A Path for Improving Stroke Recovery. Effects of MEK-ERK1/2 Inhibition

Mostajeran, Maryam

2018

Document Version:
Publisher's PDF, also known as Version of record

A Path for Improving Stroke Recovery

Effects of MEK-ERK1/2 Inhibition

MARYAM MOSTAJERAN
DEPARTMENT OF CLINICAL SCIENCES | FACULTY OF MEDICINE | LUND UNIVERSITY

A Path for Improving Stroke Recovery

Effects of MEK-ERK1/2 Inhibition

Maryam Mostajeran

LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Segerfalksalen, Wallenberg Neuroscience Center, BMC, Lund, Sweden. Date: Thursday the 20th of December, 2018, at 09:00.

Faculty opponent

Associate Professor Rayna Gonzales
Department of Basic Medical Sciences,
College of Medicine, The University of Arizona, Phoenix, USA

Organization	Document name
LUND UNIVERSITY	DOCTORAL THESIS
Experimental Vascular Research Department of Clinical Sciences Faculty of Medicine	Date of issue 20th of December, 2018
Author: Maryam Mostajeran	Sponsoring organization: Lund University

Title and subtitle: A Path for Improving Stroke Recovery - Effects of MEK-ERK1/2 Inhibition

Abstract

The present thesis aimed to shed more light on the notion of acute inhibition of extracellular-signal regulated kinase (ERK)1/2 pathway as a treatment to improve stroke recovery. Stroke is a major cause of death and long-term disability worldwide, classified as ischemic or hemorrhagic. In ischemic stroke, interruption of blood flow and deprivation of oxygen and glucose activate death signalling pathways within the area directly affected by ischemia so called “core”. In the region adjacent to core, peri-infarct, cells are hypoperfused, functionally inactive and prone to death if therapeutic strategies do not rescue them.

Treatments for ischemic stroke are limited to thrombolysis or thrombectomy. Due to narrow time window and risk of hemorrhagic transformation, few percentages of stroke patients are eligible to receive these treatments. Another approach in stroke therapy is to rescue neurons within peri-infarct region. Despite promising effects of neuroprotective agents in experimental stroke, this approach has failed in clinical trials.

Although the ERK1/2 pathway is involved in recovery processes during later stage of stroke, it is a critical modulator of destructive mechanisms i.e. upregulation of cerebrovascular receptors during acute phase. Our previous studies showed that early inhibition of the ERK1/2 pathway reduced ischemic damage and improved functional outcome after experimental stroke. These findings are the base of the present thesis. Although promising, the beneficial effects were only observed during acute phase in male rats. Our hypothesis is that acute inhibition of ERK1/2 pathway will continue to show benefit beyond the acute phase and not negatively interfere with later recovery processes. Further, the present thesis addresses important aspects that should be considered when developing a new treatment such as sex and clinical relevant time point.

Thus, this thesis addressed acute blockade of ERK1/2 pathway with regards to important aspects when developing a new treatment. The important aspects evaluated in the present thesis are as follow: (i) Beneficial outcome beyond acute phase and related recovery mechanisms, (ii) A time-window relevant to the clinic, (iii) Acute detrimental mechanisms and effect of U0126 in female rats and (iv) repair-related molecular changes during recovery phase of stroke in female rats. The experimental setup of the thesis was based on an ischemic model on rat. Inhibition of the pathway was achieved by U0126, a inhibitor of mitogen activated protein kinase kinase (MEK)1/2 which is immediately upstream of ERK1/2. In summary, the results of the present thesis showed that acute inhibition of MEK-ERK1/2 pathway is a promising potential treatment for stroke. It has been applicable in a clinically relevant time-window and beneficial for both sexes with persistence of improved functional outcome beyond acute phase. In addition, repair-related molecular changes and activation of ERK1/2 during recovery phase in female rats further supported the idea that ERK1/2 pathway contributes to recovery processes in later stage of stroke. Thus, a path well-investigated may actually lead to better stroke recovery and a future stroke treatment.

Key words: Ischemic stroke, Extracellular-signal regulated kinase (ERK)1/2, Mitogen activated protein kinase kinase (MEK)1/2, Recovery, Functional outcome, sex

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language
Lund University, Faculty of Medicine Doctoral Dissertation Series 2018:158		English
ISSN and key title 1652-8220		ISBN 978-91-7619-727-1
Recipient’s notes	Number of pages 70	Price
	Security classification	

Signature  Date 2018-11-14

A Path for Improving Stroke Recovery

Effects of MEK-ERK1/2 Inhibition

Maryam Mostajeran

LUND
UNIVERSITY

Coverphoto adapted with permission from California Brain Injury Support Center and modified by Maryam Mostajeran.

Experimental Vascular Research
Department of Clinical Sciences
Faculty of Medicine Doctoral Dissertation Series 2018:158
Lund University

ISBN 978-91-7619-727-1
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2018

Media-Tryck is an environmentally certified and ISO 14001 certified provider of printed material.
Read more about our environmental work at www.mediatryck.lu.se

To my beloved family

Table of Contents

List of Original Articles

This doctoral thesis is based on the following articles:

I. **Mostajeran M**, Edvinsson L, Warfvinge K, Singh R, Ansar S.
Inhibition of mitogen-activated protein kinase 1/2 in the acute phase of stroke improves long-term neurological outcome and promotes recovery processes in rats".
Acta Physiol (Oxf), **(2017)** April 219(4):814-824.

II. **Mostajeran M**, Wetterling F, W Blixt F, Edvinsson L, Ansar S.
Acute mitogen-activated protein kinase 1/2 inhibition improves functional recovery and vascular changes after ischemic stroke in rat-monitored by 9.4 T magnetic resonance imaging.
Acta Physiol (Oxf), **(2018)** May 223(1):e12985. Epub 2017 Nov 12

III. Ahnstedt H, **Mostajeran M**, Blixt FW, Warfvinge K, Ansar S, Krause DN, Edvinsson L.
U0126 attenuates cerebral vasoconstriction and improves long-term neurologic outcome after stroke in female rats".
Journal of Cerebral Blood Flow and Metabolism, **(2015)** Mar 35(3):454-460

IV. **Mostajeran M**, Edvinsson L, Ahnstedt H, Arkelius K, Ansar S.
Repair-related molecular changes during recovery phase of ischemic stroke in female rats.
Submitted Manuscript

Abbreviations

ADC	Apparent diffusion coefficient
Ang	Angiopoietin
ATP	Adenosine triphosphate
CBF	Cerebral blood flow
CBV	Cerebral blood volume
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
DSC	Dynamic susceptibility contrast
ERK1/2	Extracellular-signal regulated kinase 1/2
ET-1	Endothelin-1
ET_A	Endothelin receptor type A
ET_B	Endothelin receptor type B
GFAP	Glial fibrillary acidic protein
IL-10	Interleukin-10
MAPK	Mitogen activated protein kinase
MCA	Middle cerebral artery
MEK1/2	Mitogen activated protein kinase kinase 1/2
MRI	Magnetic resonance imaging
NeuN	Neuronal specific nuclei protein
PBS	Phosphate buffered saline
SIS	Silver infarct staining
SMC	Smooth muscle cell
TGF-β	Transforming growth factor-β
Tie-2	Tyrosine kinase receptor Tie-2
tMCAO	Transient middle cerebral artery occlusion
t-PA	Tissue-type plasminogen activator

Thesis Summary

The present thesis aimed to shed more light on the notion of acute inhibition of extracellular-signal regulated kinase (ERK)1/2 pathway as a treatment to improve stroke recovery. Stroke is a major cause of death and long-term disability worldwide, classified as ischemic or hemorrhagic. In ischemic stroke, interruption of blood flow and deprivation of oxygen and glucose activate death signalling pathways within the area directly affected by ischemia so called "core". In the region adjacent to core, peri-infarct, cells are hypoperfused, functionally inactive and prone to death if therapeutic strategies do not rescue them.

Treatments for ischemic stroke are limited to thrombolysis or thrombectomy. Due to narrow time window and risk of hemorrhagic transformation, few percentages of stroke patients are eligible to receive these treatments. Another approach in stroke therapy is to rescue neurons within peri-infarct region. Despite promising effects of neuroprotective agents in experimental stroke, this approach has failed in clinical trials.

Although the ERK1/2 pathway is involved in recovery processes during later stage of stroke, it is a critical modulator of destructive mechanisms i.e. upregulation of cerebrovascular receptors during acute phase. Our previous studies showed that early inhibition of the ERK1/2 pathway reduced ischemic damage and improved functional outcome after experimental stroke. These findings are the base of the present thesis. Although promising, the beneficial effects were only observed during acute phase in male rats. Our hypothesis is that acute inhibition of ERK1/2 pathway will continue to show benefit beyond the acute phase and not negatively interfere with later recovery processes. Further, the present thesis addresses important aspects that should be considered when developing a new treatment such as sex and clinical relevant time point.

Thus, this thesis addressed acute blockade of ERK1/2 pathway with regards to important aspects when developing a new treatment. The important aspects evaluated in the present thesis are as follow: (i) Beneficial outcome beyond acute phase and related recovery mechanisms, (ii) A time-window relevant to the clinic, (iii) Acute detrimental mechanisms and effect of U0126 in female rats and (iv) repair-related molecular changes during recovery phase of stroke in female rats. The experimental setup of the thesis was based on an ischemic model on rat. Inhibition of the pathway was achieved by U0126, a inhibitor of mitogen activated protein kinase kinase (MEK)1/2 which is immediately upstream of ERK1/2. In summary, the results of the present thesis showed that acute inhibition of MEK-ERK1/2 pathway is a promising potential treatment for stroke. It has been applicable in a clinically relevant time-window and beneficial for both sexes with persistence of improved functional outcome beyond acute phase. In addition,

repair-related molecular changes and activation of ERK1/2 during recovery phase in female rats further supported the idea that ERK1/2 pathway contributes to recovery processes in later stage of stroke. Thus, a path well-investigated may actually lead to better stroke recovery and a future stroke treatment.

Background

Stroke

Stroke is a major cause of death around the world. In case of survival, majority of stroke patients stay with neurological deficits leaving stroke as a major cause of disability worldwide with a great socioeconomic burden. Stroke is defined as a neurological deficit by a cerebrovascular cause resulting from interruption of blood flow to the brain and classified as either ischemic or hemorrhagic (Figure 1). Hemorrhagic stroke is caused by bleeding into brain tissue when a blood vessel bursts. Decrease of blood supply in ischemic stroke can result from thrombosis or embolism [1, 2].

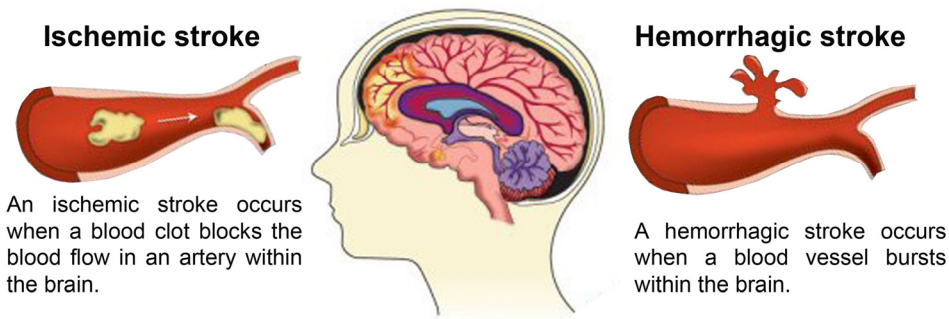

Figure 1. Different types of stroke.
Stroke is classified as either ischemic or hemorrhagic. Adapted from Centers for Disease Control and Prevention (www.cdc.gov)

Ischemic stroke

More than 80% of all strokes are ischemic [1, 3]. This thesis is based on an animal model of ischemic stroke, hence the following information address this type of stroke.

Pathophysiology

Loss of blood flow during ischemic stroke leads to oxygen and glucose deficiency. As a consequence, a cascade of cellular and molecular events are activated which result in an ischemic damage. In ischemic core, the region immediately affected by the stroke, the deficiency in oxygen and glucose leads to energy failure and lack of adenosine triphosphate (ATP). Without ATP, neurons cannot preserve their ionic gradient across the cell membrane, thereby cytoplasmic accumulation of sodium and calcium leads to the loss of membrane integrity and eventually cell death. In the peri-infarct, tissue surrounding the ischemic core, blood flow is sufficiently reduced to cause hypoxia and arrest physiological function. But, it is not severe enough to cause irreversible damage and cellular necrosis. Moreover, collateral blood flow from adjacent tissue keeps the blood flow above the threshold. Therefore, peri-infarct is a dynamic area and neuronal damage develops more slowly, leaving a time window for therapeutic opportunity to rescue dying neurons. The cell death mechanisms in the peri-infarct region is an active process largely dependent on the activation of caspase-dependent and caspase-independent apoptotic pathways, which contribute to delayed ischemic cell death mainly due to inflammation and free radical formations [3-5].

Spontaneous recovery

Interruption of blood flow during ischemic stroke leads to brain cell death and impaired brain function. Hence stroke patients may experience paralysis, loss of vision, memory loss and impaired speech[3]. However, stroke also triggers defensive mechanisms to counteract cell damage and there is evidence for a growth-promoting region in peri-infarct [6]. Thereby, most of stroke patients show some degree of spontaneous recovery over time following their initial injury. For instance, a study on arm disability showed that 80% of patients achieved their maximum arm function within 3 weeks and 90% of patients within 9 weeks of stroke onset [7]. Such studies provide information about patterns of spontaneous behavioural recovery but give limited insights into underlying molecular and cellular mechanisms [7, 8] . Furthermore, due to the loss of neurons with highly specific function, post stroke behavioral outcome is unlikely to be identical with pre-stroke patterns. Behavioral assessment protocols also cannot distinguish whether improved functions reflect true recovery, behavioral compensation or their combination. So, recovery reflects most of the time improved performance without considering the degree of compensation or true recovery [9]. It should be once more noted that post stroke recovery is incomplete. Thus, understanding the underlying mechanisms is important to develop or improve therapeutic stroke strategies. Similar patterns of improved functional outcome are also observed in animal models of stroke. Animal studies have shown that a set of highly

interactive processes such as angiogenesis, neurogenesis and upregulation of repair-related molecules i.e. inflammatory markers and growth factors act as underlying recovery mechanisms [8, 10].

Stroke phases

Based on the pathological characteristics, occurrence of spontaneous recovery and timing post-stroke, a stroke is generally classified into three clinical phases: the acute phase, subacute phase, and chronic phase [11]. In animal studies post-stroke phases have similar pattern to human patients but typically much shorter [4, 9]. The major cascades include primary neuron loss, secondary ischemic damage, brain edema, neuroinflammation, dead cell removal, endogenous plasticity, improving impairment and function. The duration and pathological severity of the three phases vary among individuals. The variation depends on the specific conditions of individuals such as location and size of lesion, the presence of cerebrovascular collateral circulation, patient's age, sex and comorbidities [12].

As mentioned above, a cascade of cellular and molecular mechanisms contribute to post stroke pathophysiology and recovery to make final stroke outcome. In the following sections, only those which have been mostly addressed in the present thesis are described more in details.

Endothelin system

Ischemic stroke affects not only neurons and glial cells but also the vasculature. Upregulation of vasoconstrictor receptors on smooth muscle cells (SMCs) and increased receptor-mediated vasoconstriction have become a novel aspect in the pathophysiology of ischemic stroke in recent years [13].

In particular, there are several studies showing a major role for the endothelin system in the pathophysiology of ischemic stroke [14]. For instance, the level of Endothelin 1 (ET-1) is increased in cerebrospinal fluid and cerebral tissue following clinical and experimental ischemic stroke [15, 16].

ET-1, a vasoactive peptide, is produced mainly by endothelial cells [17] and mediates its vasoactive effect through two types of endothelin receptors; the endothelin type A (ET_A) and the endothelin type B (ET_B) receptors [18, 19]. These receptors are very important for the plasticity of cerebral arteries. In normal condition, the ET_A receptors are expressed on the vascular smooth muscle cells and their activation leads to strong vasoconstriction while, ET_B receptors are located on the endothelial cells where their activation results in dilatation [20, 21].

However, ET_B receptors function is altered following ischemic stroke. An upregulation of ET_B receptors are observed on SMCs of occluded middle cerebral arteries resulting in increased contractile responses [22].

Astrocytes and reactive astrogliosis

Astrocytes are the most glial cell type of the central nervous system (CNS). They are very important for overall brain structure and function playing a critical role in cerebral parenchymal homeostasis [23]. Astrocytes have stellate morphology and express glial fibrillary acidic protein (GFAP) which is known as a pan-astrocyte marker [24]. Under stress and degenerative conditions like ischemic stroke, astrocytes become reactive through a process termed as reactive astrogliosis. The hallmarks of this process are proliferation, the morphological changes of astrocytes and the increased expression levels of GFAP [25, 26]. Beside upregulation of GFAP, astrocytes undergo other structural changes including upregulation of other intermediate filament proteins [27]. Nestin is a class VI intermediate filament protein which is known to be transiently expressed in proliferating stem cells during CNS development. In adult CNS, nestin is considered as a marker for new neurons in neurogenic areas [28, 29]. In addition, re-expressed or upregulated nestin have been shown in reactive astrocytes after ischemic stroke [27, 30]. The occurrence of reactive astrogliosis is essential for the formation of astrocyte scar in the tissue adjacent to the infarct delineating it from intact cerebral parenchyma [31]. The formation of astrocyte scar has been shown in both clinical and experimental stroke [32]. Formation of astrocytic scar aids axonal regeneration in central nervous system [33, 34]. There has been a pile of evidence that selective destruction of this scar leads to great spread of inflammatory cells, increased neuronal loss, greater spread of tissue damage and worse neurological function [27, 35, 36].

Angiogenesis

Angiogenesis is formation of new capillaries from existing blood vessels [37]. It occurs as a defensive mechanism in response to hypoxia including ischemic hypoxia. Its mechanism involves interaction of different components including tyrosine kinase receptor Tie-2, its ligand (angiopoietins 1 and 2), growth factors and so on [38]. Induction of these components together with increased microvessel density has been shown in animal models of stroke [39-41]. One important point is the actual role of spontaneous angiogenesis following stroke [42]. One study suggested the clean-up hypothesis in which angiogenesis facilitates macrophage infiltration to remove necrotic tissue and debris [43]. However, several studies have

pointed out a relationship between angiogenesis and better stroke outcome. For instance, it was shown that increased microvessel density was correlated with longer survival in both female and male patients after stroke [44]. In addition, a previous study also showed that new capillaries support restored perfusion in the ischemic border after cortical stroke in rats [45]. Therefore, angiogenesis may contribute to stroke recovery by restoring metabolism in surviving neurons, supplying neurotrophic factors and removal of necrotic debris. In recent years, the importance of angiogenesis has led to improvement of advanced techniques including magnetic resonance imaging (MRI) to evaluate angiogenesis after stroke. Cerebral blood flow (CBF) and cerebral blood volume (CBV) are the two hemodynamic parameters indicating microvessel formation and affected by angiogenesis that can be measured by perfusion MRI. These parameters are beneficial not only to evaluate spontaneous angiogenesis but also to study the efficacy of potential stroke treatments [46, 47].

Signalling pathways

ERK1/2 pathway

ERK1/2, also known as p42/44 mitogen activated protein kinase (MAPK), is a major subfamily of MAPK. Activation of ERK1/2 occurs via its phosphorylation by the MEK1/2, an immediate component upstream of ERK1/2 in the cascade. Activation of MEK1/2 itself depends on a MAPK kinase kinase (Raf) which needs GTP-loaded Ras to get activated [48, 49]. Activation of ERK1/2 cascade occurs in response to both mitogens and stressors stimuli such as cytokines and growth factors. Hence, this pathway plays a major role in a wide range of biological processes such as cell differentiation, proliferation as well as fate decisions and cell death [50] (Figure 2).

Dysfunction of ERK1/2 signalling is involved in several human diseases. Initial indications that activation of ERK1/2 pathway may contribute to pathogenesis of CNS disease came from using antibodies recognizing active form of ERK1/2 [49]. In particular, ERK1/2 signalling pathway contributes in the pathophysiology of stroke and activation of ERK1/2 after stroke has been reported in previous studies [51].

Oxidative stress, inflammation, increased receptor-mediated vasoconstriction and upregulation of matrix metalloproteinases are all features of the pathophysiology of ischemic stroke during acute phase, leading to exacerbate neuronal cell death and ischemic damage. These detrimental mechanisms are associated by activation of ERK1/2 signalling pathway [3, 22, 52-55]. But, ERK1/2 cascade does not have only a

dark side. It is involved in angiogenesis and neurogenesis, two main recovery mechanisms induced after stroke [56]. Survival signals i.e. growth factors also exert their beneficial effect via ERK1/2 pathway [57].

Although it is suggested that ERK1/2 has a dual effect after stroke [58] but distinguishing the effects according to the time course of stroke can mount the strategies to target the pathway for stroke therapy. Our previous studies showed that acute inhibition of this pathway could supress detrimental cascades, reduce infarct size and improve functional outcome in animal models of stroke [48, 59]. It indicates that early activation of ERK1/2 pathway after stroke is detrimental and its inhibition has acute beneficial effects. Taken all together, we have targeted this pathway as a therapeutic strategy for stroke which is the base of the present thesis.

Protein kinase B (Akt)

Akt is a serine/threonine protein kinase which plays a role in a variety of cellular processes including cell growth and survival. In CNS and more specifically during brain development, Akt plays an important role in mediating neuronal survival [60]. Activation of Akt occurs via phosphorylation. In the first step, a receptor tyrosine kinase or G-protein-coupled receptor is activated, which in turn activate phosphoinositide-3-kinase. Activated PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate into phosphatidylinositol-3,4,5-triphosphate which binds to Akt and recruit it to the inner face of plasma membrane. Then Akt is phosphorylated at Thr308 by phophoinositide-dependent protein kinase-1 and by the mammalian target of rapamycin complex-2 at Ser473 which both are needed for complete Akt activation [61]. Upon phosphorylation, Akt exerts its function via phosphorylation (and inactivation) of downstream substrate such as pro-apoptotic Bcl-2 family member Bad and caspase-9 [60](Figure 2).

Different studies have shown that Akt pathway is affected by ischemic stroke. It appears that a decrease in phosphorylation state (p) of Akt correlates with ischemic damage showing its role in ischemic stroke to be neuroprotective [62]. Additionally, protective effects of various extrinsic compounds including growth factors i.e. vascular endothelial growth factor [63] can be implicated via Akt pathway. Akt exerts its neuroprotective effects by interfering excitotoxicity, inflammation and apoptotic injury via inhibition of downstream substrates [60]. Although phosphorylation of Akt at both sites needed for its activation, fewer studies have evaluated the changes of p-Akt (Thr308) after stroke [64]. It is also worth noting that phosphorylated Akt does not always have kinase activity. This is due to the negative endogenous regulator of this pathway which is induced by ischemia. This can suggest that in the absence of neuroprotectant compounds, ischemia transiently induce phosphorylation of Akt but its kinase activity is

suppressed by negative regulator leading to activation of pro-apoptotic pathways and cell death [65].

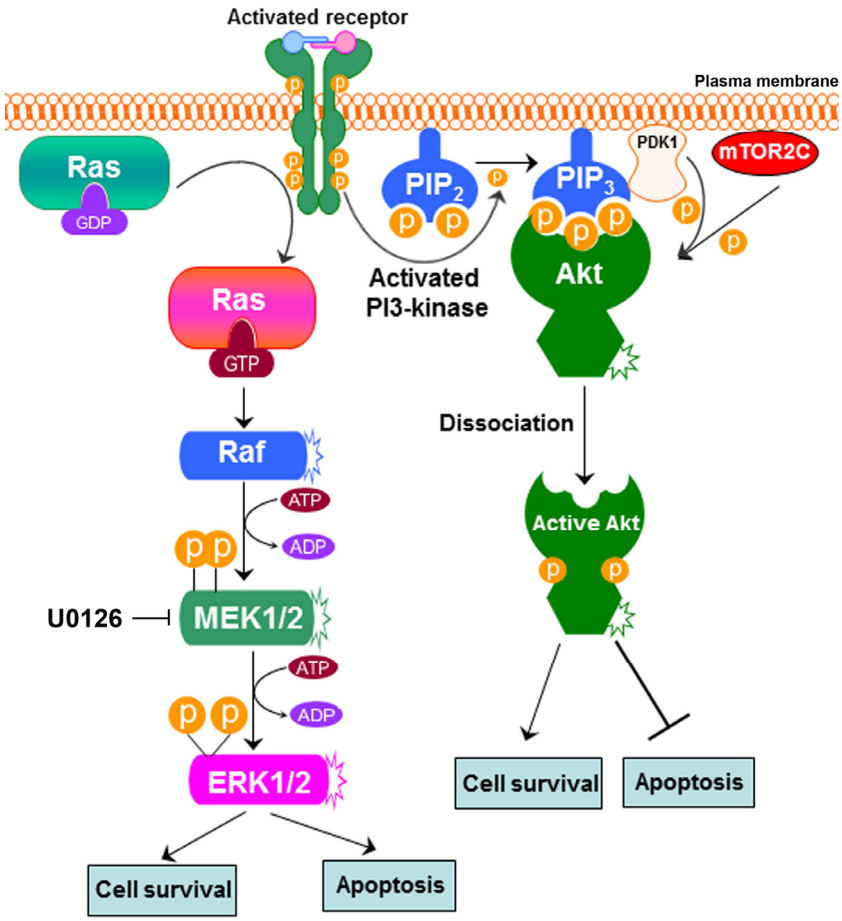

Figure 2. Activation and effects of MEK-ERK1/2 and Akt signalling pathways.
Extracellular-signal regulated kinase (ERK) 1/2, Mitogen activated protein kinase kinase (MEK) 1/2, phosphoinositide-3-kinase (PI3K), phosphatidylinositol-4,5-bisphosphate (PIP_2), phosphatidylinositol-3,4,5-triphosphate (PIP_3), phophoinositide-dependent protein kinase-1 (PDK1), the mammalian target of rapamycin complex-2 (mTOR2C). U0126, a MEK1/2 inhibitor, blocks MEK-ERK1/2 signalling pathway.

Challenges in acute ischemic stroke treatment

Over the past few decades, extensive efforts have been made in understanding the pathophysiology of ischemic stroke; however, there has not been much progress in developing new stroke treatment [12]. Today, acute treatment for ischemic stroke

consists of dealing with the cause; attempts to remove the blood clot and recanalize the vessel. Intravenous administration of a thrombolytic drug and mechanical removal of blood clot by endovascular thrombectomy are two methods to reach this aim [66].

However, there are several challenges in acute stroke treatment even after successful recanalization. Clinical evidence shows that post-stroke recanalization is accompanied by occurrence of microvascular reperfusion failure. Hence, recanalization is not always synonymous with reperfusion. Formation of secondary clot is one reason for failure in restoration of normal CBF [66].

Moreover, recanalization can be accompanied by vascular complications. For instance, the major adverse effect of tissue-type plasminogen activator (t-PA), FDA-approved thrombolytic agent, is the intracerebral hemorrhage [1, 66]. So, administration of t-PA is limited to 4.5 hours after stroke onset to reduce this haemorrhagic transformation. Because of the narrow time window for t-PA treatment and the risk of haemorrhagic transformation, in fact, only small percentage of stroke patients receive this treatment [12, 67, 68].

It is worth noting that the cause of stroke is vascular, but it has neurological consequences. Therefore, other approaches in acute stroke treatment has focused on the rescue of neuronal cells in the peri-infarct area [69]. It has been huge investment to develop new neuroprotective drugs over the past few decades but all failed in clinical trials even though showing promising results in pre-clinical studies [70]. Taken all together, there is a compelling need to develop new therapeutic strategies in the stroke field.

Hypothesis and Aims

It has previously been demonstrated that inhibition of the MEK-ERK1/2 signalling pathway reduces ischemic damage and improves functional outcome after experimental stroke [22, 53-55]. These findings are the base of the present thesis. Although promising, the beneficial effects of MEK-ERK1/2 inhibition were only observed during acute phase and only in male animals. Therefore, to be able to successfully develop new treatment strategies we need to further investigate the consequences of MEK-ERK1/2 inhibition on stroke outcome.

Our hypothesis is that acute treatment with a MEK1/2 inhibitor (U0126) will continue to show benefit beyond the acute phase and not negatively interfere with later recovery processes. Further, the present thesis addresses important aspects that should be considered when developing a new treatment such as sex and clinically relevant time window.

Several specific aims are addressed:

Paper I – II (male)

1. To evaluate if beneficial effects of early MEK-ERK1/2 blockade (0 and 24 hours post reperfusion) persist beyond acute phase.
2. To investigate whether subacute beneficial effects of U0126 persist even when it is administered in a time window relevant to the clinic (6 and 24 hours post reperfusion).
3. To investigate the expression of ERK1/2 during subacute phase.
4. To determine possible underlying recovery mechanisms for delayed beneficial effects of acute U0126 treatment.

Paper III (female)

1. To study if acute increased cerebrovascular receptor-mediated vasoconstriction occurs in female rats and if the mechanism is mediated via the MEK-ERK1/2 pathway.
2. To evaluate if acute inhibition of MEK-ERK1/2 pathway is beneficial in female rats.

Paper IV (female)

1. To investigate if spontaneous functional recovery occurs in female rats.
2. To evaluate repair-related molecular changes associated with spontaneous recovery and if ERK1/2 is activated during subacute phase.

Methods

Study design

A brief overview of the *in vivo* methods and the *in vitro* techniques used in the present thesis is given in figure 3 and 4 respectively.

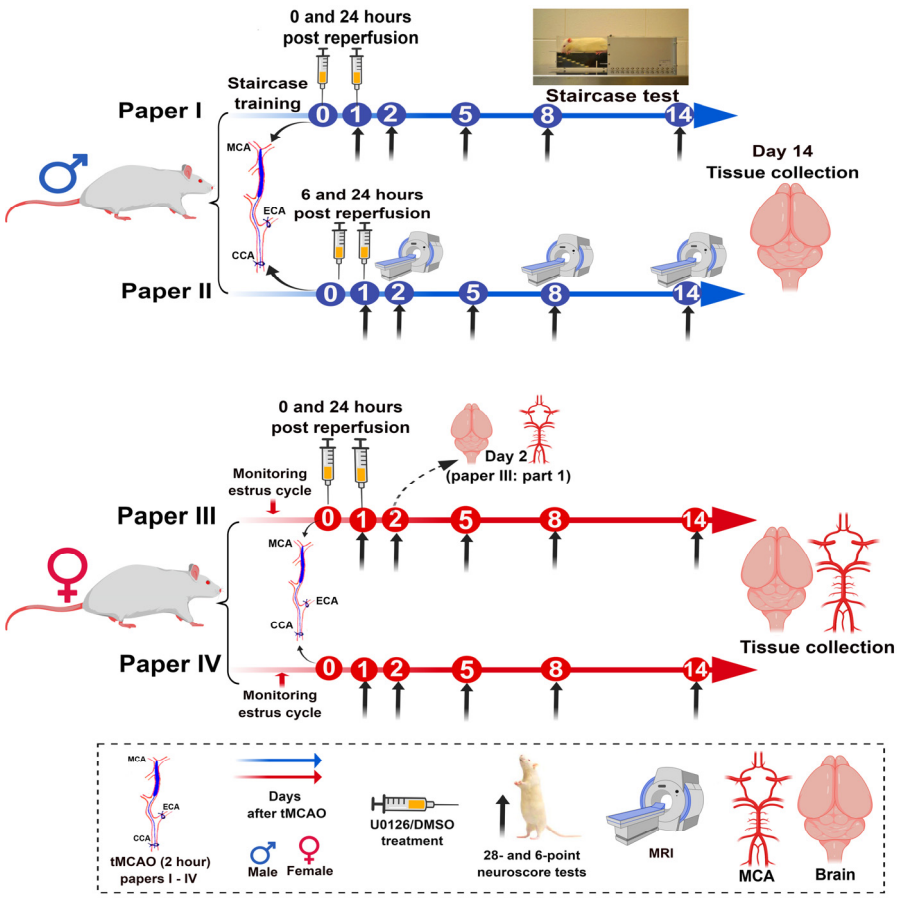

Figure 3. Overview of the study design for each project and the in vivo methods used in the thesis. tMCAO was induced at day 0 for all papres and the study was designed for a period of two weeks in each project except for paper III which has additional part with a period of two days. The animals were acclimatized before any in vivo experiments. The key to the figure is available in the dotted box. The tissue collected were further processed for in vitro techniques. MCA: middle cerebral artery, ECA: external carotid artery, CCA: common carotid artery

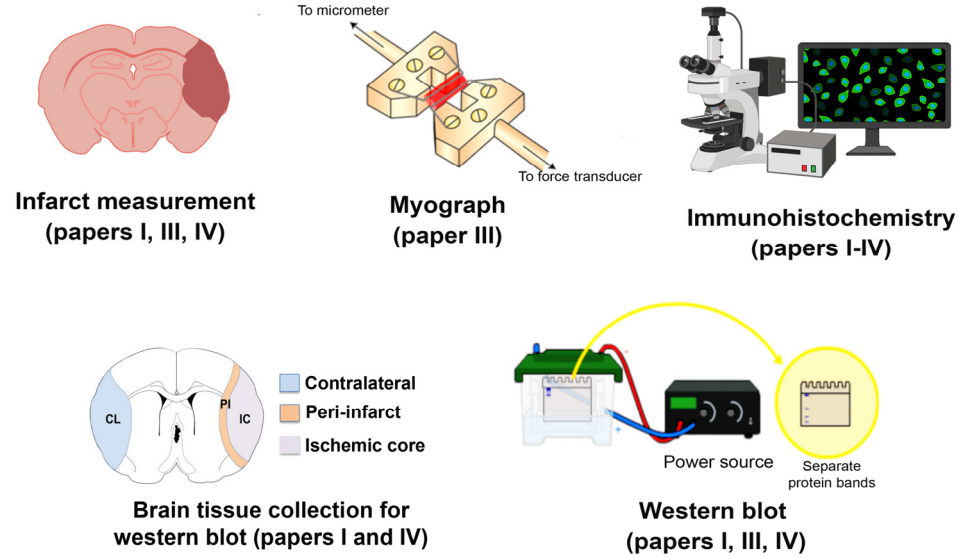

Figure 4. Overview of the in vitro techniques and their distributions in each paper.
The image from myograph is adapted from biopta.com and the image for western blot is adapted from wikipedia.com

Ethical considerations

An *in vivo* stroke model on rats has been the base for the projects of this thesis. Therefore, all of the experiments and procedures were performed in accordance with the guidelines for the European Community Council Directive (2010/63/EU) for protection of Vertebrate Animals Used for Experimental and other Scientific Purposes. The experimental procedures were approved by the Lund-Malmö Institutional Ethics Committee under the Swedish National Department of Agriculture (M188-12 and M171-14). The guidelines described in ethical permit were implemented into practice by (i) performing surgical procedure carefully according to our protocol; (ii) several precautionary steps to minimize stress and pain to the animals and (iii) following 3Rs (Replacement, Reduction and Refinement) in animal ethics where possible. By using magnetic resonance imaging the number of animals needed in paper II was reduced.

In vivo methods

Animals preparation

Male and female Wistar rats (Papers I-IV) were obtained from Charles River Laboratories, Sulfzed, Germany. Upon arrival, the rats were housed under a 12-h light/dark cycle and controlled temperature and humidity with *ad libitum* access to water and food. The rats were allowed to get acclimatized to the new environment for five days before any experiment. In paper I, male animals were trained for five days prior to stroke model to be able to perform staircase test and they were on food deprivation during this period. In papers III and IV, female animals were monitored for three consecutive estrus cycle by collection of vaginal smear and determination of cell types in smear before stroke model [71]. The surgery was only performed on the animals that were in low influence of 17-βestradiol i.e. not in proestrus phase to minimize hormonal fluctuation effects.

Transient middle cerebral artery occlusion (Papers I-IV)

Transient middle cerebral artery occlusion (tMCAO) was induced in Wistar rats using the intraluminal filament technique [72]. Anaesthesia was induced by 3.5% isoflurane in N_2O:O_2 (70:30) and then maintained by inhalation of 1.5–2% isoflurane through a facial mask during the surgical procedure. Physiological parameters (blood pressure, pH, pCO_2, pO_2 and blood glucose) were measured before occlusion by inserting a polyethylene catheter in the tail artery. Body temperature was maintained during the surgical procedure by a homoeothermic blanket connected to a rectal temperature probe. An incision was made in the midline of the neck, and the right common, internal and external carotid arteries were exposed. The right common and external carotid arteries were ligated permanently with sutures. A silicon rubber coated monofilament (Doccol Corporation, MA, USA) was inserted into the right internal carotid artery and further advanced until the tip of the filament reached the entrance of the middle cerebral artery causing an occlusion and sudden drop in cerebral blood flow. Reduction of cerebral blood flow was verified by Laser-Doppler flowmeter (AD instruments, Australia). After two hours occlusion, the animals were evaluated by 6-point neuroscore test [73, 74]. Only the animals that showed a score of 4 in this neuroscore test went through reperfusion by removing the filament. The increase of cerebral blood flow during reperfusion was also verified by Laser-Doppler flowmeter. At the end of the surgery, the rats received a subcutaneous injection of 10 ml of isotonic saline for rehydration. For sham-operated rats (papers I & II), the same procedure was applied except that no filament was advanced to occlude middle cerebral artery.

Treatment administration after tMCAO (Papers I-III)

The specific MEK1/2 inhibitor, U0126 (1,4-Diamino-2,3-dicyano-1,4-bis [2-aminophenylthio] butadiene) was used to investigate underlying mechanisms and the importance of MEK-ERK1/2 pathway in experimental stroke. Rats were treated with U0126 (30 mg/kg of animal body weight) or vehicle (dimethyl sulfoxide, DMSO) as intraperitoneal injection either at 0 and 24 hours (Paper I and III) or at 6 and 24 hours (Paper II) post reperfusion. The time and dosage of U0126 were chosen according to a previous study [22].

Behavioral tests (Papers I-IV)

Sensorimotor cortex or striatum of ipsilateral hemisphere is frequently damaged following the MCAO model and, the hippocampus and surrounding areas maybe partially affected with severe injury [75]. Moreover, many behavioral tests have been characterized in rodents that have undergone MCAO to evaluate neurological status and the degree of damage over a period of time after stroke. However, none of these tests can stand alone to fully characterize the various deficits after stroke; therefore, it is important to have combination of behavioral tests that are sensitive to detect an array of impairments. Composite scores like 28-point [76] and 6-point neuroscore assess a variety of motor, sensory, reflex and balance responses. Learning and memory impairments can be evaluated by cognitive tests and due to the loss of limb function after stroke, many motor and sensorimotor tests have been developed including staircase test and forelimb placing test, respectively [77]. In this thesis, we have used staircase test as well as 28-point and 6-point neuroscore tests. The evaluations were performed by an experienced observer that was blinded to treatment status.

28-point neuroscore test (Papers I-IV)

This test was performed to assess more detailed analysis of sensorimotor deficits after tMCAO and shown to be sensitive to both acute and long-term deficits. In the 28-point neuroscore test, the animals were evaluated by eleven different tasks with a cumulative maximum score of 28 for healthy function and 0 for severe impairment [76]. The different parameters included in the 28-point neuroscore test are listed in table 1.

Table 1.
28-point neuroscore test

Test	*Score*	*Test*	*Score*
Circling	0-4	Climbing on an Inclined platform	0-3
Motility	0-3	Grip strength	0-2
General condition	0-3	Contralateral reflex	0-1
Righting reflex when placed on back	0-1	Visual forepaw reaching	0-2
Paw placement of each paw on a table top	0-4	Contralateral rotation when held by base of tail	0-2
Ability to pull self up on a horizontal bar	0-3		

6-point neuroscore test (Papers I-IV)

This test was performed according to previous studies [73, 74, 77]. The deficits on this test might resolve in stroke models making it less useful for detection of long-term deficits but can be used for acute phase after stroke. Gross neurological function graded in six levels from 0 for no visible defects, to 5 for death overnight. The different parameters included in the 6-point neuroscore test are presented in table 2.

Table 2.
6-point neuroscore test

Test	*Score*
No disability at all, normal	0
Flexure of animals upper body while raised by the tail	1
One-side resistance to push when placed on the floor and restrained by the tail	2
Inability to walk straight	3
Tight circling	4
Death overnight	5

Staircase test (Paper I)

The staircase test, described by Montoya et al. (1991) [78], can detect long-lasting deficits after ischemic stroke in rodents [79-81]. In structure, the staircase box consists of a chamber with a central platform. It allows the animal to climb onto it and get access to a set of seven steps located on each side of the box. Food pellets are placed on each step. According to the design of the box, the animal can only reach the food pellets on right side with right forepaw and on the left side with left forepaw (Figure 5). Therefore, this test assesses the independent use of forelimbs of rats in a skilled food reaching task consisting of forelimb extension, grasping skills and side bias by observing its behaviour [77, 82]. We set up the staircase test in our laboratory in accordance with previous studies [78, 82] and our detailed staircase test protocol can be found in paper I of the present thesis.

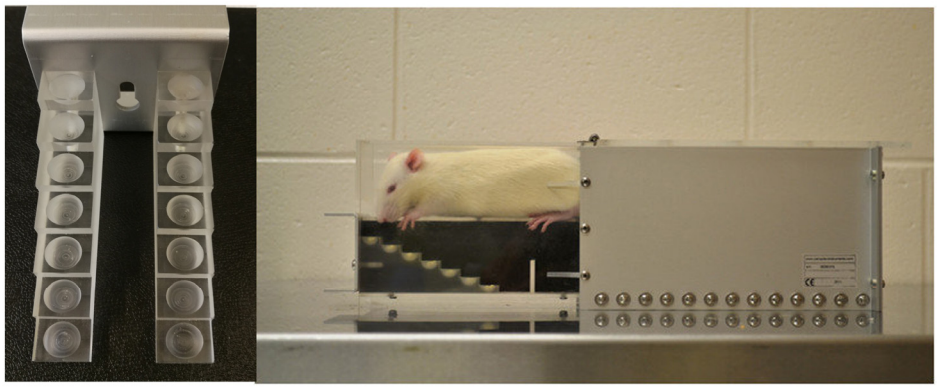

Figure 5.
Staircase test apparatus. The image for staircase box is adapted from [83] (open access with license No. CC BY 2.0).

MRI (Paper II)

MRI is a well-established technique in both experimental and clinical stroke providing extensive information about anatomy of brain with an excellent soft tissue contrast and specific physiological parameters that can be measured [84, 85]. Maybe the most important contribution of MRI in anatomical studies of brain injury has been the use of T_2-weighted images and the apparent diffusion coefficient (ADC) to define ischemic lesions. ADC is a better early marker and T_2 is a better late marker of lesion volume, but both methods reveal aspects of tissue metabolism or microstructure and can be collected together to evaluate lesion status efficiently [86]. In addition, regional CBF is a parameter of critical interest in stroke and MRI provides several ways to measure CBF. One way is dynamic susceptibility contrast imaging (DSC) in which a paramagnetic contrast agent such as gadolinium, is rapidly injected as a bolus in a peripheral vein, and rapid imaging tracks the bolus passage through the brain providing the possibility to measure regional CBF and CBV [86, 87]. The detailed MRI protocol used in this thesis to assess these parameters can be found in paper II.

In vitro techniques

Infarct size measurement (Papers I, III & IV)

Preclinical outcome measurements including infarct size should restate human clinical aspects as nearly as possible. Different methods have been developed to evaluate infarct size that each has advantages and disadvantages [88, 89]. Beside MRI as a non-invasive imaging, two other techniques were also used to measure infarct size in the present thesis.

Silver infarct staining (SIS, Papers I & IV)

In order to perform SIS, the brains were frozen in isopentane (-20 ^{o}C) and embedded by TissueTek. 12-µm brain sections were collected on a cryostat with an interval of 400 µm. The SIS was performed according to an established protocol [90]. Lack of silver staining on the sections indicated infarcted tissue which was calculated by imageJ software (http://rsb.info.nih.gov/ij/) as the infarct volume.

Neuronal specific nuclei protein staining (NeuN, Paper III)

To perform this staining, brains were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.2) overnight. After fixation, the brains were exposed to increasing concentrations of sucrose in Sorensen's phosphate buffer and then briefly washed in PBS. Brains were sectioned into 40 µm coronal sections using a microtome (Leica Biosystems, Nussloch, Germany). Sections were collected at 1 mm intervals and stained for NeuN. Lack of staining in the ipsilateral side indicated infarcted tissue. Infarct volumes were calculated by subtracting the non-lesioned volume of the ipsilateral from the non-lesioned volume of contralateral [91] by using imageJ software.

Western blot (Papers I, III & IV)

Western blot can be used to investigate protein abundance, kinase activity, cellular localization, protein-protein interactions, or monitoring of post-translational modifications [92]. In the present thesis, western blot was used to measure the expression of different proteins in the brain or middle cerebral arteries (MCAs). In this technique, a mixture of proteins were separated based on molecular weight, and thus by type, through gel electrophoresis and then transferred to a membrane producing a band for each protein. We used indirect method to detect the bands in which, the membrane was blocked and incubated with unlabelled primary

antibody specific to the protein of interest. After washing, the bound antibodies were then detected by secondary antibody conjugated with horseradish peroxidase [92, 93]. The membranes were developed using chemiluminescent substrate (Supersignal Dura kit) and protein bands were visualized by Fujifilm LAS1000 image analyser. As the antibodies only bind to the protein of interest, only one band should be visible. Quantification of band intensity was performed using imageJ software.

Immunohistochemistry (Papers I-IV)

Immunohistochemistry was used to localize and/or measure the expression of molecular targets within brain or MCA sections. The fundamental concept is the demonstration of antigens within tissue sections by means of specific antibodies [94]. There are different methods to perform immunohistochemistry. In this thesis, we used indirect or 2-step method [95] in which primary antibody is unlabelled, but the secondary antibody, raised against the primary antibody, is conjugated by reporter molecules such as fluorochromes.

Briefly, brains or MCAs were fixed in 4% PFA in 0.1 M PBS (pH 7.2) overnight. After fixation, they were exposed to increasing concentrations of sucrose in Sorensen's phosphate buffer and embedded in an egg albumin-based protein medium or TissueTEK, respectively, and then were frozen for cryosectioning. Brains (12 μm) or MCAs (10 μm) sections were obtained on a cryostat and kept in -20 ^{o}C. In order to do immunohistochemistry, the sections were permeabilized in PBS containing 0.25% triton X-100, blocked and exposed to primary antibodies overnight at 4 ^{o}C. On the following day, the sections were incubated with secondary antibodies and mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) that stains nuclei. The experiments were repeated to ensure reproducibility. Negative controls were performed where the primary antibodies were omitted.

The primary and secondary antibodies for in vitro techniques used in this work are summarized in table 3 and 4 respectively.

Table 3.
Alphabetic list of primary antibodies

Primary Antibodies	*Supplier (Catalouge No.)*	*Dilution*	*Paper*
Akt	Cell Signaling Technology (9272S)	1:1000 WB	IV
p-Akt (Ser 473)	Cell Signaling Technology (4058S)	1:1000 WB	IV
β-actin	SantaCruz (sc47778)	1:500 WB	I
β-actin:peroxidase	Sigma-Aldrich (A3854)	1:50000	IV
p-ERK1/2	Cell Signaling Technology (9101)	1:2000 WB	I, III & IV
	Abcam (ab50011)	1:200 IHC	III
t-ERK1/2	Cell Signaling Technology (9107)	1:4000 WB	I & IV
ET_B	Enzo (ALX-210-506A)	1:100 IHC	III
IL-10	Abcam (ab25073)	1:1000 WB	IV
GFAP	Dako (Z0334)	1:1500 IHC	I
Ki67	Abcam (ab16667)	1:200 IHC	II
Nestin	Abcam (ab6142)	1:500 IHC, 1:1000 WB	I & IV
NeuN	Abcam (ab104224)	1:1000 IHC, 1:10000 WB	II & IV
SM22-α	Abcam (ab10135)	1:1000 WB	III
TGF-β	Abcam (ab66043)	1:250 WB	IV
Tie-2	SantaCruz (sc-9026)	1:200 WB	I & IV

All the primary antibodies were checked to react against rat antigens. WB: western blot, IHC: immunohistochemistry.

Table 4.
Alphabetic list of secondary antibodies

Conjugate (Host)	*Against*	*Supplier (Catalouge No.)*	*Dilution*	*Paper*
Alexa-594 (donkey)	Rabbit	Jackson ImmunoResearch (711-585-152)	1:400 IHC	II
Alexa-594 (goat)	Mouse	Thermo Fisher (A11032)	1:200 IHC	I
Cy^3 (donkey)	Rabbit	Jackson ImmunoResearch (711-165-152)	1:200 IHC	IV
Dylight 594 (donkey)	Sheep	Jackson ImmunoResearch (713-516-147)	1:50 IHC	III
FITC (donkey)	Mouse	Jackson ImmunoResearch (715-095-151)	1:100 IHC	II & III
FITC (goat)	Rabbit	Cayman Chemical (10006588)	1:100 IHC	I
IgG:peroxidase (donkey)	Goat	Pierce Biotechnology (A16005)	1:10000 WB	III
IgG:peroxidase (goat)	Rabbit	Cell Signaling (7074S)	1:2000 WB	IV
IgG:peroxidase (horse)	Mouse	Cell signaling (7076)	1:2000 WB	IV

WB: western blot, IHC: immunohistochemistry.

Analysis of immunohistochemistry sections (Papers I-IV)

Immunoreactivity of the sections was visualized at the appropriate wavelengths with an epifluorescence microscope (Nikon 80i; Tokyo, Japan) and photographed with an attached Nikon DS-2MV camera equipped with appropriate lenses.

MCAs sections (Papers III-IV):

Qualitative evaluation was performed by an expert in the field blinded to the study details in order to localize positive immunoreactivity. Moreover, the expression

was evaluated by measuring fluorescence intensity in the smooth muscle layer using the imageJ software.

Brain sections (Papers I-II):

Large image of the sections was taken using NIS basic research software (Nikon). The ischemic core on each section was marked according to SIS or double staining with NeuN as reference. The expansion of areas including the molecular target of interest in peri-infarct region or ischemic core was determined using magnification in the screen until separate cells could be distinguished. Thereafter, positive cells in these determined areas were counted using automated counting option of imageJ software. All the images were taken at the same day for all the sections, and the image analysis was blinded to the investigator.

Wire-myograph (Paper III)

Contractile properties of rat cerebral arteries were evaluated *in vitro* by mounting of cylindrical arterial segments in wire myograph [96, 97]. Two parallel wires were inserted into the lumen; one being connected to an adjustable micrometer screw setting the distance between the wires and hence the vascular tone. Initially, the arteries were progressively stretched to 90% of the diameter each vessel would have if fully relaxed under a transmural pressure of 100 mm Hg. The arterial segments were immersed in a temperature controlled (37^{o}C) bicarbonate buffer solution. The buffer was continuously aerated with oxygen enriched with 5% CO_2 resulting in a pH of 7.4. Viability and contractile capacity were determined by exposure to high potassium bicarbonate buffer solution that causes smooth muscle cell contraction via membrane depolarization and influx of calcium [98].

Endothelial influences were blocked by L-NG-Nitroarginine methyl ester (L-NAME) and indomethacin. Individual receptor-mediated responses were evaluated by cumulative application of sarafotoxin 6c (S6c, ET_B receptor agonist) and ET-1 (ET_A and ET_B receptors agonist). In order to measure ET_A receptor-mediated responses specifically, ET_B receptors were either desensitized by S6c, or by incubation with the ET_B receptor antagonist BQ788, prior to ET-1 application. The vasomotor responses are expressed as percentage of the K^+-evoked response. E_{MAX} represents the maximum contraction induced by an agonist and the pEC_{50} value refers to the negative logarithm of the concentration needed to elicit 50% of the maximum response.

Illustrations and statistics

Graphs and figures were created and arranged using Microsoft Excel, GraphPad Prism (Version 7 for Windows, La Jolla California, USA) and Adobe Photoshop CS (Adobe systems, Mountain View, CA, USA). Statistical analyses were performed by SPSS (versions 22-25, IBM Corp. Released 2013-2017, Armonk, NY, USA) and GraphPad Prism (version 4). Detailed statistical tests performed for each study are described in each paper under the materials and methods section. The statistical analysis and the use of different tests were performed according to the type of data, normality and n number and were discussed by professional statisticians where necessary. In all statistical tests, $p<0.05$ were considered as significant and n refers to the number of animals.

Results and Comments

Beneficial effects of MEK-ERK1/2 inhibition persist beyond acute phase in male rats (Papers I-II)

Previous studies demonstrated that acute inhibition of MEK-ERK1/2 pathway using specific MEK1/2 inhibitor, U0126, could block the detrimental events, reduced infarct size and improved functional outcome after experimental stroke [13, 22, 52-54, 99]. Although very promising, these effects were only observed in the acute phase. Moving forward with the hypothesis of MEK-ERK1/2 inhibition to the clinic, further investigations are needed.

Stroke patients stay with different degrees of neurological deficits when they enter subacute phase. Therefore, the beneficial effects of new potential treatments should not only rescue the patients from acute detrimental events but also persist beyond acute phase and aid stroke recovery mechanisms [5, 12]. In addition, only few percentages of stroke patients are actually able to receive available stroke treatments due to the narrow time-window and risk of haemorrhagic transformations. Thus, new potential treatment should be developed and be applicable in a time-window relevant to the clinic [1, 12, 67, 68]. Considering the contribution of MEK-ERK1/2 pathway in recovery processes beyond the acute phase [56, 58], it is also necessary to investigate that acute inhibition of the pathway does not suppress the late expression of ERK1/2 and recovery processes.

In order to address these points and to further investigate the hypothesis of MEK-ERK1/2 inhibition as a therapeutic strategy for ischemic stroke, papers I and II were designed. We subjected male Wistar rats to 2-hour MCAO treated with U0126 or vehicle at 0 and 24 hours (paper I) or 6 and 24 hours (paper II) post reperfusion. The neurological functions of the rats after tMCAO were followed beyond acute phase (a period of two weeks) using staircase test (paper I) and/or composite neuroscore tests (paper I-II). MRI was applied to monitor ischemic damage over time and to evaluate regional cerebral circulation (paper II). The animals were terminated 14 days after tMCAO and brains were then collected for further histological evaluations.

In addition, sham-operated animals were included in these studies. The aim was to exclude any possible effect of surgical procedure on the interpretation of the data

and the effect of the treatment. It was observed that surgical procedure did not cause any neurological deficit, and the sham animals did not show any ischemic damage. In addition, U0126- or vehicle-treated sham animals did not show any difference in performing behavioral tests (Figure 6).

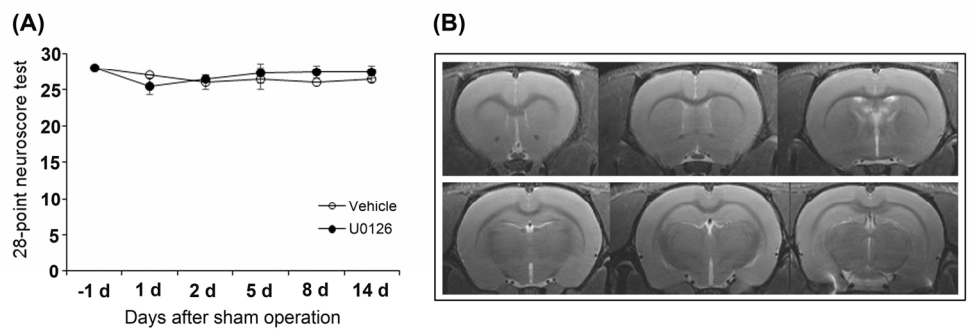

Figure 6. U0126 or vehicle had no effect on sham-operated animals.
(A) Sham-operated animals showed no neurological deficits compared to their pre-operation score in both U0126- and vehicle-treated groups. Vehicle; n = 3 and U0126; n = 4. Data are expressed as mean ± SEM; **(B)** Representative images of T_2-weighted MRI in sham-operated animal showed no sign of damage.

Administration of U0126 at both of the time-points (0 and 24 hours or 6 and 24 hours post reperfusion) significantly improved functional outcome in male rats after tMCAO over a period of two weeks. Improvement of functional outcome in U0126 group (6 and 24 hours post reperfusion) compared to vehicle group for 28-point neuroscore test is shown in figure 7 as a representative of composite neuroscore tests performed.

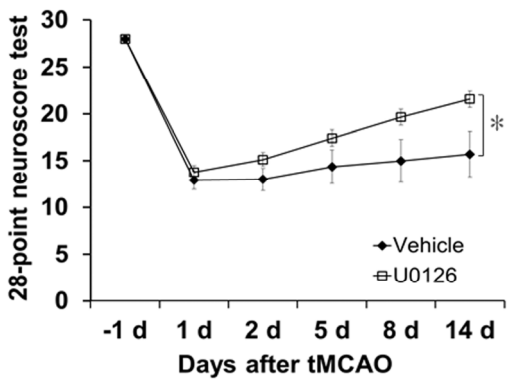

Figure 7. Inhibition of MEK-ERK1/2 pathway using U0126 at 6 and 24 hours post reperfusion resulted in improved neurological function after tMCAO.
28-point neuroscore test, Vehicle; n = 11 and U0126; n = 14. Data are expressed as mean ± SEM; * indicates significant differences between U0126- and vehicle-treated rats over time ($p < 0.05$)

As mentioned before, animals are restricted to reach the food pellets on right side with right forepaw and on the left side with left forepaw in the staircase test [82]. Therefore, it was possible to evaluate motor function of each forelimb separately in a skilled reaching task. Surgical procedure did not affect the ability of left forepaw in the sham group. However, vehicle-treated animals showed significant deficit in their success rate after tMCAO and were unable to reach their pre-stroke success rate as the time passed. U0126 significantly improved animals' success rate of left forepaw compared to vehicle group. Sham, vehicle and U0126 treated animals all could achieve their pre-stroke success rate of the right forepaw and there was no difference in their success rate (Figure 8).

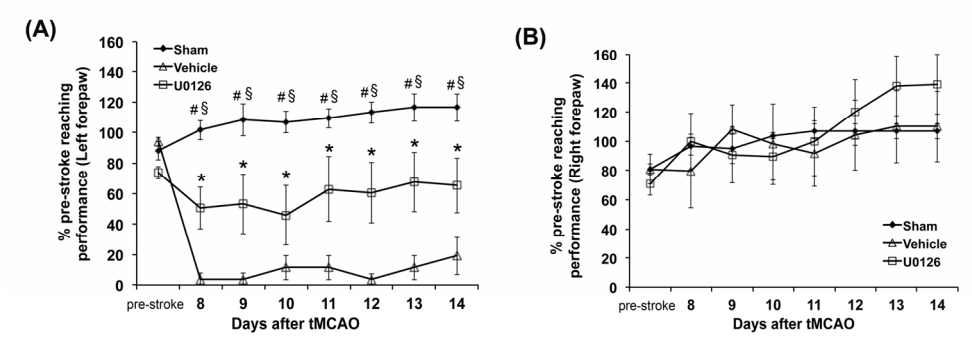

Figure 8. U0126 improved long-term neurological function after tMCAO.
(A) Success rate for left forepaw (Contralateral to stroke) of the rats in staircase test compared to their prestroke reaching performance. **(B)** Success rate for right forepaw (Ipsilateral to stroke) of the rats in staircase test compared to their pre-stroke reaching performance. Sham; n = 6, Vehicle; n = 4 and U0126; n = 8. Data are expressed as mean ± SEM, * indicates significant differences between U0126 and vehicle-treated rats. # indicates significant differences between sham- and vehicle-treated rats. § indicates significant differences between sham- and U0126-treated rats. *, # and § show significance level at 0.05 ($p < 0.05$).

U0126 significantly reduced infarct size at day 14 after tMCAO in male rats compared to vehicle group given at 0 and 24 hours post reperfusion (84.3 ± 14.3 mm^3 and 144.8 ± 11.6 mm^3, respectively). Furthermore, implementing MRI into the project for paper II allowed us to monitor animals for the changes of ischemic damage over time. T_2-weighted images were acquired at day 2, 8 and 14 after tMCAO for both U0126- and vehicle-treated animals. Data showed that U0126 significantly decreased infarct size over time compared to vehicle group (Figure 9).

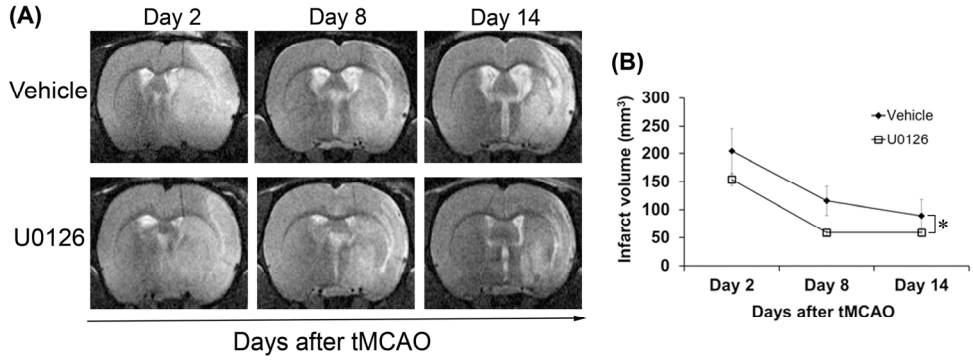

Figure 9. Inhibition of MEK1/2 significantly reduced infarct size over time after tMCAO.
(A) Representative images of T_2-weighted MRI are shown for vehicle (upper panel)- and U0126 (lower panel)-treated animals at days 2, 8 and 14 after tMCAO. **(B)** Administration of U0126 significantly reduced infarct size over time compared to vehicle-treated animals. Vehicle; n = 5-6 and U0126; n = 6-8. Data are expressed as mean ± SEM, * indicates significant differences between U0126- and vehicle-treated rats ($p < 0.05$).

As mentioned before, therapeutic interventions targeting MEK-ERK1/2 pathway should minimize the destructive potential of the pathway during the acute phase but not interfering with its beneficial contributions to tissue repair in the later stage of cerebral ischemia. Therefore, the status of p-ERK1/2 in peri-infarct, ischemic core and contralateral region of the brain at day 14 post-stroke was investigated. The expression of total ERK1/2 did not differ between U0126- and vehicle-treated animals in the three different pre-defined regions. There was also no difference for the phosphorylated state of ERK1/2 between vehicle and U0126-treated animals in any of the regions (Figure 10).

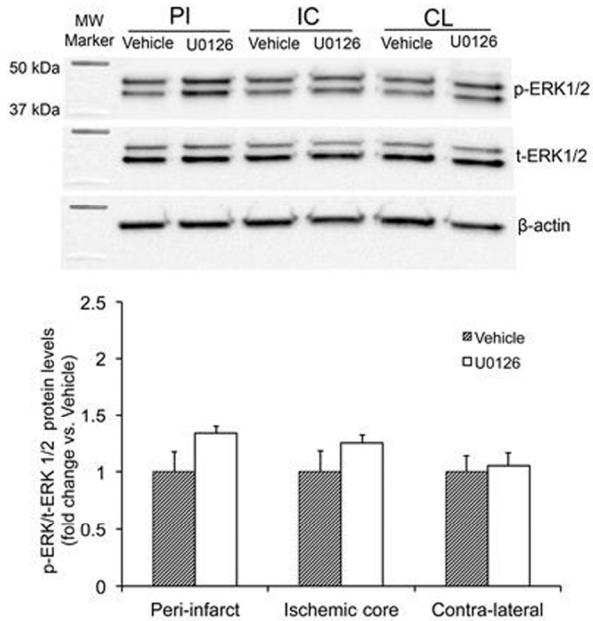

Figure 10. Representative Western blots and bar graphs areshowing protein expression of p-ERK 1/2 in IC (ischemic core), PI (peri-infarct) and CL (contralateral side) of brains for both vehicle- and U0126-treated rats. The expression of p-ERK 1/2 was normalized to total ERK1/2 and presented as percentage activity relative to the average value for vehicle group in each pre-defined regions. The average value for vehicle group was set to 100%. The molecular weight of p-ERK1/2 and total-ERK1/2 is 42/44 kDa and bactin is 43 kDa. β-actin was used as loading control. Molecular weight (MW) marker shows the bands of the standard molecular weight. Data are presented as mean±SEM. vehicle; n = 4 and U0126;n = 5.

To further investigate if observed beneficial effects of MEK-ERK1/2 inhibition are associated with recovery mechanisms 14 days after tMCAO, we looked at gliotic scar formation, angiogenesis and cerebrovascular changes.

Dynamic repair processes occur in the peri-infarct area [6]. Reactive astrocytes delineate the infarct border by forming a gliotic scar, a well described phenomenon. Gliotic scar prevents infarct to expand and contributes to recovery of neurological functions [33-35]. Increased expression of GFAP and re-expression of nestin in astrocytes are the hall marks of this phenomenon [27]. Our data demonstrated a significant increase in the number of cells expressing nestin at day 14 after cerebral ischaemia in the U0126-treated group compared to the vehicle group (Figure 11). Double immunostaining of GFAP and nestin confirmed astrocytes as the cell type expressing nestin and it was mainly found in the border zone of the cerebral infarction. Thus, nestin expression occurs in glial cells that participate in synaptic remodelling at the border of ischemic infarct.

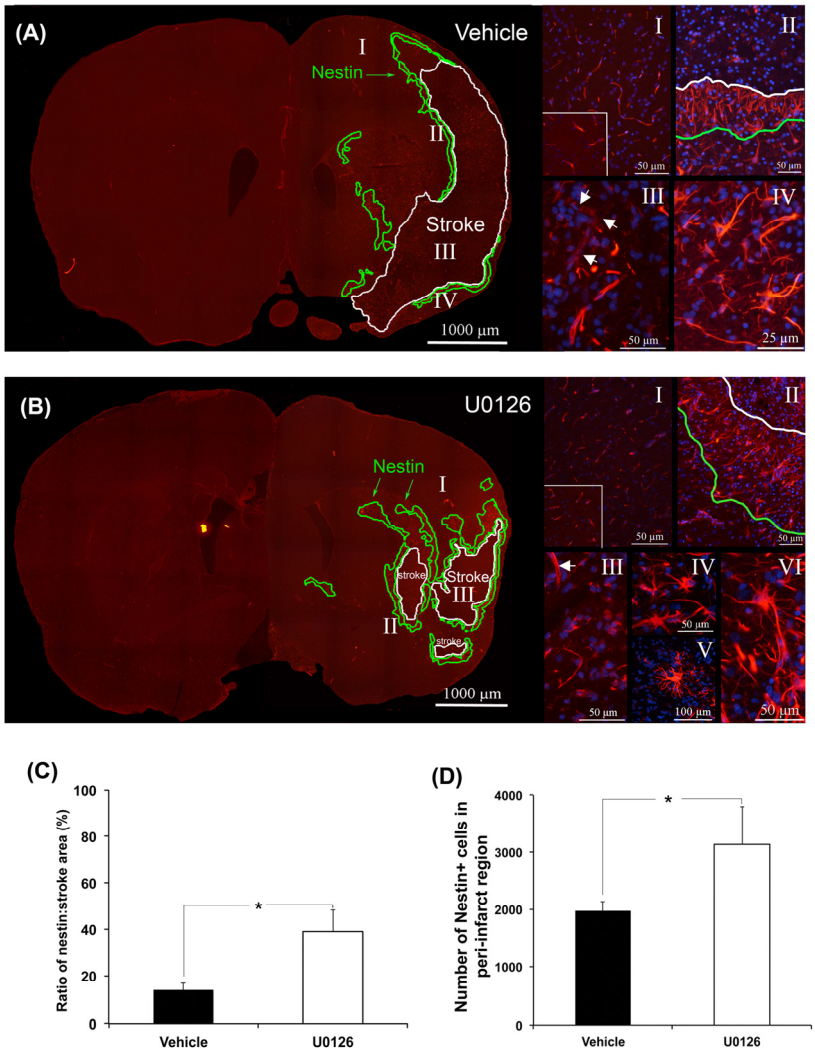

Figure 11. Expression of nestin in brain tissue of vehicle- and U0126-treated rats.
(A) Vehicle-treated animal; areas of stroke (outlined in white) and nestin immunoreactivity in the boundary of stroke region as so called peri-infarct (outlined in green, arrow) are illustrated in a large image. I–IV refer to the given illustrations. I. Regions of the brain considered not to involve in stroke. Insert demonstrates a corresponding region of the control side of the brain as well. Only vessels showed immunoreactivity. II. The image demonstrates the delineation of the nestin immunoreactivity in peri-infarct region. III. Nestin immunoreactivity in ischaemic core. IV. Glial-like cells expressing nestin. **(B)** U0126-treated animal; white-outlined stroke areas and green-outlined nestin immunoreactivity in peri-infarct regions are illustrated in a large image. I-VI refer to the given illustrations. I. only vessels show immunoreactivity in the regions of the brain considered not to involve in stroke. Insert demonstrates a corresponding region of the control side. II. The image demonstrates the delineation of the nestin immunoreactivity in peri infarct regions. III. Nestin immunoreactivity in ischaemic core. IV–VI. Demonstrate immunoreactive cells expressing nestin of different shapes and activation. Blue dots are nuclei stained with DAPI. Nestin expression is visualized in red. **(C)** Average of ratio for areas including nestin-labelled cells (outlined in green on large images): stroke (outlined in white on large images) area for vehicle- and U0126-treated rats. **(D)** Number of nestin-positive cells in peri-infarct area (outlined in green on large images) for vehicle- and U0126- treated rats. Data are presented as mean ± SEM. *P < 0.05, n = 3 for vehicle- and U0126-treated rats.

Angiogenesis is a well-known mechanism occurring in patients as well as in animal models of ischemic stroke by correlation of higher blood vessel counts with longer survival and improved functional outcome [44, 45, 100]. Induction of angiogenic factors such as Tie-2, Ang-1 and 2 after experimental stroke has been shown in previous studies [39-41, 101]. Administration of U0126 (0 and 24 hours post reperfusion) significantly increased expression of Tie-2 in peri-infarct area of animals compared to vehicle group at day 14 after tMCAO in paper I (Figure 12).

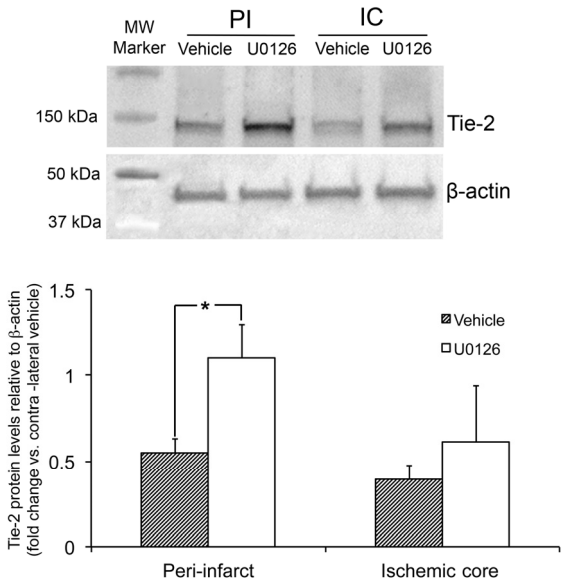

Figure 12. Expression of Tie-2 significantly increased in peri-infarct area of brain in U0126-treated male rats compared to vehicle group.
The expression of Tie-2 was normalized to β-actin and presented as percentage activity according to contralateral vehicle. Expected molecular weight of Tie-2 is 140 kDa. IC (ischemic core) and PI (peri-infarct). Molecular weight (MW) marker shows the bands of the standard molecular weight. Data are presented as mean ± SEM. *P < 0.05, vehicle; n = 4 and U0126; n = 5.

The increased expression of Tie-2 might be an indication of elevated angiogenesis in the ischemic border of U0126 treated animals; however, it made us interested to further investigate if inhibition of MEK-ERK1/2 pathway could actually improve cerebral blood circulation. Therefore, DSC-MRI was applied in paper II and data showed improved perfusion and enhanced regional CBV (caudate putamen) in U0126-treated compared to vehicle-treated animals (treatment at 6 and 24 hours post reperfusion) at day 14 after tMCAO. Moreover, ADC maps corresponded well with infarct area and CBV for both vehicle- and U0126-treated animals (Figure 13).

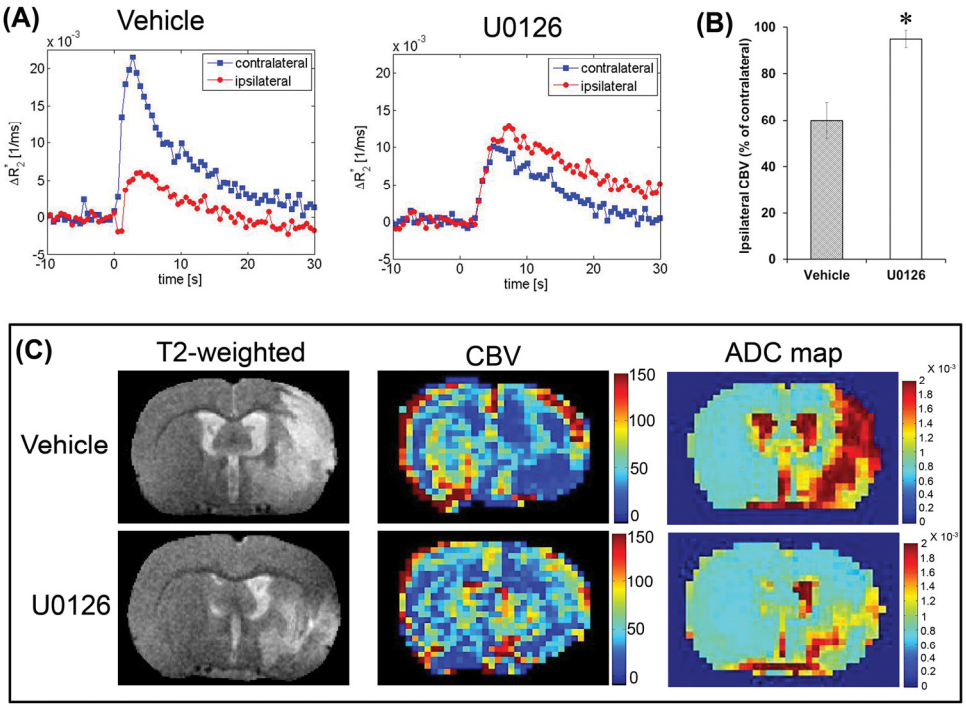

Figure 13. MEK-ERK1/2 inhibition significantly improved cerebral perfusion at day 14 after tMCAO.
(A) Representative perfusion plot for U0126- and vehicle-treated animal in caudate putamen. There is a lack of perfusion in the vehicle-treated animal compared to U0126-treated. **(B)** Graph shows regional CBV in ipsilateral caudate putamen relative to its corresponding region in contralateral side for both U0126- and vehicle-treated animals. Regional CBV was improved in U0126-treated animals compared to vehicle-treated group at day 14 after tMCAO. Vehicle; n = 3 and U0126; n = 7. Data are expressed as mean ± SEM; * indicates significant differences ($P < 0.05$). **(C)** Representative images for T2-weighted (leftpanel), relative cerebral blood volume map (middle panel) and apparent diffusion coefficient (ADC) map for vehicle- and U0126-treated animals.

In our data, CBV was negatively correlated by infarct size indicating that enhancement of CBV was accompanied by smaller infarct size. Moreover, smaller infarct size was positively correlated by better behavioral outcome in paper I. Therefore, we concluded that beneficial effects of acute MEK-ERK1/2 inhibition persist beyond acute phase and, the elevated CBV, vessel stability and protective effect of scar formation could be underlying mechanisms of reduced infarct size leading to improved functional outcome. Data from paper I and II support the notion of acute MEK-ERK1/2 inhibition as a potential therapeutic strategy for stroke.

Acute inhibition of MEK-ERK1/2 pathway and functional outcomes in females (Paper III)

It is well documented that sex differences play a role in stroke incidence, outcome and response to potential treatments [102]. The overall incidence of stroke is higher in men throughout the lifespan; however, women account for 60.6% of stroke deaths [102]. Functional recovery after stroke and stroke severity is also worse in women, even in age-matched data with men, leaving women as the major burden of stroke-related disability. A recent study showed that female sex significantly increases the predisposition to stroke, even independent of age [103, 104]. Despite more severe stroke symptoms, t-PA treatment shows more favorable outcome in women, and consequently less intracerebral hemorrhage risk than men [105, 106].

One of the main reasons for the "translational roadblock" in the development of stroke treatments is that the majority of pre-clinical studies are performed in male rodents, a clear discrepancy when considering the clinical situation [2, 106]. Therefore, it is important to evaluate females when developing new potential treatments and paper III was designed to address this issue.

Upregulation of vascular ET_B receptor was shown previously in cerebral arteries after stroke in rat [107, 108] as well as human [109] resulting in increased vasoconstriction and further reduced CBF. In parallel, MEK-ERK1/2 pathway was also activated in SMCs of occluded MCA after experimental stroke. U0126 reduced endothelin receptor-mediated vasoconstriction showing ERK1/2 pathway as intracellular pathway underlying this mechanism [13, 22, 99]. However, it was not known whether similar mechanism contributes to the effect of ischemic stroke in females. Therefore, in the first part of paper III, female rats were subjected to 2-hour tMCAO, treated by either U0126 or vehicle (at 0 and 24 hours post reperfusion), terminated after 48 hours and brains and MCAs were removed for further analysis.

Wire-Myograph experiments showed increased ET_B-mediated vasoconstriction in occluded MCA compared to non-occluded side. As similar to previous results in male rats, we also observed by immunohistochemistry that expression of ET_B receptor was increased in SMCs of occluded MCA compared to non-occluded side. Increased expression of ET_B receptor was associated by activation of MEK-ERK1/2 pathway via ERK1/2 phosphorylation in SMCs of occluded MCAs. Administration of U0126 could block activation of MEK-ERK1/2 pathway, suppress upregulation of ET_B receptor and hinder ET_B receptor-mediated vasoconstriction. These data indicate that similar to males, upregulation of ET_B

and its vasoconstrictive effect after tMCAO are also via MEK-ERK1/2 pathway in female rats (Figure 14).

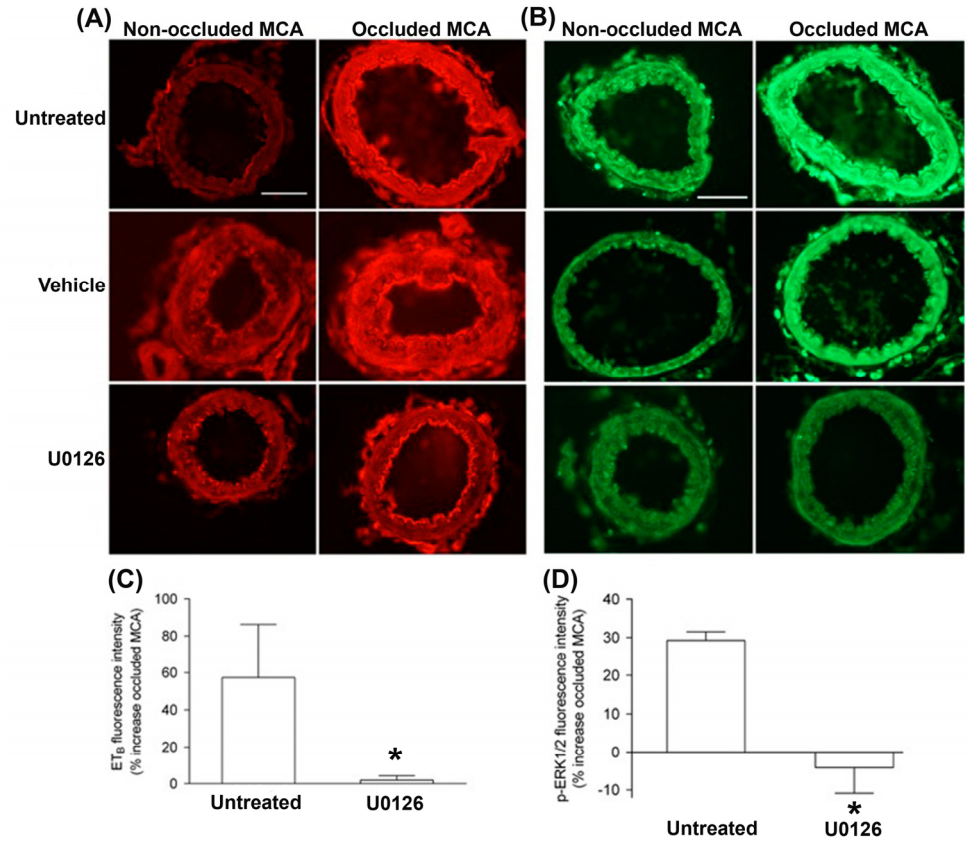

Figure 14. Ischemia-induced increase in smooth muscle ET_B receptor and p-ERK1/2 expression after tMCAO is prevented by U0126 treatment.
(A) Representative images of ET_B receptor and **(B)** p-ERK1/2 expression at 48 hours of reperfusion after tMCAO in the occluded and non-occluded MCA in untreated, vehicle, and U0126 animals. Scale bar: 50 µm. **(C)** Quantification (mean fluorescence intensity in the smooth muscle cell layer of the occluded MCA normalized to the non-occluded MCA) of ET_B receptor and **(D)** p-ERK1/2 expression. Untreated: n=7, vehicle: n=3, U0126: n=6. *p<0.05.

Knowing that similar mechanisms occur in female rats, we moved forward to evaluate if the hypothesis of MEK-ERK1/2 blockade was also affecting functional outcome of females in the second part of paper III. Therefore, female rats were subjected to (2-hour) tMCAO, treated by either U0126 or vehicle (at 0 and 24 hours post reperfusion) and tested by composite neuroscore tests over a period of two weeks. U0126 could significantly improve functional outcome of the female animals at day 2 and 14 after tMCAO (Figure 15). In male rats, inhibition of MEK-ERK1/2 pathway not only improved neurological functions but also

decreased infarct size at these time-points [53] (and papers I-II). However, there was no difference in the infarct size between vehicle- and U0126-treated female rats neither at day 2 nor at day 14 (data not published) after tMCAO. It should be noted that the ultimate goal in stroke therapy is that patients recover from their functional and neurological deficits [5] and similar to our data; improved motor function without affecting infarct size was also reported by previous studies [110].

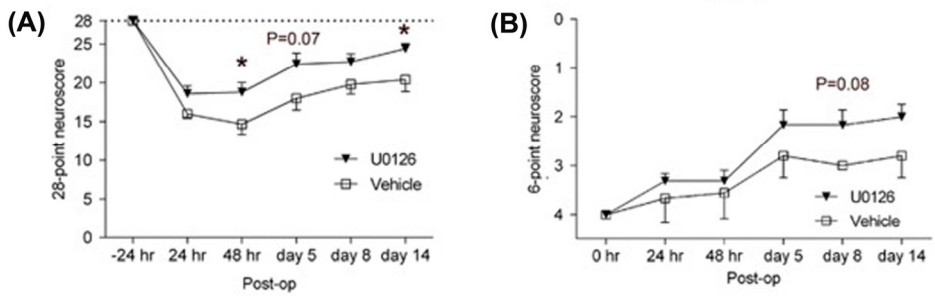

Figure 15. MEK1/2 inhibition results in improved neurologic function after tMCAO in female rats.
(A) Recovery of sensorimotor function up to 14 days after tMCAO with the 28-point neuroscore. **(B)** Gross neurologic function graded in six levels from 0—no visible defects to 5—death. Behavioral analysis up to 2 days, 9 to 15 animals per group; 5 to 14 days, 5 to 7 animals per group.

U0126 also affects cerebrovasculature of females in a similar way as in males. However, female cerebral arteries are under larger influence of vasodilatory mechanisms and thereby increased ET_B mediated vasoconstriction might have less detrimental effect in females [111-113]. Therefore, we suggest that the effects are not large enough to change infarct volume but significant enough to improve functional outcome in female rats. In paper III, we concluded that early activation of ERK1/2 is associated with detrimental effect of ET_B receptor upregulation and vasoconstriction. Inhibition of MEK-ERK1/2 pathway in acute phase, in return leads to improved post-stroke neurologic function during acute phase and beyond that in female rats.

Spontaneous recovery in females and repair-related molecular changes (Paper IV)

Some degree of spontaneous functional recovery occurs in stroke patients [8, 114, 115]. Although this recovery is limited, studying its underlying mechanisms can provide knowledge to build hypothesis for promoting recovery and brain remodelling [10]. Animal studies have provided details about molecular and cellular mechanisms associated with spontaneous functional recovery such as scar formation,

angiogenesis and inflammation as well as intracellular signalling pathways i.e. ERK1/2 and Akt pathways [8].

However, most of these mechanistic studies have been performed in male animals and studies on females are few [76, 106, 116]. As remarked earlier, sex has a major impact on stroke outcome [102]. For instance, U0126 improved functional outcome in both female and male rats but, it did not affect infarct size in females at day 2 or at day 14 after tMCAO. This data is in contrast to previous male studies where U0126 significantly decreased infarct size [22, 53] (and papers I-II). These differences show that a more complete understanding of sex-specific underlying mechanisms during subacute phase could result in improved treatment strategies. Therefore, we kept our interest to study females more in details.

To achieve that, paper IV was designed and female rats were subjected to 2-hour MCAO and monitored by 28-point and 6-point neuroscore tests in a period of two weeks. At day 14, animals were terminated and brains and MCAs were removed to investigate molecular mechanisms of interest. Our data showed that female rats had significant neurological deficits during the first two days after tMCAO; however, these deficits spontaneously and gradually recovered up to two weeks after experimental stroke (Figure 16). Since neurological function did not reach plateau at day 14, an active functional recovery might be still be on-going in female rats at this time-point.

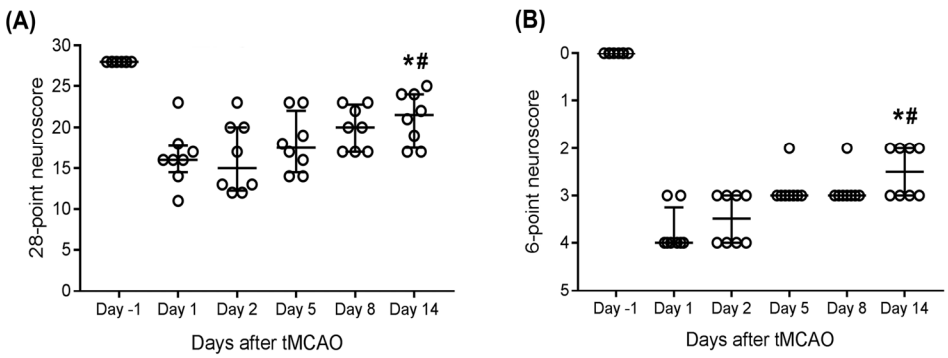

Figure 16. Female rats showed significant spontaneous functional recovery after ischemic stroke.
(A) Animals showed spontaneous recovery from neurological deficits in 28-point test at day 2, until day 14 after tMCAO. **(B)** neurological score of animals from 0 for death overnight to 5 for healthy function is shown. Neurological function of the animals was significantly affected by tMCAO, however, the animals showed significant functional improvement at day 14 after tMCAO. * shows significance level at $p<0.05$ for comparison between day -1 (pre-stroke) and day 14. # shows significance level at $p<0.05$ for comparison between day 1 (for 6-point test) and 2 (for 28-point test) vs day 14. Number of rats in both tests; n = 8.

To shed more lights on molecular mechanisms associated with spontaneous functional recovery in female rats, intracellular signalling pathways of our interest, MEK-ERK1/2 pathway, and Akt pathway were first investigated (Figure 17). The result showed that ERK1/2 pathway is significantly activated (elevated p-ERK1/2) in peri-infarct region of brain compared to control side at day 14 in female rats. This data even more support the idea that activation of ERK1/2 pathway beyond acute phase plays a role in different recovery mechanisms like angiogenesis or beneficial effects of growth factors [58]. However, early activation of ERK1/2 after ischemic stroke is detrimental and that acute inhibition of the pathway has been beneficial after ischemic stroke [22, 53] in both female and male rats (papers I-III).

The status of Akt after ischemic stroke was also investigated in paper IV. Increased expression of p-Akt (Ser-473) but decreased expression of t-Akt was observed in ischemic region compared to control side. This might suggest that Akt-473 promotes a Lys-48-linked polyubiquitination of Akt which leads to its degradation as previously stated [117]. However, previous studies showed that the role of Akt in ischemic stroke is neuroprotective; for instance, growth factors exert their beneficial effects via this pathway [60, 63, 64]. Therefore, we propose that in absence of survival factors, Akt can undergo degradation via phosphorylation of Ser-473 at the later stage of stroke in female rats. However, survival factors like growth factor can switch this phosphorylation towards Akt stability and activation.

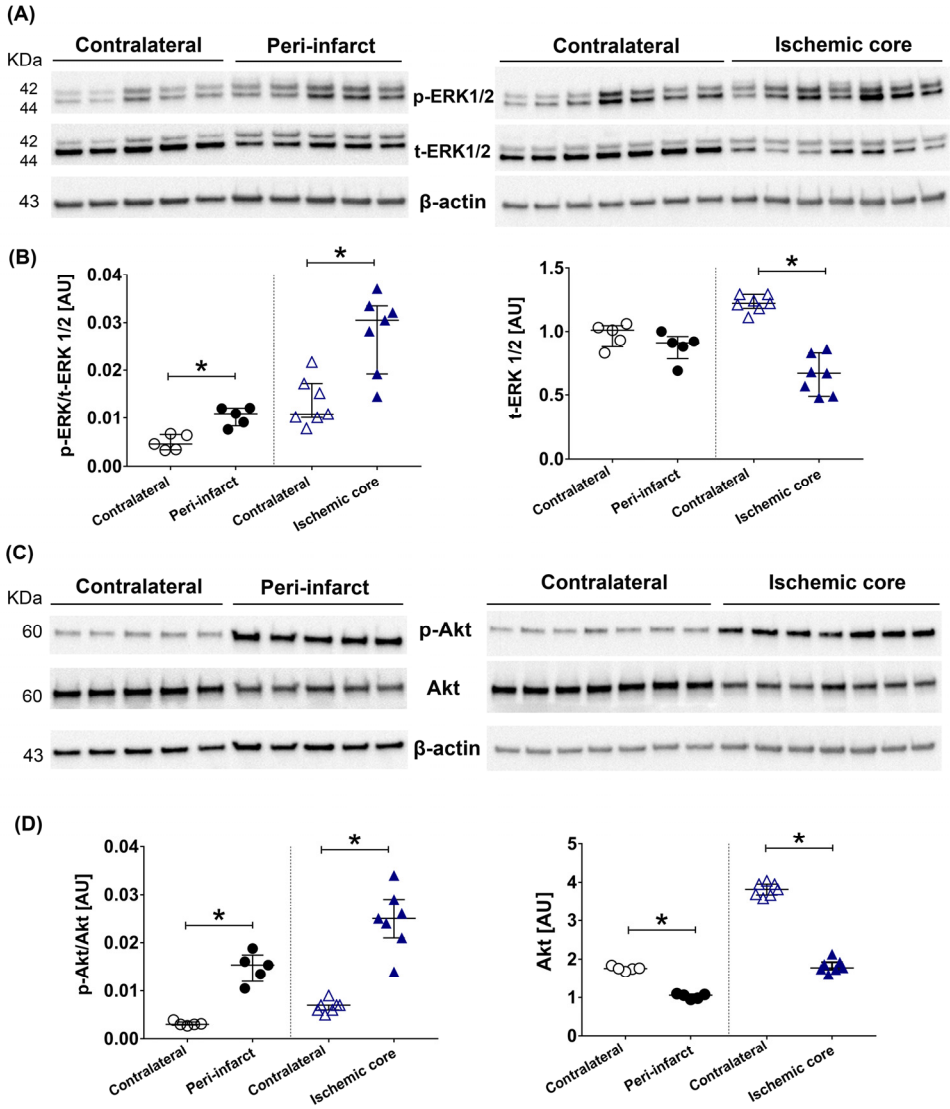

Figure 17. ERK1/2 and Akt pathways are affected at day 14 after tMCAO in female rats.
(A) Representative western blots of p-ERK1/2 and t-ERK1/2 for peri-infarct and ischemic core vs. contralateral samples. **(B)** Quantification of p-ERK1/2 (normalized to t-ERK1/2 and β-actin - left graph) and t-ERK1/2 (normalized to β-actin – right graph) in peri-infarct and ischemic core compared to contralateral. **(C)** Representative western blots of p-Akt and Akt for peri-infarct vs. contralateral (left panel) and ischemic core vs. contralateral (right panel). **(D)** Quantification of p-Akt (normalized to Akt and β-actin) in peri-infarct and ischemic core showed significant increase compared to their related contralateral samples (left graph). Akt expression was significantly lower in peri-infarct and ischemic core compared to contralateral (right graph). Number of animals for ischemic core; n=7, number of animals for peri-infarct; n=5. [AU]: arbitrary units, * indicates significant differences ($p<0.05$).

In addition to signalling pathways that are critical modulator during stroke recovery, our study was preceded by evaluation of other repair-related biomarkers involved in scar formation, protective effects of trophic factors, angiogenesis and inflammation in female rats.

Transforming growth factor-β (TGF-β) proteins are multifunctional cytokines. Krupinski et al. (1996) showed an increased expression of TGF-β in the ischemic brain areas of stroke patients [118]. Since then, TGF-β has elicited clinical interest in stroke field. A neuroprotective role of the induced TGF-β was suggested by its correlation with the reduction of the infarcted area. The possible mechanisms for neuroprotective role of TGF-β include anti-inflammatory actions, promotion of scar formation, anti-apoptotic actions, and protection against excitotoxicity. In addition, local activation of TGF-β *in vivo* promotes angiogenesis and maybe is critical to vessel remodelling and stability [119-122]. These effects are exerted through pathways such as Akt and ERK1/2; however, most of the mechanistic studies evaluated male animals and studies addressing females are few.

Data in paper IV showed that expression of TGF-β was significantly increased in ischemic core compared to control side of the brain in female rats at day 14 after tMCAO. Immunohistochemical analysis also showed significant expression of TGF-β in occluded MCA vs non-occluded side (Figure 18).

Our observation is supported by a previous study showing astrocytes control neuroinflammation via TGF-β signalling and preserve brain function 2-3 days after stroke in female mice [123]. Moreover, another study showed that TGF-β signalling in the brain after stroke is equivalent in males and females, increased by 2 fold, peaking on day 7 but not threeafter [124]. Therefore, we suggest that TGF-β is also implementing its possible roles through MEK-ERK1/2 pathway either in angiogenesis or neuroprotection via anti-apoptotic mechanisms in female rats. However, the direct interaction of these components after stroke in favour of functional improvement in female rats should be further investigated.

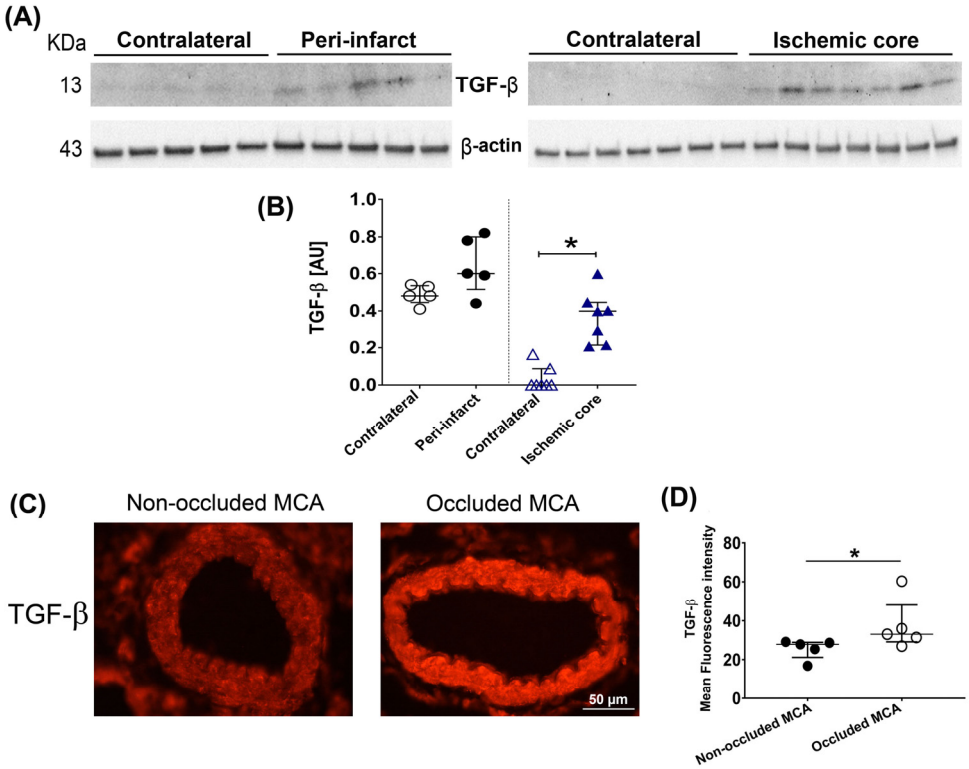

Figure 18. Expression of TGF-β was significantly increased in occlud MCA and ischemic region of the brain 14 days after tMCAO in female rats.
(A-B) Representative western blots and quantification of TGF-β in peri-infarct and ischemic core vs. contralateral side. Expression of TGF-β was normalized to β-actin. Ischemic core; n=7, peri-infarct; n=5. [AU]: arbitrary units, * indicates significant differences between peri-infarct or ischemic core and contralateral ($p<0.05$). **(C-D)** Ischemia induced a significant increase of TGF-β expression in the smooth muscle cell layer in the occluded vs non-occluded MCAs in female rats. **(C)** Representative image of TGF-β expression at day 14 after tMCAO in the occluded and non-occluded MCAs. **(D)** Quantification of TGF-β mean fluorescence intensity in occluded vs non-occluded MCAs at day 14 after tMCAO; n=5, * $p<0.05$

Nestin, an intermediate filament protein is a marker for new neurons [125] and has also been expressed in astrocytes surrounding infarct with formation of gliotic scar, preventing infarct to expand and contributing to recovery of neurological functions [24, 27, 32]. Formation of astrocytic scar also aids axonal regeneration in central nervous system [33, 34]. Expression of nestin was previously observed in the peri-infarct area surrounding ischemic core in male rats (paper I). Increased expression of nestin in ischemic regions of brains was also demonstrated for female rats in paper IV (Figure 19). These set of data suggest the expression of nestin at this time-point in female rats may also contribute in formation of gliotic scar and neuroregeneration after stroke.

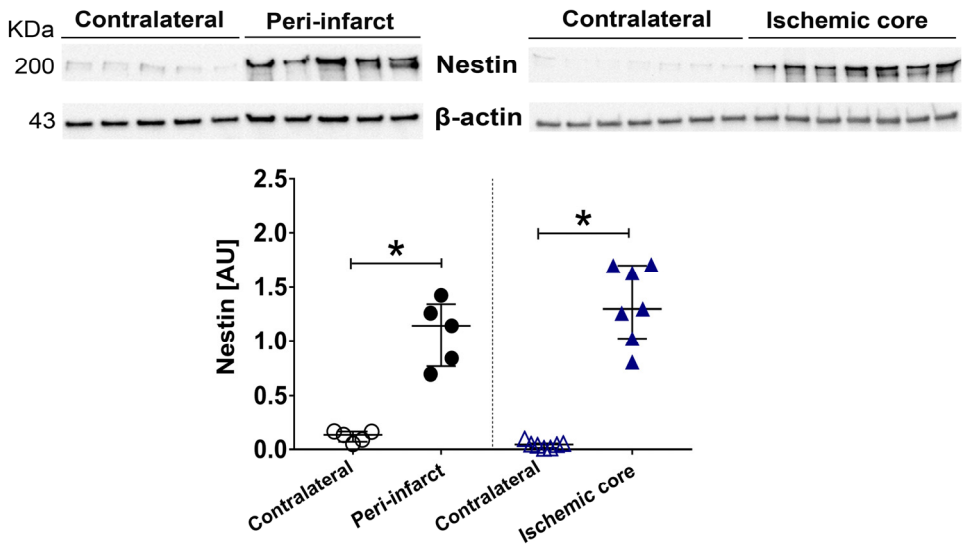

Figure 19. Increased expression of nestin in ischemic regions of brain at day 14 after tMCAO in female rats. Representative western blots and quantification of nestin in peri-infarct and ischemic core vs. contralateral side. Ischemic core; n=7, peri-infarct; n=5. [AU]: arbitrary units, * indicates significant differences between peri-infarct or ischemic core and contralateral ($p<0.05$).

Induction of angiogenic markers like Tie-2, Ang-1 and 2 in males has been shown for animal models of ischemic stroke in previous studies [40, 41, 101] as we also showed in case of Tie-2 in paper I. A more recent study illustrated three angiogenic stages following ischemic stroke in male rats each with distinct mRNA expression profile. In this study, no change in Tie-2 mRNA was shown for the studied time-points following MCAO compared to previous studies[39]. Despite controversy in male data, we also did not observe any difference for Tie-2 in ischemic region compared to control side of the brain in female rats at day 14 after tMCAO. However, angiogenesis is a well-known mechanism occurring in both female and male patients after stroke [44]. In addition, there is evidence that new capillaries support restored perfusion in the ischemic border after cortical stroke in experimental stroke and more specific in female rats [45]. Therefore, it has been proposed in paper IV that occurrence of angiogenesis upon ischemia is accompanied by changes in endogenous expression of other angiogenic markers but not Tie-2.

Inflammatory responses in brain after cerebral ischemia have been studied extensively in male but not female. Interleukin (IL)-10 is an important anti-inflammatory cytokine expressed in response to brain injury. Clinical and preclinical studies, mostly performed in male animals, show opposite results on the effect of IL-10 [126, 127]. The controversy exists in the role of IL-10 in ischemic

stroke and fewer studies in females show the necessities of more targeted studies to evaluate precise contribution of IL-10 following acute brain injuries. A previous study revealed smaller infarct volume in female vs male mice at day 4 after tMCAO, suggesting increased distinct population of IL-10-secreting $CD8^{+}CD122^{+}$ T-suppressor cells in females compared to male mice as underlying mechanism [128]. More evidence for the role of IL-10 in female was shown in a study that higher level of IL-10 was associated with poor outcome in female patients but not in males [129]. Our data show show no significant difference of IL-10 between ischemic and non-ischemic sides in female rat brains during recovery phase of ischemic stroke. Further, the data also suggested a big variation of IL-10 between individuals. One reason for this finding could be the sample size and that evaluation of IL-10 has been performed only in one time-point during recovery phase and may have missed the peak IL-10 response since we saw an increase of IL-10 in ischemic region but not statistically significant (Figure 20).

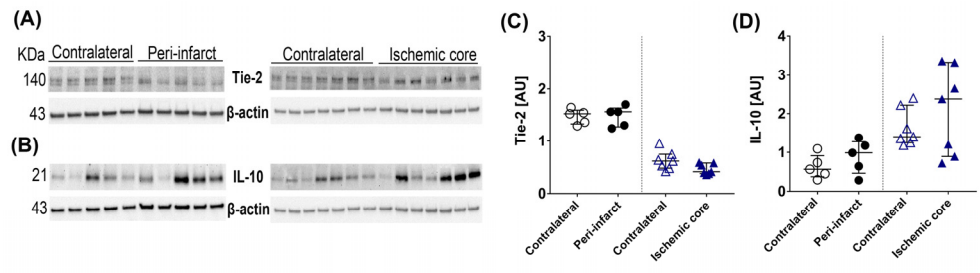

Figure 20. Expression of Tie-2 and IL-10 in brain 14 days after tMCAO.
(A-B) Representative western blots of Tie-2 and IL-10 in peri-infarct and ischemic core vs. contralateral side. **(C-D)** Quantification of Tie-2 and IL-10 in peri-infarct and ischemic core vs. contralateral side. All markers were normalized to β-actin. Ischemic core; n=7, peri-infarct; n=5. [AU]: arbitrary units, * indicates significant differences between peri-infarct or ischemic core and contralateral ($p<0.05$).

In conclusion, we clearly demonstrated that spontaneous functional recovery in female rats is accompanied by a significantly higher expression of nestin, p-Akt, p-ERK1/2 and TGF-β in ischemic regions compared to contralateral side at day 14 post-stroke. These findings might be part of the underlying mechanisms during the recovery phase of stroke and can be addressed to promote brain remodelling, aid stroke recovery and develop new therapeutic strategies in females.

Concluding Remarks

Protection of viable tissue within peri-infarct region from unleashed mechanisms that lead to cell and tissue demise is a challenge in ischemic stroke [69, 70]. Previous studies showed that early inhibition of MEK-ERK1/2 pathway is beneficial during acute phase [53, 54, 99]. Therefore, we have targeted this pathway as a potential stroke treatment to be further investigated.

The goal of acute ischemic stroke treatment is not only to rescue brain cells from acute detrimental events but also to improve patient outcome in later stage of the event [5]. Our data in papers I and II showed that acute inhibition of MEK-ERK1/2 using U0126 improved functional outcome and reduced infarct size beyond acute phase. It is also important to highlight the results in paper II where treatment was given in a clinically relevant time-window and beneficial effects were still observed beyond acute phase. The importance arises from the point that there is "door to needle" duration in every stroke case. Therefore, developing acute treatments which can be applicable in a more expanded time-window from stroke onset could be beneficial to greater percentage of stroke patients. In paper I, the late status of ERK1/2 was not affected by acute inhibition of this pathway. This is important since this pathway has beneficial contributions to tissue repair in the later stage of cerebral ischemia. In conclusion, we proposed increased expression of Tie-2, enhanced CBV and astrocyte scar formation as part of underlying mechanisms for delayed beneficial effect of acute U0126 treatment.

Studies on females in stroke field are few [76, 106, 116] although sex has a big impact on stroke incidence, outcome and response to the treatments [102]. Thus, the second part of the thesis addressed females in regard to our initial hypothesis. Data in paper III showed that ET_B receptor was upregulated on smooth muscle cells of occluded MCAs and induced vasoconstriction via MEK-ERK1/2 pathway during acute phase in females as similar to males. In addition, acute inhibition of MEK-ERK1/2 pathway supressed increased receptor-mediated vasoconstriction and also improved functional outcome in female rats.

Stroke patients show some degree of spontaneous recovery after initial injury [8, 114, 115]. Similar patterns of recovery are also observed in animal studies [8]. In paper IV, we demonstrated that spontaneous functional recovery in female rats was coincided by higher expression of nestin, p-Akt, TGF-β and activation of ERK1/2 in ischemic region during recovery phase. These data can be addressed to promote

brain remodelling and aid stroke recovery in females. It also supports the idea that although early activation of MEK-ERK1/2 pathway is destructive but in later stage, it may involve in recovery processes as observed in males.

Taken all together, the results of the present thesis shed more light on the notion of acute inhibition of MEK-ERK1/2 pathway as a therapeutic strategy for stroke. It has been applicable in a clinically relevant time-window and beneficial for both sexes with persistence of improved functional outcome beyond acute phase. So, a path well-investigated may actually lead to better stroke recovery and a future stroke treatment.

Svensk Sammanfattning

Stroke är en ledande dödsorsak samt den vanligaste orsaken till funktionshinder i västvärlden och uppstår på grund av försämrat blodflöde till en del av hjärnan som resulterar i nedsatt syre och näringstillförsel. Syrebristen orsakas antingen av en blodpropp (ischemisk hjärninfarkt) eller bristning av ett blodkärl (blödning). I det mest centrala området (kärnan) som är i direkt anknytning till ischemin aktiveras signalvägar som leder till nervcells död. Det område som ligger omkring kärnan får blodtillförsel från omkringliggande kärl och kallas för peri-infarkt området. Hjärncellerna i detta område kan överleva under gynnsamma förhållanden, men riskerar att dö vid brist på behandling. Denna avhandling har fokuserat på hjärninfarkt.

Behandlings alternativen är begränsade till trombolys eller trombectomi. Trombolysbehandling har många restriktioner, måste ges inom 4,5 timmar efter insjuknandet annars uppstår komplikationer som blödning. Detta leder till att väldigt få patienter får behandlingen. En alternativ behandling är att försöka att rädda nevceller i peri-infarkt området. Trots att man har sett positiva resultat med den typen av behandling på en experimentell nivå så har kliniska studier inte kunnat påvisa övertygande resultat. Vad det beror på är ännu okänt, men att forskningen inte har inkluderat könsskillnader, ålder, eller behandlingstid kan vara bidragande faktorer. Det finns ett stort behov av en ny effektiv behandling för stroke.

Våra tidigare studier har påvisat att en blockering av MEK-ERK1/2 signalmolekylen minskar hjärnskadan och förbättrar den neurologiska funktionen hos hanråttor. Det är dock bara undersökt i det akuta skedet samt i hanråttor. För att kunna utveckla ny behandlings strategi, behöver vi bättre kunna klargöra effekterna av MEK-ERK blockering.

Syftet med denna avhandling är att klargöra effekterna vid akut hämningen av MEK-ERK1/2 signalvägen efter stroke genom att undersöka följande i en experimentell modell av stroke (i) Positiv effekt utöver det akuta stadiet och anknutna återhämtningsmekanismer, (ii) en tidsram som är mer kliniskt anpassad, (iii) akuta negativa mekanismer och effekten av hämningen i honråttor (iv) reparerande mekanismer i återhämtningsfasen efter stroke i honråttor.

Sammanfattningsvis bevisar denna avhandling att akut hämning av MEK-ERK1/2 signalvägen är ett lovande behandlingssätt för stroke. Det har dessutom påvisat lovande resultat inom en behandlings tid som är kliniskt relevant med positiva effekter på båda könen, samt en långvarig positiv funktionell effekt. Dessutom har reparations och återhämtningsmekanismer i det senare stadiet av stroke bevisats hos honråttor vilket understryker att ERK1/2 signalvägen är inblandad i naturliga återhämtningsprocesser utanför det akuta stadiet. Därför är detta ett behandlingsalternativ som kan leda till en bättre återhämtning för stroke patienter och till en framtids terapi för stroke.

Acknowledgements

Then I get to the part of the thesis which I have been waiting for! It is the part that I can express my pure feelings and words. In my opinion, PhD study is a challenging journey with ups and downs, tears, happiness and excitements. But it should not be forgotten that beyond that life goes on! And the value of life is priceless. Then every second of such a journey will be part of this priceless gift, and therefore would be appreciated. Having said that, I would like to express my deep gratitude to the people who were part of this journey with me and without them I could not reach to this point.

First of all, my supervisors; ***Saema Ansar***, my main supervisor, what a journey we have had together! Now we can lay back and be happy on the big day! I am honoured to defend as your first PhD student. Thank you for your openness for discussions that I could freely ask you whatever and your comments made my PhD life easier! You truly believe that PhD program is a learning process along which you come together with your students until reaching the finish line. Thank you for your great supervision throughout the projects; especially on the last project giving me a fantastic opportunity to further develop as a scientist and improve my writing skills in an absolute professional manner. That is not very common for PhD students to share office with supervisors; however, I truly enjoyed it and really missed you when you were away.

Lars Edvinsson, the head of Experimental Vascular Research and my co-supervisor, Thank you for giving me the opportunity to be part of your group to pursue my PhD study. With no doubt, you are a professional and experienced scientist with great scientific achievements that have always been inspiring me. Thank you for all your precise and concise comments on my work and also for your supportive words to keep me motivated.

Then I would like to thank all the present members of Experimental Vascular Research; the people whom I spent majority of my time with during these years. We have had an incredible environment and I will never forget our crazy and fun discussions during lunch, fikas and so on. ***Karin***, thank you for teaching me immunohistochemistry, for your contribution and great help in my first project and for being interested in listening to me when I needed your advice as a senior! ***Frank***, for your help during my second project while badly needed, for our friendship, numerous research-related and unrelated discussions, being an amazing

buddy during our PhD study in the group and being a wonderful invader over the top-shelf territory borders in the office! ***Lena***, for your kindness and care whenever I showed up by your office for a talk or advice. I admire your positive attitude and your desire for sport and training. ***Kajsa,*** my travel buddy during the conferences! I hope for a future trip to have our long breakfasts, lunches and so on! Thank you for your contribution in female project and I wish you the very best for your PhD study! ***Mimmi, Philip, Andre and Marie-Louise***, for our great discussions and providing a nice atmosphere whenever you were around.

Then I would like to thank people outside Experimental Vascular Research who contributed to my projects; ***Hilda***, for making me part of your project during your post-doc here in Lund. For all your prompt answers and comments whenever I was blocked with female project. ***Friedrich***, a big thank you for your amazing knowledge in the field of MRI, without that my second project would have not been possible to be performed. ***Diana***, for your constructive comments on my projects during your visits to the lab. ***Gunilla***; for taking all my questions patiently on various aspects of in vivo experiments. You made me feel confident with in vivo work. ***Rushani***, for your contribution to the first project. I would also like to thank ***Lund University Bioimaging Center*** (LBIC), for providing us access to MRI facility. Especially to ***Adnan***, for your help running the MRI experiments and taking care of my animals while I had to run back and forth between different places during my second project.

My former colleagues at Experimental Vascular Research; ***Aida***, for introducing me to the group and your fabulous support in the beginning of my PhD journey, ***Melinda***, for your sincere friendship and all life discussions we have had together. ***Sajedeh***, for our sweet Persian conversations in the lab or after work and for your advice on immuno experiments. ***Elisabeth***, the lab felt like home when you were around with all your care, laughter and reminder to take care of ourselves. All the former and present members in our *sister lab* at Glostrup; ***Sarah, Stine, Simona, Kristian, Simon, Jacob***, for our fun Christmas parties, enjoyable lab retreats, and great discussions. Especially ***Anne-Sofie***, I truly enjoyed being a part of your projects, also for our family-related discussions whenever you have been in Lund. You were always in the right time here (fika!).

All the former and present people in A13; ***Karsten***, I have a big respect for you as a scientist! Your questions and comments always made me think deeper about my work and try to learn more. ***Miriana***, for your kind help as a senior especially while being prepared for my half-time. ***Kerstin, Enida, Daniela, Katrin, Eleonor, Sarah, Andrea, Jakob and Carin*** for our breakfast and fika times and for creating a nice atmosphere.

I would like to thank my friends outside the work environment especially; ***Shirin and Liana***, for your wonderful friendship over the last 20 years. You two know

me better than myself! ***Ingrid***, for your patience to listen to me whenever needed and for your valuable comments and friendly advice. ***Mrs. Ara***, the head coach of Iran national youth volleyball team and the former coach of Isfahan University team. You taught me not only volleyball but also life lessons during the years I was part of your team. ***Sahar***, for our numerous discussions, our family gatherings and being together to finish our PhD journeys. ***Afsaneh, Masoomeh, Shahrzad, Hossein, Mahdi, Mehdi, Mojgan, Maryam, Reza and Matin*** for our nice time together and also for the support you provided me like a second family during tough times.

Special thanks to my family, my parents; ***Akbar and Fatemeh***, you are adorable! Dad, you are my role model, mentor and best friend in my life. With your vast knowledge and experience both in academia and management, you have taught me a lot! You have supported me in every single personal and professional decision I made in my life. Mom, you have always believed in me and being excited with my every achievement, for your fabulous support throughout my education. How could I manage the tough time during my complicated pregnancy and after that without you? You are an angel walking on the earth!

My brothers; ***Ali***, for all your love, care and sweetness to your little sister during childhood and even now. ***Ehsan***, the fun and charming guy in the family, Thank you for always making me happy, smile and laugh with your positive attitude even from distance. My sister in law, ***Mahsa***, time is forgotten when we are together. Thank you for all crazy and fun times we have had together and your kindness. My nephew and niece; ***Navid and Nadia***, for all your sweet and cute wording to support aunty Maryam when she was tired and stressed from work. I would like also to thank ***the family Sina*** for your nice hospitality during our trips to Iran, especially my father in law, for your care and interest about the progress of my PhD and always believing in me.

And last but not least, ***my dearest Hossein***, it is impossible to describe how much I love you! Seeing you by chance back in time during my stay in Singapore changed the whole equation of my life. Your honesty and kindness were apparent in your first words. You have a big heart filled with love, passion and care for your family! Thank you for being by my side every moment I needed and for your fabulous professional, scientific and emotional support during my PhD. Here we are, reaching our eight anniversaries and we cannot be happier than now with our beautiful princess, ***Nikki!***

References

1. Donnan GA, Fisher M, Macleod M, Davis SM. Stroke. *Lancet (London, England)*. 2008;371:1612-1623
2. Mergenthaler P, Meisel A. Do stroke models model stroke? *Disease models & mechanisms*. 2012;5:718-725
3. Moskowitz MA, Lo EH, Iadecola C. The science of stroke: Mechanisms in search of treatments. *Neuron*. 2010;67:181-198
4. Carmichael ST. The 3 rs of stroke biology: Radial, relayed, and regenerative. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics*. 2016;13:348-359
5. Fisher M, Saver JL. Future directions of acute ischaemic stroke therapy. *The Lancet. Neurology*. 2015;14:758-767
6. Carmichael ST, Archibeque I, Luke L, Nolan T, Momiy J, Li S. Growth-associated gene expression after stroke: Evidence for a growth-promoting region in peri-infarct cortex. *Experimental neurology*. 2005;193:291-311
7. Nakayama H, Jorgensen HS, Raaschou HO, Olsen TS. Recovery of upper extremity function in stroke patients: The copenhagen stroke study. *Archives of physical medicine and rehabilitation*. 1994;75:394-398
8. Cramer SC. Repairing the human brain after stroke: I. Mechanisms of spontaneous recovery. *Annals of neurology*. 2008;63:272-287
9. Murphy TH, Corbett D. Plasticity during stroke recovery: From synapse to behaviour. *Nat Rev Neurosci*. 2009;10:861-872
10. Zhang ZG, Chopp M. Promoting brain remodeling to aid in stroke recovery. *Trends Mol Med*. 2015;21:543-548
11. Bernhardt J, Hayward KS, Kwakkel G, Ward NS, Wolf SL, Borschmann K, et al. Agreed definitions and a shared vision for new standards in stroke recovery research: The stroke recovery and rehabilitation roundtable taskforce. *International journal of stroke : official journal of the International Stroke Society*. 2017;12:444-450
12. Zhao LR, Willing A. Enhancing endogenous capacity to repair a stroke-damaged brain: An evolving field for stroke research. *Progress in neurobiology*. 2018;163-164:5-26
13. Edvinsson LI, Povlsen GK. Vascular plasticity in cerebrovascular disorders. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2011;31:1554-1571
14. Edvinsson L. Cerebrovascular endothelin receptor upregulation in cerebral ischemia. *Current vascular pharmacology*. 2009;7:26-33

15. Lampl Y, Fleminger G, Gilad R, Galron R, Sarova-Pinhas I, Sokolovsky M. Endothelin in cerebrospinal fluid and plasma of patients in the early stage of ischemic stroke. *Stroke*. 1997;28:1951-1955
16. Viossat I, Duverger D, Chapelat M, Pirotzky E, Chabrier PE, Braquet P. Elevated tissue endothelin content during focal cerebral ischemia in the rat. *Journal of cardiovascular pharmacology*. 1993;22 Suppl 8:S306-309
17. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*. 1988;332:411-415
18. Endoh M, Fujita S, Yang HT, Talukder MA, Maruya J, Norota I. Endothelin: Receptor subtypes, signal transduction, regulation of ca2+ transients and contractility in rabbit ventricular myocardium. *Life sciences*. 1998;62:1485-1489
19. Koyama Y. Endothelin systems in the brain: Involvement in pathophysiological responses of damaged nerve tissues. *Biomolecular concepts*. 2013;4:335-347
20. Davenport AP, O'Reilly G, Kuc RE. Endothelin eta and etb mrna and receptors expressed by smooth muscle in the human vasculature: Majority of the eta sub-type. *British journal of pharmacology*. 1995;114:1110-1116
21. Szok D, Hansen-Schwartz J, Edvinsson L. In depth pharmacological characterization of endothelin b receptors in the rat middle cerebral artery. *Neuroscience letters*. 2001;314:69-72
22. Maddahi A, Edvinsson L. Enhanced expressions of microvascular smooth muscle receptors after focal cerebral ischemia occur via the mapk mek/erk pathway. *BMC neuroscience*. 2008;9:85
23. Choudhury GR, Ding S. Reactive astrocytes and therapeutic potential in focal ischemic stroke. *Neurobiol Dis*. 2016;85:234-244
24. Becerra-Calixto A, Cardona-Gomez GP. The role of astrocytes in neuroprotection after brain stroke: Potential in cell therapy. *Front Mol Neurosci*. 2017;10:88
25. Eng LF, Ghirnikar RS, Lee YL. Glial fibrillary acidic protein: Gfap-thirty-one years (1969-2000). *Neurochemical research*. 2000;25:1439-1451
26. Sims NR, Yew WP. Reactive astrogliosis in stroke: Contributions of astrocytes to recovery of neurological function. *Neurochem Int*. 2017;107:88-103
27. Sofroniew MV. Molecular dissection of reactive astrogliosis and glial scar formation. *Trends Neurosci*. 2009;32:638-647
28. Liem RK. Molecular biology of neuronal intermediate filaments. *Current opinion in cell biology*. 1993;5:12-16
29. Wiese C, Rolletschek A, Kania G, Blyszczuk P, Tarasov KV, Tarasova Y, et al. Nestin expression--a property of multi-lineage progenitor cells? *Cellular and molecular life sciences : CMLS*. 2004;61:2510-2522
30. Duggal N, Schmidt-Kastner R, Hakim AM. Nestin expression in reactive astrocytes following focal cerebral ischemia in rats. *Brain research*. 1997;768:1-9

31. Zamanian JL, Xu L, Foo LC, Nouri N, Zhou L, Giffard RG, et al. Genomic analysis of reactive astrogliosis. *J Neurosci*. 2012;32:6391-6410
32. Huang L, Wu ZB, Zhuge Q, Zheng W, Shao B, Wang B, et al. Glial scar formation occurs in the human brain after ischemic stroke. *Int J Med Sci*. 2014;11:344-348
33. Anderson MA, Burda JE, Ren Y, Ao Y, O'Shea TM, Kawaguchi R, et al. Astrocyte scar formation aids central nervous system axon regeneration. *Nature*. 2016;532:195-200
34. Liddelow SA, Barres BA. Regeneration: Not everything is scary about a glial scar. *Nature*. 2016;532:182-183
35. Bush TG, Puvanachandra N, Horner CH, Polito A, Ostenfeld T, Svendsen CN, et al. Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. *Neuron*. 1999;23:297-308
36. Pekny M, Nilsson M. Astrocyte activation and reactive gliosis. *Glia*. 2005;50:427-434
37. Risau W. Mechanisms of angiogenesis. *Nature*. 1997;386:671-674
38. Navaratna D, Guo S, Arai K, Lo EH. Mechanisms and targets for angiogenic therapy after stroke. *Cell Adhesion & Migration*. 2014;3:216-223
39. Moisan A, Favre IM, Rome C, Grillon E, Naegele B, Barbieux M, et al. Microvascular plasticity after experimental stroke: A molecular and mri study. *Cerebrovascular diseases (Basel, Switzerland)*. 2014;38:344-353
40. Lin TN, Wang CK, Cheung WM, Hsu CY. Induction of angiopoietin and tie receptor mrna expression after cerebral ischemia-reperfusion. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2000;20:387-395
41. Lin TN, Nian GM, Chen SF, Cheung WM, Chang C, Lin WC, et al. Induction of tie-1 and tie-2 receptor protein expression after cerebral ischemia-reperfusion. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2001;21:690-701
42. Greenberg DA. Poststroke angiogenesis, pro: Making the desert bloom. *Stroke*. 2015;46:e101-102
43. Manoonkitiwongsa PS, Jackson-Friedman C, McMillan PJ, Schultz RL, Lyden PD. Angiogenesis after stroke is correlated with increased numbers of macrophages: The clean-up hypothesis. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2001;21:1223-1231
44. Krupinski J, Kaluza J, Kumar P, Kumar S, Wang JM. Role of angiogenesis in patients with cerebral ischemic stroke. *Stroke*. 1994;25:1794-1798
45. Wei L, Erinjeri JP, Rovainen CM, Woolsey TA. Collateral growth and angiogenesis around cortical stroke. *Stroke*. 2001;32:2179-2184

46. Seevinck PR, Deddens LH, Dijkhuizen RM. Magnetic resonance imaging of brain angiogenesis after stroke. *Angiogenesis*. 2010;13:101-111
47. Chopp M, Zhang ZG, Jiang Q. Neurogenesis, angiogenesis, and mri indices of functional recovery from stroke. *Stroke*. 2007;38:827-831
48. Sun J, Nan G. The mitogen-activated protein kinase (mapk) signaling pathway as a discovery target in stroke. *Journal of molecular neuroscience : MN*. 2016;59:90-98
49. Chu CT, Levinthal DJ, Kulich SM, Chalovich EM, DeFranco DB. Oxidative neuronal injury. The dark side of erk1/2. *European journal of biochemistry*. 2004;271:2060-2066
50. Irving EA, Bamford M. Role of mitogen- and stress-activated kinases in ischemic injury. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2002;22:631-647
51. Krupinski J, Slevin M, Marti E, Catena E, Rubio F, Gaffney J. Time-course phosphorylation of the mitogen activated protein (map) kinase group of signalling proteins and related molecules following middle cerebral artery occlusion (mcao) in rats. *Neuropathology and applied neurobiology*. 2003;29:144-158
52. Iadecola C, Anrather J. The immunology of stroke: From mechanisms to translation. *Nature medicine*. 2011;17:796-808
53. Maddahi A, Edvinsson L. Cerebral ischemia induces microvascular pro-inflammatory cytokine expression via the mek/erk pathway. *Journal of neuroinflammation*. 2010;7:14
54. Maddahi A, Chen Q, Edvinsson L. Enhanced cerebrovascular expression of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 via the mek/erk pathway during cerebral ischemia in the rat. *BMC neuroscience*. 2009;10:56
55. Maddahi A, Povlsen GK, Edvinsson L. Regulation of enhanced cerebrovascular expression of proinflammatory mediators in experimental subarachnoid hemorrhage via the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathway. *Journal of neuroinflammation*. 2012;9:274
56. Shioda N, Han F, Fukunaga K. Role of akt and erk signaling in the neurogenesis following brain ischemia. *International review of neurobiology*. 2009;85:375-387
57. Hetman M, Gozdz A. Role of extracellular signal regulated kinases 1 and 2 in neuronal survival. *European journal of biochemistry*. 2004;271:2050-2055
58. Sawe N, Steinberg G, Zhao H. Dual roles of the mapk/erk1/2 cell signaling pathway after stroke. *Journal of neuroscience research*. 2008;86:1659-1669
59. Sun J, Nan G. The extracellular signal-regulated kinase 1/2 pathway in neurological diseases: A potential therapeutic target (review). *International journal of molecular medicine*. 2017;39:1338-1346

60. Zhao EY, Efendizade A, Cai L, Ding Y. The role of akt (protein kinase b) and protein kinase c in ischemia-reperfusion injury. *Neurol Res*. 2016;38:301-308
61. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of akt/pkb by the rictor-mtor complex. *Science (New York, N.Y.)*. 2005;307:1098-1101
62. Kim YS, Yoo A, Son JW, Kim HY, Lee YJ, Hwang S, et al. Early activation of phosphatidylinositol 3-kinase after ischemic stroke reduces infarct volume and improves long-term behavior. *Mol Neurobiol*. 2017;54:5375-5384
63. Kilic E, Kilic U, Wang Y, Bassetti CL, Marti HH, Hermann DM. The phosphatidylinositol-3 kinase/akt pathway mediates vegf's neuroprotective activity and induces blood brain barrier permeability after focal cerebral ischemia. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2006;20:1185-1187
64. Zhao H, Sapolsky RM, Steinberg GK. Phosphoinositide-3-kinase/akt survival signal pathways are implicated in neuronal survival after stroke. *Mol Neurobiol*. 2006;34:249-270
65. Miyawaki T, Ofengeim D, Noh KM, Latuszek-Barrantes A, Hemmings BA, Follenzi A, et al. The endogenous inhibitor of akt, ctmp, is critical to ischemia-induced neuronal death. *Nature neuroscience*. 2009;12:618-626
66. El Amki M, Wegener S. Improving cerebral blood flow after arterial recanalization: A novel therapeutic strategy in stroke. *International journal of molecular sciences*. 2017;18
67. Emberson J, Lees KR, Lyden P, Blackwell L, Albers G, Bluhmki E, et al. Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute ischaemic stroke: A meta-analysis of individual patient data from randomised trials. *Lancet (London, England)*. 2014;384:1929-1935
68. Hacke W, Kaste M, Bluhmki E, Brozman M, Davalos A, Guidetti D, et al. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. *The New England journal of medicine*. 2008;359:1317-1329
69. Paciaroni M, Caso V, Agnelli G. The concept of ischemic penumbra in acute stroke and therapeutic opportunities. *European neurology*. 2009;61:321-330
70. O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW. 1,026 experimental treatments in acute stroke. *Annals of neurology*. 2006;59:467-477
71. Goldman JM, Murr AS, Cooper RL. The rodent estrous cycle: Characterization of vaginal cytology and its utility in toxicological studies. *Birth defects research. Part B, Developmental and reproductive toxicology*. 2007;80:84-97
72. Koizumi J, Yoshida Y, Nakazawa T, Ooneda G. Experimental studies of ischemic brain edema. I. A new experimental model of cerebral embolism

in rats in which recirculation can be introduced in the ischemic area. *Japanese Stroke Journal*. 1986;8:1-8
73. Engelhorn T, Goerike S, Doerfler A, Okorn C, Forsting M, Heusch G, et al. The angiotensin ii type 1-receptor blocker candesartan increases cerebral blood flow, reduces infarct size, and improves neurologic outcome after transient cerebral ischemia in rats. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2004;24:467-474
74. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: Evaluation of the model and development of a neurologic examination. *Stroke*. 1986;17:472-476
75. Schallert T. Behavioral tests for preclinical intervention assessment. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics*. 2006;3:497-504
76. Encarnacion A, Horie N, Keren-Gill H, Bliss TM, Steinberg GK, Shamloo M. Long-term behavioral assessment of function in an experimental model for ischemic stroke. *Journal of neuroscience methods*. 2011;196:247-257
77. Schaar KL, Brenneman MM, Savitz SI. Functional assessments in the rodent stroke model. *Experimental & translational stroke medicine*. 2010;2:13
78. Montoya CP, Campbell-Hope LJ, Pemberton KD, Dunnett SB. The "staircase test": A measure of independent forelimb reaching and grasping abilities in rats. *Journal of neuroscience methods*. 1991;36:219-228
79. Grabowski M, Brundin P, Johansson BB. Paw-reaching, sensorimotor, and rotational behavior after brain infarction in rats. *Stroke*. 1993;24:889-895
80. Pagnussat Ade S, Michaelsen SM, Achaval M, Netto CA. Skilled forelimb reaching in wistar rats: Evaluation by means of montoya staircase test. *Journal of neuroscience methods*. 2009;177:115-121
81. Baird AL, Meldrum A, Dunnett SB. The staircase test of skilled reaching in mice. *Brain research bulletin*. 2001;54:243-250
82. Klein A, Dunnett SB. Analysis of skilled forelimb movement in rats: The single pellet reaching test and staircase test. *Current protocols in neuroscience*. 2012;Chapter 8:Unit8.28
83. Livingston-Thomas JM, Tasker RA. Animal models of post-ischemic forced use rehabilitation: Methods, considerations, and limitations. *Experimental & translational stroke medicine*. 2013;5:2
84. Koretsky AP. New developments in magnetic resonance imaging of the brain. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics*. 2004;1:155-164
85. Albensi BC, Knoblach SM, Chew BG, O'Reilly MP, Faden AI, Pekar JJ. Diffusion and high resolution mri of traumatic brain injury in rats: Time course and correlation with histology. *Experimental neurology*. 2000;162:61-72

86. Mandeville ET, Ayata C, Zheng Y, Mandeville JB. Translational mr neuroimaging of stroke and recovery. *Translational stroke research*. 2017;8:22-32
87. Duong TQ. Mri in experimental stroke. *Methods in molecular biology (Clifton, N.J.)*. 2011;711:473-485
88. Liu F, Schafer DP, McCullough LD. Ttc, fluoro-jade b and neun staining confirm evolving phases of infarction induced by middle cerebral artery occlusion. *Journal of neuroscience methods*. 2009;179:1-8
89. Zille M, Farr TD, Przesdzing I, Muller J, Sommer C, Dirnagl U, et al. Visualizing cell death in experimental focal cerebral ischemia: Promises, problems, and perspectives. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2012;32:213-231
90. Vogel J, Mobius C, Kuschinsky W. Early delineation of ischemic tissue in rat brain cryosections by high-contrast staining. *Stroke*. 1999;30:1134-1141
91. Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR. A semiautomated method for measuring brain infarct volume. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 1990;10:290-293
92. Bass JJ, Wilkinson DJ, Rankin D, Phillips BE, Szewczyk NJ, Smith K, et al. An overview of technical considerations for western blotting applications to physiological research. *Scandinavian journal of medicine & science in sports*. 2017;27:4-25
93. Mahmood T, Yang PC. Western blot: Technique, theory, and trouble shooting. *North American journal of medical sciences*. 2012;4:429-434
94. Ramos-Vara JA, Miller MA. When tissue antigens and antibodies get along: Revisiting the technical aspects of immunohistochemistry--the red, brown, and blue technique. *Veterinary pathology*. 2014;51:42-87
95. Coons AH, Leduc EH, Connolly JM. Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *The Journal of experimental medicine*. 1955;102:49-60
96. Hogestatt ED, Andersson KE, Edvinsson L. Mechanical properties of rat cerebral arteries as studied by a sensitive device for recording of mechanical activity in isolated small blood vessels. *Acta physiologica Scandinavica*. 1983;117:49-61
97. Mulvany MJ, Halpern W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circulation research*. 1977;41:19-26
98. Ratz PH, Berg KM, Urban NH, Miner AS. Regulation of smooth muscle calcium sensitivity: Kcl as a calcium-sensitizing stimulus. *American journal of physiology. Cell physiology*. 2005;288:C769-783

99. Henriksson M, Stenman E, Vikman P, Edvinsson L. Mek1/2 inhibition attenuates vascular eta and etb receptor alterations after cerebral ischaemia. *Experimental brain research*. 2007;178:470-476
100. Navarro-Sobrino M, Rosell A, Hernandez-Guillamon M, Penalba A, Boada C, Domingues-Montanari S, et al. A large screening of angiogenesis biomarkers and their association with neurological outcome after ischemic stroke. *Atherosclerosis*. 2011;216:205-211
101. Beck H, Acker T, Wiessner C, Allegrini PR, Plate KH. Expression of angiopoietin-1, angiopoietin-2, and tie receptors after middle cerebral artery occlusion in the rat. *The American journal of pathology*. 2000;157:1473-1483
102. Ahnstedt H, McCullough LD, Cipolla MJ. The importance of considering sex differences in translational stroke research. *Translational stroke research*. 2016;7:261-273
103. Manwani B, McCullough LD. Sexual dimorphism in ischemic stroke: Lessons from the laboratory. *Women's health (London, England)*. 2011;7:319-339
104. Falsetti L, Viticchi G, Buratti L, Balucani C, Marra AM, Silvestrini M. From head to toe: Sex and gender differences in the treatment of ischemic cerebral disease. *Pharmacological research*. 2017;121:240-250
105. Hill MD, Kent DM, Hinchey J, Rowley H, Buchan AM, Wechsler LR, et al. Sex-based differences in the effect of intra-arterial treatment of stroke: Analysis of the proact-2 study. *Stroke*. 2006;37:2322-2325
106. Liu F, McCullough LD. Middle cerebral artery occlusion model in rodents: Methods and potential pitfalls. *Journal of biomedicine & biotechnology*. 2011;2011:464701
107. Stenman E, Malmsjo M, Uddman E, Gido G, Wieloch T, Edvinsson L. Cerebral ischemia upregulates vascular endothelin et(b) receptors in rat. *Stroke*. 2002;33:2311-2316
108. Hansen-Schwartz J, Hoel NL, Zhou M, Xu CB, Svendgaard NA, Edvinsson L. Subarachnoid hemorrhage enhances endothelin receptor expression and function in rat cerebral arteries. *Neurosurgery*. 2003;52:1188-1194; 1194-1185
109. Vikman P, Edvinsson L. Gene expression profiling in the human middle cerebral artery after cerebral ischemia. *European journal of neurology*. 2006;13:1324-1332
110. Lopez-Valdes HE, Clarkson AN, Ao Y, Charles AC, Carmichael ST, Sofroniew MV, et al. Memantine enhances recovery from stroke. *Stroke*. 2014;45:2093-2100
111. Geary GG, Krause DN, Duckles SP. Estrogen reduces myogenic tone through a nitric oxide-dependent mechanism in rat cerebral arteries. *The American journal of physiology*. 1998;275:H292-300
112. Ospina JA, Duckles SP, Krause DN. 17beta-estradiol decreases vascular tone in cerebral arteries by shifting cox-dependent vasoconstriction to

vasodilation. *American journal of physiology. Heart and circulatory physiology*. 2003;285:H241-250
113. Ospina JA, Krause DN, Duckles SP. 17beta-estradiol increases rat cerebrovascular prostacyclin synthesis by elevating cyclooxygenase-1 and prostacyclin synthase. *Stroke*. 2002;33:600-605
114. Verheyden G, Nieuwboer A, De Wit L, Thijs V, Dobbelaere J, Devos H, et al. Time course of trunk, arm, leg, and functional recovery after ischemic stroke. *Neurorehabil Neural Repair*. 2008;22:173-179
115. Delavaran H, Aked J, Sjunnesson H, Lindvall O, Norrving B, Kokaia Z, et al. Spontaneous recovery of upper extremity motor impairment after ischemic stroke: Implications for stem cell-based therapeutic approaches. *Translational stroke research*. 2017;8:351-361
116. Bosetti F, Koenig JI, Ayata C, Back SA, Becker K, Broderick JP, et al. Translational stroke research: Vision and opportunities. *Stroke*. 2017;48:2632-2637
117. Wu YT, Ouyang W, Lazorchak AS, Liu D, Shen HM, Su B. Mtor complex 2 targets akt for proteasomal degradation via phosphorylation at the hydrophobic motif. *J Biol Chem*. 2011;286:14190-14198
118. Krupinski J, Kumar P, Kumar S, Kaluza J. Increased expression of tgf-beta 1 in brain tissue after ischemic stroke in humans. *Stroke*. 1996;27:852-857
119. Dobolyi A, Vincze C, Pal G, Lovas G. The neuroprotective functions of transforming growth factor beta proteins. *International journal of molecular sciences*. 2012;13:8219-8258
120. Islam A, Choudhury ME, Kigami Y, Utsunomiya R, Matsumoto S, Watanabe H, et al. Sustained anti-inflammatory effects of tgf-beta1 on microglia/macrophages. *Biochim Biophys Acta Mol Basis Dis*. 2018;1864:721-734
121. Lee HS, Han J, Bai HJ, Kim KW. Brain angiogenesis in developmental and pathological processes: Regulation, molecular and cellular communication at the neurovascular interface. *FEBS J*. 2009;276:4622-4635
122. Buisson A, Lesne S, Docagne F, Ali C, Nicole O, MacKenzie ET, et al. Transforming growth factor-beta and ischemic brain injury. *Cellular and molecular neurobiology*. 2003;23:539-550
123. Cekanaviciute E, Fathali N, Doyle KP, Williams AM, Han J, Buckwalter MS. Astrocytic transforming growth factor-beta signaling reduces subacute neuroinflammation after stroke in mice. *Glia*. 2014;62:1227-1240
124. Doyle KP, Cekanaviciute E, Mamer LE, Buckwalter MS. Tgfbeta signaling in the brain increases with aging and signals to astrocytes and innate immune cells in the weeks after stroke. *Journal of neuroinflammation*. 2010;7:62

125. Michalczyk K, Ziman M. Nestin structure and predicted function in cellular cytoskeletal organisation. *Histology and histopathology*. 2005;20:665-671
126. Garcia JM, Stillings SA, Leclerc JL, Phillips H, Edwards NJ, Robicsek SA, et al. Role of interleukin-10 in acute brain injuries. *Frontiers in neurology*. 2017;8:244
127. Spychala MS, Honarpisheh P, McCullough LD. Sex differences in neuroinflammation and neuroprotection in ischemic stroke. *Journal of neuroscience research*. 2017;95:462-471
128. Banerjee A, Wang J, Bodhankar S, Vandenbark AA, Murphy SJ, Offner H. Phenotypic changes in immune cell subsets reflect increased infarct volume in male vs. Female mice. *Translational stroke research*. 2013;4:554-563
129. Conway SE, Roy-O'Reilly M, Friedler B, Staff I, Fortunato G, McCullough LD. Sex differences and the role of il-10 in ischemic stroke recovery. *Biology of sex differences*. 2015;6:17

Printed by Media-Tryck, Lund 2018 NORDIC SWAN ECOLABEL 3041 0903

A Path for Improving Stroke Recovery

Maryam Mostajeran

Extremely self-motivated for research as my childhood dream was to become a scientist! I was born in Isfahan (Iran), did my Bachelor and Master of Science in Cell and Molecular Biology – Microbiology at Isfahan University. Then I moved to Singapore to begin my research journey abroad. Later on, I started a PhD in Division of Experimental Vascular Research at Lund University, Sweden which will be defended on December 20th, 2018.

My PhD thesis aimed to shed more light on the notion of acute inhibition of MEK-ERK1/2 pathway as a therapeutic strategy for ischemic stroke. Our results showed that acute blocking of this pathway has been applicable in a clinically relevant time-window and beneficial for both sexes with persistence of improved functional outcome beyond acute phase. Thus, a path well-investigated may actually lead to better stroke recovery and a future stroke treatment.

LUND UNIVERSITY

FACULTY OF MEDICINE

Experimental Vascular Research
Department of Clinical Sciences

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2018:158
ISBN 978-91-7619-727-1
ISSN 1652-8220