+} influx into the axoplasm during retrograde degeneration does not play major role in the secondary pathology occurring after an ischemic lesion.

The secondary pathology in the thalamus after cerebral ischemia was a consistent phenomenon in rats and mice, but it was not detected in non-human primates. This is in agreement with *post mortem* studies in patients with cerebrovascular lesions. The reason for the different pathology remains to be elucidated.

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Anu Lipsanen

Secondary Neuropathology after Experimental Stroke

With Special Emphasis on Calcium, Amyloid-β and Inflammation

Stroke and Alzheimer's disease (AD) are the leading causes of disability. This thesis aims to study the secondary neuropathology after experimental stroke, which is strikingly similar to that in AD. It appears that non-specific calcium channel blocker, bepridil, prevents calcium and amyloid-beta accumulation in the thalamus after stroke and this improves functional recovery. The thesis also showed that secondary neuropathology in rodents after stroke was not observed in non-human primates, which complicates the translation of experimental data to clinical practice.

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