DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Impact of CXCl12 inhibition on gene expression patterns after stroke

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Professor Associado Karsten Ruscher (Wallenberg Neuroscience Center, Lund University, Sweden) e do Professor Carlos Duarte (Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, Portugal)

Ana Rita Pombo Antunes

2015

The experimental work described in this thesis was performed at the Laboratory for Experimental Brain Research, Wallenberg Neuroscience Center, Lund University, under the supervision of the Associate Professor Karsten Ruscher.

This work was funded by the Swedish Research Council, the Swedish Brain Fund and the Craaford Foundation.

Statement of originality

I hereby declare that the present thesis is the product of my own work. I certify that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that authors and references in use are properly cited in the text and are all listed in the references section. I declare that this is a true copy of my thesis, including any final revisions, as approved by my supervisor, and that this thesis has not been submitted for a higher degree to any other University or Institution.

Acknowledgments

First of all, I would like to thank my supervisor Karsten Ruscher for all the guidance and support during all this year. Thank you for all your patience while teaching me everything and most of all, thank you for sharing your knowledge with me. It was essential to accomplish this thesis to have such a present and attentive supervisor. I could not ask for a better supervisor and person to guide me during this year!

I also want to thank Professor Tadeusz Wieloch, if he had not answered my email last summer I probably would not have had this amazing experience in his lab. Moreover, thanks so much for caring and always asking "How is the work going Ana Rita?".

To Miri that became more than a colleague, a friend. Thank you for all the care and help in the lab even when it would be the hundredth time that I would call you to check my slices in the "criotome". Also for all the amazing gympa power classes (I am not there but don't give up on going). And most important, thank you for all the talks and true advice. I miss you already!

Kerstin, besides the technical support that was amazing, thank you for always having a friendly and kind word. Thanks for making my days in the lab happier.

Jakob and Jens thanks so much for all the fun that you brought to my working days in lab. It was a pleasure to share the office and the lab with you.

To Sarah, for me the joy of the A13 floor. Thank you for all the pep talks in the lab and for sharing with me your passion about travelling. I hope one day we can travel together. Thanks also to the rest of the mito lab.

To my beautiful, amazing and outstanding basketball girls Charlotte, Annika and Friederike: thank you for being the best friends that I could ask for. Words will never be enough to describe how your friendship, support and happiness brought joy to my life in Lund. Love you girls! See you soon (I am sure)!

Thank you so much to all the "mölle team"! Living in this students' dorm was the best thing that could happen to me. I met awesome and amazing people that I will never forget. Thanks to you I never felt lonely. A special thanks to my flat mates Jordi, Maxime, Olivier, Rohit and Jasper for always being there for me even when I would behave like a huge diva, you were essential in many ways! Also to my amazing BMC and gym mate Patricia, for being such a beautiful person with whom I could always share my concerns and joys.

I also need to thank my Swedish basketball team, Lugi and Kalmar nation basketball team for allowing me to keep playing my favorite sport and for showing me how Swedish people can be nice and warm.

Não posso deixar de agradecer à Carla, a minha predecessora portuguesa no lab. Sem ela a minha chegada a Lund teria sido bem mais difícil. Obrigada por todas as dicas e conselhos.

Aos meus amigos que me acompanharam nesta aventura que é ser estudante universitário: Helena, Dani, Anita, JP, Ruben, Rui Silva, Rui Simões, Tadeu, Ana Raquel, Elvira e Bruno. Obrigada por terem estado sempre presentes mesmo a quilómetros de distância e por me fazerem sentir sempre tão querida. Vocês são os melhores!

Às minhas amigas: Helena, Xana, Inês, Joana e Bárbara. Por me mostrarem que uma amizade não se mede pelo tempo de existência mas pela intensidade com que é vivida. Obrigada!

À minha espanhola favorita Bruna por me fazer acreditar que numa verdadeira amizade a distância não é nada. Obrigada por a teres encurtado este ano…foi maravilhoso poder ter partilhado um pouco deste meu ano fantástico contigo. Tu e Barcelona que me esperem, deste ano não passa!

Um obrigado especial às minhas amigas de sempre e para sempre Joana e Agreira, porque por mais anos que passem e as coisas mudem vocês permanecem. Joana, obrigada ainda a ti e também ao Carlos por terem querido partilhar esta aventura comigo. Poder mostrar-vos um pouco do meu mundo este ano foi maravilhoso. Lund adorou-vos!

Não posso deixar de fazer um agradecimento ao Professor Alfredo e à Gracita pela ajuda que me deram aquando da escrita desta tese.

Por último, mas com toda a importância, tenho de agradecer à minha família por ser a melhor família de sempre (e não é por ser a minha). O vosso apoio incondicional durante esta aventura foi essencial. Obrigada por estarem sempre à distância de uma chamada, mensagem ou carta. Um obrigado em especial aos meus pais por serem um grande exemplo na minha vida e por me terem dado as ferramentas e apoio necessários para conseguir ultrapassar todos os obstáculos que a vida pessoal e académica me impõem. E, claro à minha irmã Catarina por ser linda e maravilhosa, mas principalmente por ter um dos melhores corações que conheço.

Gostaria de dedicar esta tese à minha mãe por ter sido um exemplo de força, persistência e de amor à família e ao próximo durante toda a sua vida. Sem o seu amor, carinho, compreensão e dedicação não teria sido possível chegar onde já cheguei. Obrigada mãe!

Index

Abbreviations

ADP - Adenosine diphosphate

AMD3100 - 1-[4-(1,4,8,11-tetrazacyclotetradec-1-ylmethyl)phenyl]methyl]-1,4,8,11-tetrazacyclo-tetradecan

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

APS - Ammonium Persulfate

ATP - Adenosine triphosphate

BBB - Blood brain barrier

BW - Body weight

CBF - Cerebral blood flow

CD4 - Cluster of differentiation 4

CD8 - Cluster of differentiation 8

CINC - Cytokine-induced neutrophil chemoattractant

CNS - Central nervous system

CXCL12 - C-X-C motif chemokine ligand 12

CXCR4 - C-X-C chemokine receptor type 4

CXCR7 - C-X-C chemokine receptor type 4

CX3CL1 - C-X3-C chemokine ligand 1

CX3CR1 - C-X3-C chemokine receptor 1

DEPC - Diethylpyrocarbonate

DNA - Deoxyribonucleic acid

EDTA - Ethylenediamine tetraacetic acid

FDA - Food and Drug Administration

FKN - Fractalkine

GABA - Gamma-AminoButyric Acid

GFP - Green fluorescence protein

Iba1 - ionized calcium binding adaptor molecule 1

IC - Infarct core

ICAM-1 - Intercellular adhesion molecule 1

ICAM-2 - Intercellular adhesion molecule 2

IL-1 - Interleukin-1

IL-6 - Interleukin-6

IL-17 - Interleukin 17

i-LTP - Ischemic long term potentiation

Ip - Intraperitoneally

LTP - Long term potentiation

MC - Mast cells

MCAO - Middle cerebral artery occlusion

MCP-1 - Monocyte chemoattractant protein-1

MHC I - Major histocompatibility complex class I

MHC II - Major histocompatibility complex class II

NMDA - N-methyl-D-aspartate receptor

MMPs - Matrix metalloproteases

mRNA - Messenger Ribonucleic acid

NAD - Nicotinamide Adenine Dinucleotide

NEM - Neuromuscular electrical stimulation

NOS - Nitric oxide synthase

NTN - Neurotactin

PARP-1 - Poly(ADP-ribose)polymerase-1

PIDs - Peri-infarct depolarizations

PT - photothrombosis

RIN - RNA integrity number

RNA - Ribonucleic acid

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

rRNA - Ribosomal Ribonucleic acid

SDS - Sodium Dodecyl Sulfate

tDCM - transcranial Direct Current Stimulation

TEMED - N,N,N',N'-Tetramethyllenediamine

TGF-β - Transforming growth factor beta

tMCAO - transient Middle Cerebral Artery Occlusion

TMS - Transcranial Magnetic Stimulation

TNF-α - Tumor necrosis factor alpha

t-PA - tissue Plasminogen Activator

VCAM-1 - Vascular cell adhesion molecule 1

VEGF - vascular endothelial growth factor

Abstract

Ischemic Stroke is a cerebrovascular disease that critically diminishes brain functions due to acute brain tissue loss caused by lack of blood supply. Although, acute treatments (thrombolysis) have been successfully implemented in clinical practise only a very smal percentage of stroke patient is eligible for such an intervention. The majority of patients, however, is left with some degree of diability. Therefore, the development of adjuvant stroke treatments applicable in the rehabilitation phase would be a benefit for most of the patients to improve neurological deficits and enhance quality of life. In rodent models of stroke mechanisms of functional recovery of lost neurological function can be studied and potential recovery enhancing drugs can be tested. Here we have investigated if gene expression patterns change in the nonlesioned contralateral hemisphere i.e. the corpus callosum and adjacent cortex in heterozygous CX3CR1$^{+/GFP}$ mice subjected to permanent focal ischemia induced in the primary motor cortex by photothrombosis. Moreover, we studied if treatment with 1-[4-(1,4,8,11-tetrazacyclotetradec-1-ylmethyl)phenyl]methyl]-1,4,8,11-tetrazacyclo-tetradecan (AMD3100; 0.5 mg/kg) for 5 days starting on day 2 after PT influenced these patterns. Seven days after photothrombosis, we found an accumulation of GFP positive cells co-expressing major histocompatibility complex class II and Ionized calcium binding adaptor molecule 1 (Iba1) accumulating in the corpus callosum contralateral to the lesioned hemisphere. Mice displayed significant neurological deficits assessed by a composite neuroscore not affect by treatment with AMD3100. First results from gene expression analysis showed the regulation of genes related to the poststroke immune response, poststroke inflammation as well as neuronla plasticity. Moreover, our data indicate that treatment with AMD3100 downregulated GABAergic neurotransmission suggestive for a crosstalk between inflammatory processes and mechanisms of neuronal plasticity. Further studies are needed to elucidate exact function of candidate genes regulated in the postischemic brain.

Keywords: Stroke recovery, inflammation, microglia, gene array.

Resumo

O acidente vascular cerebral (AVC) é uma doença vascular cerebral que é caracterizada pela diminuição crítica das funções do cérebro devido a uma perda aguda dos tecidos causada por falta do normal fornecimento de sangue. Apesar dos tratamentos agudos (trambolíticos) terem sido implementados com sucesso na clínica, apenas uma pequena percentagem dos pacientes estão nas condições necessárias para os receber. A maioria dos pacientes fica com algum grau de deficiência. Assim sendo, o desenvolvimento de tratamentos adjuvantes aplicáveis à fase de reabilitação seria um benefício para a maioria dos pacientes para que melhorem as capacidades neurológicas e ganhem qualidade de vida. Em modelos animais de AVC é possível o estudo de mecanismos funcionais da recuperação da função neurológica perdida, bem como, o teste de drogas capazes de aumentar a recuperação. Neste trabalho é investigado se ocorre alteração no padrão de expressão genética no hemisfério não lesionado, mais especificamente no corpus callosum e no cortex adjacente num ratinho heterozigótico para GFP sujeito a isquémia focal permanente, induzida no cortex motor primário, por fototrombose. Mais ainda, é estudado se o tratamento com AMD3100 (0.5 mg/kg) iniciado no dia 2 após a fototrombose e mantido durante 5 dias influencia essas alterações. Sete dias após a fototrombose, encontrou-se células GFP co-expressando o complex maior de histocompatibilidade classe II e a molécula adaptadora ligante de cálcio ionizado-1 acumuladas no corpus callosum, contralateralmente ao hemisfério lesionado. Os ratinhos apresentaram significantes défices neurológicos determinados por uma composição de testes neurológicos mas aparentam não ter sido afetados pelo AMD3100. Os primeiros resultados da análise da expressão genética mostram a regulação de genes relacionados com a resposta imune e inflamatória pós-AVC mas também relacionados com plasticidade sináptica. Mais ainda, os dados indicam que o tratamento com AMD3100 diminuiu a regulação da neurotransmissão GABAérgica sugerindo um cruzamento entre os processos inflamatórios e os mecanismos de plasticidade neuronal. Mais estudos são necessários para elucidar a função exacta de cada gene candidato na regulação cerebral após isquémia.

Palavras chave: Recuperação pós AVC, inflamação, microglia, *array* genético.

Chapter 1 - Introduction

1. Introduction

Stroke is a cerebrovascular disease that affects the neurological function of the brain due to an imbalance in the blood flow. Several pathophysiological mechanisms have been identified with an occlusion or rupture of a blood supplying brain artery as most prevalent causes of ischemia. Lack of blood supply to any part of the brain is a very serious event since the energy consumption of the brain completely relies on oxydative phosphorylation and the brain consumes around 20% of the body oxygen (75 litres per day) to sustain normal function (American Heart Association). Without oxygen and nutrients the brain cells start to die within the first minutes after stroke onset.

Depending on the duration, severity and location of the ischemia, stroke can lead to permanent brain tissue damage resulting in loss of function, disability or even death (Maas & Safdieh, 2009). Loss of neurological functions and disability patterns can be observed for example as paralysis of one side of the body or both, vision problems, memory loss, speech problems and others (American Heart Association). In addition, stroke may deeply affect people's life, their social interaction and integration in the society.

1.1 Epidemiological data on stroke

Stroke has alarming statistics that need to concern the society and justifies the time and money spent searching for new ways of treatments and prevention.

Every year, 15 million people suffer from stroke and out of those about 6 million people die worldwide (WHO, 2002). It is a disease that affects primarily elderly people (>85 years) with a higher incidence in developed countries (Feigin, Lawes, Bennett, & Anderson, 2003; Morris & Schroeder, n.d.)

In the United States of America (USA) a stroke occurs every 40 seconds and one stroke patient dies every 4th minute, being the fourth cause of death behind heart diseases, cancer and chronic lower respiratory diseases (Go et al., 2014). In 2002, Europe reached approximately 650000 stroke deaths each year with Portugal having one of the higher mortality rates (Morris & Schroeder, n.d.; WHO, 2002). In Portugal, every hour six people suffer from a stroke and two in three people die from this disease (Sá, 2009).

In addition to high mortality rates, globally, stroke is the second leading cause of disability, following dementia, and the leading cause in the USA (Go et al., 2014). Each year, 5 million of people become permanently disabled (WHO, 2002). As one of the consequences, stroke

represents a high economic burden to health care systems and societies to provide sufficient rehabilitation and re-integration programmes. Only in Europe, the annual costs for stroke patient management amounts to approximately 20 billion euros.

1.2 Risk factors for stroke

A number of factors have been identified that significantly increase the risk of stroke. Based on these studies modifiable and non-modifiable risk factors have been categorized. Most of all and as age advances the probability of experiencing a stroke increases and it has been found that after the age of 55 the chance of having a stroke duplicates each ten years of life (Panel et al., 1997). Concerning race, African-Americans are more prone to suffer on a stroke compared to Caucasians, due to different life style habits (Panel et al., 1997). Also the stroke incidence in some asian countries, i.e. China, has reached epidemic proportions (Kim & Johnston, 2013; Panel et al., 1997). Postmenopausal women are more affected by strokes also with an increased incidence due to the lack of estrogens. Nevertheless, men are more susceptible when compared to women (Appelros, Stegmayr, & Terent, 2009). In addition, there are some rare genetic predispositions such as mutations in the Notch3 gene or mutations in the mitochondrial DNA which are associated with a higher risk to suffer a stroke (Chinnery, Elliott, Syed, & Rothwell, 2010; Francis, Raghunathan, & Khanna, 2007; Hassan & Markus, 2000; Meschia, Worrall, & Rich, 2014). Along with those, the occurrence of stroke is more likely in families in which 1st grade relatives suffered a stroke, however, life style factors may influence this type of risk (Panel et al., 1997).

On the other hand, there are the controllable or modifiable risk factors, such as high blood pressure, heart disease, diabetes, high blood cholesterol, carotid or other artery disease, depression, obesity, attributed mainly to the lack of physical exercise, unhealthy diets, a stressful life and smoking (Mukherjee & Patil, 2011; Panel et al., 1997). Finally, the risk to suffer a stroke is higher in patients who already experienced a stroke or transient ischemic attack. Usually the new stroke is more severe and harmful (American Heart Association).

1.3 Stroke Causes and types

The main cause of stroke is the occlusion or rupture of a blood vessel. Based on these two main causes strokes are sub-divided in two categories: ischemic and hemorrhagic, each having its own causes (Doyle, Simon, & Stenzel-Poore, 2008; Shah, n.d.).

The ischemic stroke can happen due to atherosclerosis in the blood supplying brain arteries, obstructing them. From the pathophysiological and clinical point of view and with different consequences for secondary prevention this type of stroke is divided in two classes: ischemia caused by the formation of a blood clot locally in an atherosclerotic brain artery and blood clots occluding a brain artery by embolism (Shah, n.d.). Here, the blood clot has been formed in other places of circulatory system (heart and upper chest), however, the main cause for clot formation is an intermittend or permanent atrial fibrillation (American Heart Association). Transient ischemic attacks (TIAs) are characterized by transient loss of neurological functions which persist less than 24 hours (Johnston, 2002). This type of stroke can be seen as "ministroke" or "warming stroke". It reversibly impairs brain function and represents a warning signal for a subsequent and more dangerous stroke (American Heart Association).

The hemorrhagic stroke is characterized by the rupture of vessel and the leak of blood (Shah, n.d.). Taking into account the place of the rupture and the causes, hemorrhages can be categorized into intracerebral and subarachnoid hemorrhages. The blood is spread in the brain tissue in intracerebral hemorrhage, damaging brain cells. It might be caused by high blood pressure, head trauma, vascular malformations, the use of blood-thinning medications, among others. One the other hand, the leak of subarachnoid hemorrhage happens into the space between the brain and the skull. This hemorrhage is caused by the rupture of vessels in a zone of weakened walls (aneurysm).

Based pathophysiological considerations, stroke can also be classified into permanent or transient occlusion of a brain artery. Both types of stroke can be seen in the clinics, in particular in respect to acute treatments.

1.4 Approved Treatment options for ischemic stroke

1.4.1. 'Better Safe Than Sorry' - It is better to prevent

According to the National Stroke Association approximately 80% of all strokes could be prevented eliminating modifiable risk factors (National Stroke Association). If people would adapt a healthy life style by doing some sports and having a healthy diet the probability of experiencing a stroke would be reduced significantly.

1.4.2. Approved Stroke treatments

Acute treatments of ischemic stroke available are confined to a specific time window, from the moment of symptom onset. The four accepted medical treatments are the use of intravenous or intra-arterial tissue Plasminogen Activator (t-PA), the MERCI retrieval device and the penumbra aspiration system, each one used within different time intervals after stroke onset.

The administration intravenously of t-PA is the only pharmacological treatment approved by the Food and Drug Administration (FDA) (Taussky, Tawk, Daugherty, & Hanel, 2011). It was approved in 1996 and this approval was based on the National Institute of Neurological Disorders and stroke rt-PA study (Stroke study group, 1995; Khaja & Grotta, 2007; Taussky et al., 2011). Tissue plasminogen activator is a serine protease that leads to the elimination of the clot (thrombolytic) and the restoration of the blood flow to the ischemic area. The use of this method is highly reliable and safe but only during the first 4.5 hours after symptom onset (Hacke et al., 2008). Thereafter, clinical studies have shown an increased risk of bleeding (hemorrhagic transformation) (Khaja & Grotta, 2007).

The t-PA can be also administrated intra-arterially using a micro-catheter. Although this technique is not FDA approved, it is accepted by the scientific community and in the clinics. The time window in this case is increased, with the drug being used until 6h after symptom onset (del Zoppo et al., 1998). As mentioned above for intravenous administration of t-PA the risk of hemorrhagic transformation has to be calculated against potential benefits the stroke patient will have from this maneuvre (Khaja & Grotta, 2007).

The other two methods are not pharmacological but mechanic. The removal of the clot is achieved through the use of a catheter. The MERCI retrieval device and the penumbra aspiration system can be used until 8 hours after symptom onset (Smith et al., 2008; The Penumbra Pivotal Stroke Trial Investigators, 2009).

As can be noted there are some methods to eliminate the clots and to re-establish the blood flow in the case of ischemic stroke. However they are confined to a very narrow time window (at its best less than 10% of the stroke patients are eligible for this treatment (Tenser, 2012)) and serious adverse effects like hemorrhagic transformation may occur. Also, recovery of lost neurological function is not guaranteed by these procedures. These issues show that there is the quest to study alternative approaches either to improve the existing techniques and to come up with new treatment strategies applicable for the majority of stroke patients.

1.5 Experimental treatments for stroke

Besides the attempt to guarantee the blood flow of an occluded brain artery there is rising a concern with the cells adjacent to the clot. Some therapies are under investigation to increase survival, to give neuroprotection and higher probabilities of surviving to these cells (Broderick, 2002). Two examples of this approach are the use of aspirin even until 48h after the first symptoms and the use of hypothermia as therapy actually tested in a European multicenter trial (EuroHYP-1).

1.6 Rehabilitation

Due to very narrow time window of acute treatments the majority of patients are not eligible to the treatment when they arrive to the hospital. This will lead possibly to permament neurological deficits such as aphasia, loss of motor control, loss of social skills and cognitive dysfunction, as already mention above. There are some rehabilitation treatments available which are crucial to assist the patients in having a normal life and being reintegrated in the work world.

It is accepted that spontaneous reorganization of the cerebral tissue occurs in the postischemic brain after stroke. Rehabilitation can enhance and increase the formation of the neural connections and consequently improve the impairments caused by the stroke (Cramer & Nudo, 2010).

During rehabilitation, physical, speech, occupational and recreational therapists and psychologists are fundamental and have numerous techniques available to help patients (American stroke Association, 2004).

Among these techniques, constraint-induced movement therapy is becoming widely used together with classical physical therapy. Remarkable results in the improvement of motor control are observed by this approach. Patients are not allow to use theirs unaffected limbs, being obligated to use the affected limb for a long period of time (American stroke Association, 2004). By repetitive exercises the limb regains the function due to the formation of new neural pathways. This technique is also used in the treatment of aphasia by forbidding the use of sign language (Koenig-bruhin, Kolonko, At, Annoni, & Hunziker, 2013).

Also there are some electromagnetic or electric approaches, such as, transcranial magnetic stimulation (TMS), transcranial direct current stimulation (tDCM) and neuromuscular electrical stimulation (NEM) (Cramer & Nudo, 2010). Transcranial magnetic stimulation and tDCM consists in electric or magnetic stimulations of zones of the brain that improve the cognitive

function and sensory-motor control (Schlaug & Renga, 2008). Neuromuscular electrical stimulation is the use of electric stimuli in the affected muscles to make them contract (Cramer & Nudo, 2010). Through this procedure signals are sent towards the central nervous system (CNS) with the aim to provide a feedback loop from the CNS (American stroke Association, 2004; Lin & Yan, 2011).

Other techniques used by therapists to improve movement control are biofeedback, treadmill training with partial body support and functional tone management arm training program (American stroke Association, 2004).

Besides electromagnetic, robotic and technological approaches there are the pharmacological treatments to enhance recovery of lost neurological function. It is studied that neurotransmitters have some influence, positive or negative, on the recuperation of motor control.

For example, the increase of the presence of glutamate, noradrenaline and dopamine in the synaptic cleft after injury is beneficial to the recovery of motor control. On the other hand, increased levels of the inhibitory neurotransmitter GABA have the opposite effect. So it is possible to modulate the presence of neurotransmitters in the environment through the use of drugs, such as, amphetamines, which increase the release of noradrenaline and dopamine or levodopa, which is decarboxylated into dopamine increasing the levels of the same (Cramer & Nudo, 2010).

Moreover, enhancing the levels growth factors and the use of stem cells are approaches that are extensively studied. Growth factors control the cellular pathways that are involved in the formation of neuronal circuits during brain development. In the adult brain, these factors regulate mechanisms of plasticity as well as the cellular survival (Schäbitz et al., 2007). Studies show that growth factors prevent neuronal death and promote brain recovery after injury. Stem cells in turn are being studied to restore neuronal pathways due to their ability to self-renew (Kornblum, 2007) and may contribute to mechanisms of recovery by attenuation of poststroke inflammation as well as the secretion of growth factors (Chen, Venkat, Zacharek, & Chopp, 2014).

In conclusion, several approaches have been identified and tested in experimental models of stroke rehabilitation treatments, however, more research is needed to fully understand the underlying mechanisms and to translate preclinical finding into clinical trials in order to make them safe and reliable to the patients.

2. Stroke pathophysiology

2.1 The ischemic core and the penumbra concept

As mentioned above, ischemic stroke is characterized by a sudden drop of cerebral blood flow in a defined area of the brain. To minimize the damage, the brain increases vasodilatation, extraction of the oxygen and glucose from the blood and with high importance of collateral blood flow and diffusion of oxygen and nutrients (Shah, n.d.). Therefore, based on the current concepts, it exists an infarct core in the center and in particular at the border of the ischemic territory different degrees of damage can be observed with time of ischemia.

Taking advantage of imaging technics, it is possible to distinguish two main zones after an ischemic stroke: the infarct core and the ischemic penumbra (Heiss, 2010).

Within the infarct core the cerebral blood flow (CBF) is below the threshold necessary to keep the basic metabolism of the cells (approximately 10ml/100g/min) (Shah, n.d.). In this area the damage is irreversible and due to the lack of the ATP the cells die quickly by necrosis (Doyle et al., 2008).

On the other hand, in the ischemic penumbra the tissue is functionally and metabolically impaired but the damage is reversible (Heiss, 2012) The CBF is below 20ml/100g/min and to preserve the energetic stores of ATP, brain tissue in this region becomes electrically silent and the activity of synapses is diminished (Shah, n.d.). If the CBF does not return to normal levels (reperfusion) the damage in penumbra becomes irreversible, consequently causing a bigger infarct core .(Kunz, Dirnagl, & Mergenthaler, 2010) In conclusion, the penumbra is a salvageable tissue but only during a short period of time and its rescue is depended on the residual CBF and the restoration of a sufficient blood perfusion.

2.2 Mechanisms of cell death

Unlike in the infarct core in which cells die by necrosis, cells in the penumbra start to die in a different way and display features of acute (necrosis) and delayed (apoptosis, autophagy) cell death. How exactly cells die is dependent on the type of cell, their integration in the local tissue environment, on the time after the insult (minutes, hours or even days after the vessel occlusion) and the distance to the infarct core. Importantly, it has to be considered that pure acute or delayed cell death exist but that characteristics of different types of cell death even can be observed in a single cell. Several mechanisms have been identified to contribute to cell

death. Among them are excitotoxicity, peri-infarct depolarization, oxidative and nitrative stress and inflammation(Doyle et al., 2008; Kunz et al., 2010).

2.2.1 Excitotoxicity

Without oxygen the brain is not able to generate ATP, a vital molecule for its survival. The lack of ATP leads to an increase of intracellular sodium and a decrease of intracellular potassium (ionic imbalance) once the Na+/K+ pumps are not capable of maintaining the ionic gradients without energy (Doyle et al., 2008). The loss of the gradients promotes membrane depolarization and consequently the release of neurotransmitters to the synaptic cleft. The neurotransmitter involved in the pathophysiology of ischemia is glutamate and this event is then called excitotoxicity. Also glutamate transporters (membranous proteins in neurons and glial cells), responsible for taking the glutamate out of the cleft, undergo a reversal allowing glutamate to exit the cell and thereby exacerbating the damage (Doyle et al., 2008). The increase of extracellular glutamate activates excessively ionotropic glutamate receptors (NMDA and AMPA) leading to an increase of intracellular calcium. Calcium acts as a second or third messenger activating downstream enzymes such as phospholipases, proteases and DNases, which degrade membranes, proteins and DNA essential for cell survival (Kunz et al., 2010). Intracellular calcium concentration is also controlled by Ca^{2+} ATPase, which is not able to maintain the normal concentration due to the lack of ATP and increased acidosis, that activates channels permeable to calcium (Doyle et al., 2008).

The entire scenario can be seen as a vicious cycle once the increase of glutamate causes membrane depolarization, thereby exacerbating the cell damage.

2.2.2 Peri-infarct depolarizations

The release of glutamate and potassium from the focus of the ischemia causes spontaneous and successive depolarizations repolarizations throughout the affected tissue. Due to the origin, these are called peri-infarct depolarizations (PIDs) and contribute to enhance the damage (Dirnagl, Iadecola, & Moskowitz, 1999).

2.2.3 Oxidative and nitrative stress

The brain has low levels of antioxidants; thereby it is extremely vulnerable to uncontrolled increase of reactive species of oxygen or nitrogen levels (ROS/RNS) (Doyle et al., 2008).

During ischemia, together with increased concentrations of Na^+, Ca^{2+} and ADP, mitochondria produce high levels of ROS and RNS, which in turn damage the mitochondria itself, initiating a vicious cycle (Kunz et al., 2010). The damage leads to the mitochondria swelling and release of pro-apoptotic molecules resulting cell death. In addition, the expression of isoforms of the nitric oxide synthase is increased leading to an increased production of nitric oxide, which in turn reacts with superoxide to produce peroxynitrite, a potent oxidant (Dirnagl et al., 1999). Nitric oxide is also involved in the overactivation of poly(ADP-ribose)polymerase-1 (PARP-1), a DNA repair enzyme. Over-activating PARP-1 consumes NAD+, the last not being available for essential processes such as glycolysis and mitochondrial respiration (Doyle et al., 2008).

After ischemia and during reperfusion, concentrations of RNS and NOS increases in the vicinity of blood vessels and activate matrix metalloproteases (MMPs), which leads to blood brain barrier (BBB) disruption and together with the expression of adhesion molecules promote the recruitment and migration of different types of leucocytes into the postischemic brain tissue. These events are critical since they also may cause parenchymal hemorrhage and vasogenic brain edema (Doyle et al., 2008).

2.3 The early inflammatory response after stroke

The inflammatory response in the postischemic brain has to be considerd as beneficial and detrimental depending on the spatiotemporal activation and the magnitude of action. In this section, detrimental actions of early inflammatory cascades are addressed:

The inflammatory response is very well organized and time patterned and represents a continuum of events primarily sealing the infarcted brain tissue and phagocytize cellular debris potentially with aggravation of brain damage. As macrophages microglia actively phagocytize and eliminate cellular debris and dead cells and thereby contribute to the clearance of the tissue environment. In addition, the activation of downstream events by Ca^{2+}, the increase of ROS/RNS and hypoxia lead to the expression of several pro-inflammatory genes (Dirnagl et al., 1999; Doyle et al., 2008). In turn, resident brain cells like microglia and astrocytes become activated increasing the expression of pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α, TGF-β and chemokines such as CINC and MCP-1 (Doyle et al., 2008; Lakhan, Kirchgessner, & Hofer, 2009). Endothelial cells also express pro-inflammatory molecules and in addition, express cell adhesion molecules on its surface, important for the adhesion of peripheral immune cells (Kunz et al., 2010; Lakhan et al., 2009; Siniscalchi et al., 2014). This release of

pro-inflammatory factors is a non-specific immune/inflammatory response and occurs in the first hours after the vessel occlusion.

In summary, several mechanisms contribute to neuronal damage and promote cell death during ischemia and reperfusion. They are all somehow interconnected and most of the times once they have been initiated, irreversible cascades are activated which can not be stopped and finally are harmful for the brain. This also is one reason why all of these events by principle are targets for potential therapies, however, the extremely short therapeutic window practically limits acute therapeutic options.

Beyond the acute phase after stroke onset, it has been recognized that, in addition to the reorganization of neuronal circuitries, non-neuronal cells play an important role in restoring lost neurological functions affected by ischemia. In particular microglia, astrocytes and peripheral leucocytes have an active role in the development of cell damage but also in the recovery.

2.4 Reorganization of the salvageable tissue

The brain has the capability of self-reorganization after injury. Soon after the damage mechanisms of recovery start mainly in penumbra and peri-infarct area.

One of the processes that contribute to the reconnection of the brain circuits after injury is synaptic plasticity. Plasticity is a process where there is a change in the connection between neurons, in other words, transmission of signals can be established or abolished, synaptic transmission can become stronger or weaker (Citri & Malenka, 2008). Moreover, synaptic plasticity is the molecular and cellular base of learning and memory (Ho, Lee, & Martin, 2011). During adulthood, contrary to what occurs during development, synaptic plasticity is inhibited. However, brain injury such as ischemia triggers the activation of this phenomenon and after a stroke there is an extensive remodeling in connectivity (Hermann & Chopp, 2012). Post-ischemic long term potentiation (i-LTP) is an example of synaptic plasticity that is triggered by ischemia (Di Filippo et al., 2008). It has a similar molecular basis to physiological LTP, being the NMDA receptors essential for their development and maintenance (Di Filippo et al., 2008). Importantly and opposite to detrimental activation of NMDA receptors in the early phase after stroke, NMDA receptors activation excitatory to the cell, is one the key processes for synaptic plasticity during the recovery phase.

2.5 The role of cell genesis in stroke recovery

The formation of new cells, their proliferation and migration including neurogenesis and angiogenesis have been considered as important processes to brain recovery Neuronal precursor cells possibly integrating into neuronal circuits proliferate in the subventricular zone close to the lateral ventricle and the subgranular layer of the dentate gyrus of hippocampus and may migrate to the place of injury and may differentiate (Hermann & Chopp, 2012; Ming & Song, 2011). In damaged tissue newborn neurons release factors that contribute to a recovery permissive tissue environment by the release of trophic factors, such as vascular endothelial growth factor (VEGF), an angiogenic factor. Although neurogenesis seems an important process for brain remodeling, however, the contribution to recovery is ambigious. For instance, the rate of survival of the newborn neurons is very low (Hermann & Chopp, 2012).

Angiogenesis is crucial for brain recovery once the new vessels promote an environment for the migration and differentiation of the newborn neurons, through the release of several factors, such as, stromal derived factor 1, VEGF, BDNF and metalloproteinases 2 and 9 (Hermann & Chopp, 2012). In addition, previous studies have shown that poststroke angiogenesis and formation of capillaries occur temporally inside and close to the infarct core to allow the invasion of peripheral immune cells to phagocytize cellular debris (clean-up hypothesis) (Manoonkitiwongsa, Jackson-Friedman, McMillan, Schultz, & Lyden, 2001).

Another important processes in brain recovery are oligodendrogenesis and gliosis. Newborn oligodendrocytes will be important in the formation of myelin sheaths of the sprouting axons (Zhang et al., 2013). Gliosis consists of the change in morphology and function of glial cells, mainly astrocytes in response to CNS damage (Burda and Sofroniew, 2014).

2.6 Astrogliosis after stroke

Dependent on the location of astroglial cells these cells partially proliferate and become hypertrophic, however, are bound to their astrocytic domain if the insult is not deleterious mainly in the distal peri-infarct area (Wilhelmsson et al., 2006). Close to the infarct core reactive astrocytes change their morphology to “seal” necrotic tissue in the infarct core forming the glial scar (Sofroniew, 2009). The glial scar is important to isolate the damaged region and to protect the living cells from noxious factors released from the infarct core (Anderson, Blomstrand, Blomstrand, Eriksson, & Nilsson, 2003; Sofroniew & Vinters, 2010). On

the other hand, the glial scar is also a barrier for the regeneration of neuronal circuits, being an obstacle to recovery (Anderson et al., 2003). Proteins involved in limiting neuronal sprouting and synaptic plasticity are members of the Nogo family (Schmandke, Schmandke, & Schwab, 2014; Zemmar et al., 2014).

In addition, reactive astroglia release important factors for the neuronal remodeling, such as, proteoglycans acting as guidance cues for axons and dendrites (Hermann & Chopp, 2012). All these events are potential targets for restorative therapies, which have the goal of saving and promote tissue reorganization in the peri-infarct area (Cramer, 2008). Interestingly, dependent on their activation some of the molecules and signalling cascades have a dual role after stroke. While an activation in the acute phase is detrimental and results in damage, the same molecules or activation of cascades have recovery enhancing functions. It will be crucial to understand the molecular basis and the timing of all the recovery processes in order to enhance benefical and to diminish detrimental actions.

2.7 Recovery of neuronal networks

Changes in cells and tissue happen mainly in peri-infarct area, however, adaptive changes also have been detected in remote brain regions. Brain areas homotypic to the lesioned hemisphere and remote brain regions that have been connected to the damaged area and the peri-infarct region also show adaptations (Cramer, 2008; Hermann & Chopp, 2012) to the changes caused by stroke. Due to the injury, original connections have been disrupted resulting in a lack of communications between brain areas. Changes in remote brain areas have been described after stroke and can be interpreted as potential adaptive mechanisms finally to overcome the loss of neurological functions. In general, three mechanisms have been described: first, an increase of brain activity can be observed in regions remote to the injury area which are connected and which potentially may take over lost function. Second, there is an increase of activity in the contralateral hemisphere to the lesion with the goal to reduce laterality. It is thought that possibly the hemisphere not affected by the stroke takes over lost functions of the lesion hemisphere or the lesioned hemisphere is no able to inhibit the non affected hemisphere. In any of the cases the objective is to maintain as much as normal the brain function and try to not affect the output signals. Hence, the dominance of the healthy hemisphere significantly inhibits brain activity in the lesioned hemisphere. An increased neuronal activity, however, is essential for mechanisms of neuronal plasticity in the ischemic hemisphere including increased levels of excitatory neurotransmitters such as glutamate

(Dhawan et al., 2011) or dopamine (Ruscher, Kuric, & Wieloch, 2012), decreased levels of GABA (Clarkson, Huang, MacIsaac, Mody, & Carmichael, 2010), respective receptors and increased levels of growth promoting molecules (Kuric et al., 2013). All paths cumulate in a third mechanism of reorganization of brain function in newly formed somatotopic maps (Winship & Murphy, 2008).

3. Immunology of stroke

3.1 Introduction

The immune system is a biological defense system of an organism against pathogens and allows the distinction between what is self and what is foreign. This system can be subdivided into an innate and adaptive system. Innate immunity encompasses several mechanisms, such as inflammation, activation of the complement system, the action of innate leukocytes allows immediate but non-specific host defense. On the other hand, adaptive and specific immunity exists and develops within several days or weeks upon exposure to specific pathogens (antigens). This response consists of an antigen-specific response through subpopulations of T- and B-lymphocytes. The adaptive immune system creates an “immunological” memory that enables a faster and stronger response to a subsequent attack of the same pathogen, which initiates the response (Parkin & Cohen, 2001). The immune system is an important defense against infections. This system, however, can also be activated without the exposure to pathogens and this is what takes place after stroke.

3.2 The inflammatory response after stroke

After stroke, the inflammatory response encompasses the complex crosstalk between the expression and release of pro-inflammatory molecules together with a regulated accumulation and activation of immune cells in the ischemic territory, however, the activation of inflammatory cascades and cells can also be observed in areas remote to the injury site. During the early phase (hours after stroke onset), inflammation is one of the responses of innate immune system and as mention above it is a well organized, time patterned and at first ends with cell death. Importantly, both the humoral and cellular inflammatory response always exists together and never are uncoupled.

3.3 The humoral inflammatory response

Initially, inflammation is activated by hypoxia, changes in the shear stress and production of ROS due to occlusion of a blood supplying artery. These alterations will lead to the activation of the coagulation cascade, complement system as well as platelets and endothelial cells (Iadecola & Anrather, 2011). All these events promote vessel occlusion and an augmentation of inflammation. In parallel, the translocation of P-selectin and E-selectin to the surface of the platelets and endothelial cells can be observed and further leads to the aggregation of platelet and leukocytes (Iadecola & Anrather, 2011; Wang, Tang, & Yenari, 2007). Due to oxidative stress the bioavailability of NO in endothelial cells decreases due to oxidative stress. Since NO promotes vasodilatation and inhibits platelet aggregation and leukocyte adhesion, its decrease enhances the ischemic injury (Davignon & Ganz, 2004; Kubes, Suzuki, & Granger, 1991). In addition to selectins, other adhesion molecules such as members of immunoglobulin family (ICAM-1 and 2 and VCMA-1) and integrins also allow the interaction between endothelial cells and leukocytes, enhancing leukocytes adhesion (Wang et al., 2007; Yilmaz & Granger, 2010). The permeability of the BBB is increased due to extracellularly active proteases such as metalloproteinases. The release of proteases by leukocytes and histamine, proteases and TNF alpha (TNF-α) by mast cells facilitate leukocyte invasion into the brain. At the same time, pro-inflammatory factors like the cytokines IL-1 and TNF-α but also chemokines like fractalkine are released by mast cells (MC) and resident brain cells like reactive microglia and neurons. The release of inflammatory molecules and the expression of respective receptors by immune cells but also brain resident cells give the first insights into a scenario of complex communication between neurons and other brain resident cells with immune and endothelial cells (Judy Huang, Upadhyay, & Tamargo, 2006; Siniscalchi et al., 2014; Wang et al., 2007).

3.4 The cellular inflammatory response

Minutes after the beginning of the ischemic incident, microglial cells, a source of pro-inflammatory mediators, are activated and can contribute to tissue damage. The maximum of microglia accumulation in the ischemic territory peaks at 48h to 72h after the occlusion and the levels can maintain for several weeks (Jin, Yang, & Li, 2010; Nakajima & Kohsaka, 2001). Although detrimental actions of microglia cells significantly contribute to tissue damage in the early phase after stroke onset their spatiotemporal accumulation in the ischemic territory strongly suggest their involvement in the formation of a preliminary scar to seal the ischemic infarct core.

In addition, inflammatory cell populations invade the brain parenchyma in a sequenced and time-dependent manner. The first population detectable are Mast cells which reach the brain approximately 4h after ischemia (Strbian, Kovanen, Karjalainen-Lindsberg, Tatlisumak, & Lindsberg, 2009). Upon activation, Mast cells release vasoactive substances, cytokines, anticoagulants and proteases. Moreover, these cells can phagocyte, be involved in the modulation of the adaptive immune response and in antigen presentation (Iadecola & Anrather, 2011). In addition, Mast cells modulate neutrophil accumulation after transient MCAO in mouse (Strbian et al., 2009).

Neutrophils can be detected one day after stroke and reach a peak on day 3 (Jin et al., 2010; Weston, Jones, Jarrott, & Callaway, 2007). Thereafter, the number of neutrophils are reduced in the ischemic territory, however, maintain themselves in the brain until day 7 to 15, camouflaged by reactive microglia (Weston et al., 2007). Neutrophils exacerbate the injury by releasing cytokines and chemokines, MMPs, producing additional ROS and expressing leukocyte integrins and adhesion molecules (Jin et al., 2010). According to Gelderblom et al, dendritic cells, as neutrophils, infiltrate the postischemic brain and can be detected one day after stroke. Their accumulation reaches a peak on day 3 after transient MCAO. These cells have a function in antigen processing presenting and therefore they are one of the cell types acting as an interface between innate and adaptive immunity (Iadecola & Anrather, 2011; Jin et al., 2010).

Blood-derived monocyte/macrophages infiltrate the brain 3 to 7 days after the stroke and just like microglia produce several pro-inflammatory molecules and contribute to enhance cerebral damage (Jin et al., 2010; Schilling et al., 2005). Importantly, to date and based on the expression of surface and intracellular marker proteins an exact differentiation between brain derived microglia and infiltrating macrophages is not possible.

Lymphocyte populations are the key cells of the adaptive immunity and an accumulation in the postischemic brain can be found at later time points following stroke. However, unlike other cells, the time of infiltration is not well determined. Jander et al found that infiltration of T-cells occurs on day 3, in contrast, other studies already observed T-cells as early as 24h after stroke (Jander, Kraemer, Schroeter, Witte, & Stoll, 1995; Yilmaz, Arumugam, Stokes, & Granger, 2006). The different findings can be explained by the existence of different types of T-cells invading into the postischemic brain and it also needs to be considered that T-cells can be found under physiological conditions in the brain (Jin et al., 2010).

Controversial data have been obtained concerning the effects of T-cells on brain damage, hence also here effects seem to depend on the type of lymphocyte and on the time point of accumulation in the brain.

CD4+ and CD8+ T-cells seem to contribute to the brain damage. Liesz et al have shown that the inhibition of lymphocyte adhesion to the endothelial cells prevents trafficking of these cells to the brain parenchyma leading to a reduction of lesion size and an improvement of the behavior 7 days after moderate ischemia (Liesz et al., 2011). In addition, the absence of CD4+ and CD8+ during the first 24h after stroke improves brain damage in T-cell deficient mice (Yilmaz et al., 2006). In contrast, it has been demonstrated that CD4+ TH2 cells, unlike TH1 cells, have a protective role against injury, releasing anti-inflammatory cytokines (Arumugam, Granger, & Mattson, 2005).

Regulatory T cells (Treg cells) are involved in the modulation of the immune response, by controlling the invasion of other lymphocytes and releasing anti-inflammatory cytokines (Liesz et al., 2009). In contrast, subsequent investigations could not confirm these findings showing that Treg cells did not affect lesion size and functional outcome after MCAO (Ren, Akiyoshi, Vandenbark, Hurn, & Offner, 2011). Moreover, other studies also found that Treg cells promote ischemic neurodegeneration and are already harmful 24h after stroke (Kleinschnitz et al., 2013). These contradictory results might be explained by different experimental conditions and evaluation of the function of cells at different time points. While cells have been analyzed in a more delayed phase after ischemia in the Liesz paper, Kleinschnitz et al studied them in the early phase, 24h after the stroke.

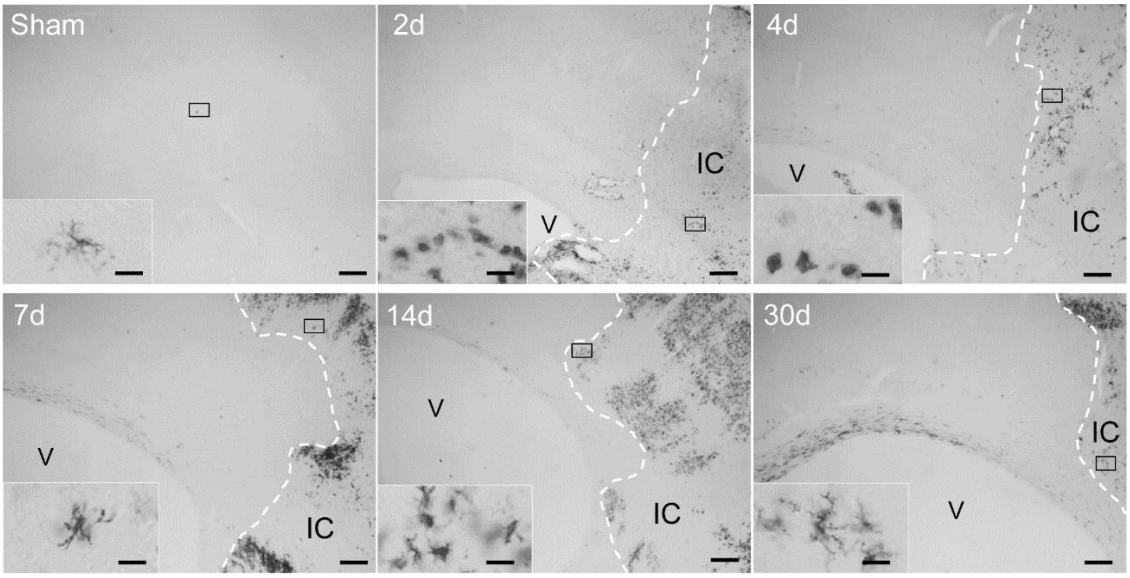
Another important type of lymphocytes are γδT cells and B cells. Both of these types are less studied among lymphocytes. γδT cells seems to enhance delayed brain damage through release of Il-17 (Shichita et al., 2009). Hence, these data have been obtained in a Il-17 deficient mice and several outcome measures such as lesion size and behavioral outcome in this study are dependent on vessel configuration which is abberant in these mice. Interestingly, it has been shown that regulatory B cells are involved in the protection of the brain against injury (Akiyoshi et al., 2011).

Despite lymphocytes improve lost neurological function and diminish ischemic brain injury after stroke, currently, it can not be clearly estimated if the adpative immune system is involved in outcome after stroke. Lymphocytes may enhance damage through an antigen-independent manner (Iadecola & Anrather, 2011; Kleinschnitz et al., 2010). In addition, Kleinschnitz et al showed that the damage caused by T-cells 24h after experimental stroke is

not dependent on antigen recognition and co-stimulatory molecules (Kleinschnitz et al., 2010). Moreover, the damage occuring 24h after stroke is not compatible with the time needed for antigen presentation and clonal expansion (Hurn et al., 2007; Kleinschnitz et al., 2010). On the other hand, some researchers argue that the adaptive immune system appears to be necessary to the enhance the damage. To scavenge detrimental actions of T-cells a recombinant T-cell receptor ligand RTL551 (an I-A(b) molecule linked to MOG-35-55 peptide) convalently linked to myelin peptides (antigen) has been tested in experimental stroke studies. Application of RTL551 reduced stroke lesions and reduced the number of different immune cell populations in the postischemic brain (Subramanian et al., 2009). The importance of adaptive immunity in ischemic injury has been demonstrated by the development of protective tolerance to CNS antigens in experimental stroke (Becker et al., 1997). Together, it can be concluded that lymphocytes act in an antigen independent manner in an early phase of the injury. In the delayed phase (weeks or months) after stroke, antigen presentation is needed to improve functional outcome after stroke (Iadecola & Anrather, 2011).

3.5 On the role of MHC processing and presentation in the postischemic brain

Activation of a specific immune response requires the recognition of specific epitopes (antigens). Antigens are processed and presented via major histocompatibility complexes (MHC I) on all nucleated cells or MHC II on antigen presenting cells such as dendritic cells, microglia/macrophages and certain populations of B-cells (Biddison & Martin, 2000).



Firure1. (see legend on next page)

(see figure on previous page)

Figure 1. Distribution of MHC II expressing cells in the ipsilateral cortex of a sham operated animal and at the indicated time points after tMCAO. The border of the infarct core is delineated by a dotted line. Abbreviations: V – lateral ventricle, IC – infarct core. Scale bar – low magnification - 200 µm, higher magnification - 20 µm.

Interestingly, an accumulation of MHC II positive cells can be observed in the ischemic hemisphere (Kuric & Ruscher, 2014) (Figure 1). Dependent on the location MHC II positive cells appear amoeboid in the infarct core during the first days after stroke onset and thereafter disappear, while stellate cells in the peri-infarct area can be observed for weeks following the injury. In addition, an accumulation of MHC II positive cells has been observed in remote white matter tracts upon chronic cerebral hypoperfusion (Wakita, Tomimoto, Akiguchi, & Kimura, 1994), in the spinal cord of stroke patients with a longer survival time (5 weeks to 4 months) (Schmitt et al., 1998) but also in white matter lesions and nonlesional white matter of ageing brains (Simpson et al., 2007). Importantly, the expression and presentation of MHC II in descending tracts of the spinal cord were not associated with a T-cell response (Schmitt et al., 2000). Starting on day 4 we found a delayed accumulation of MHC II positive cells in the corpus callosum ipsilateral but also contralateral to the lesioned hemisphere of rodents (mice and rats) subjected to experimental stroke (Figure 2).

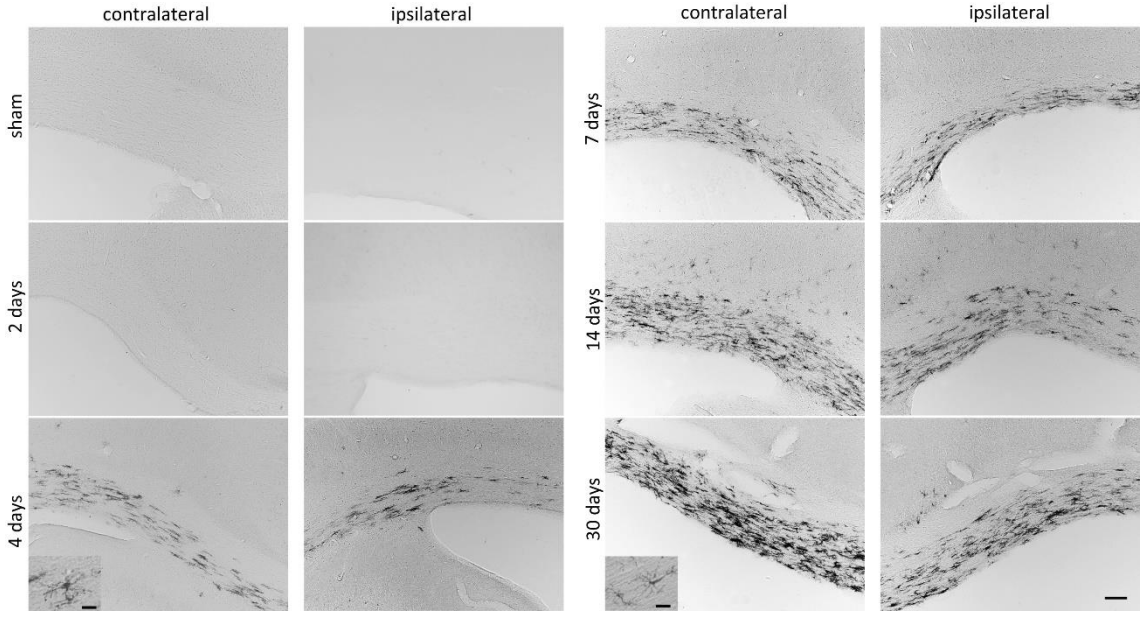


Figure 2. Temporal profile of MHC II positive cells in the corpus callosum contralateral and ipsilateral to the lesioned hemisphere at different time points after tMCAO and a sham operated animal, respectively. Scale bars: lower magnification - 100 µM, higher magnification - 20 µM.

A maximum of cells was observed on day 30 after MCAO (Kuric & Ruscher, 2014) and even 9 weeks after transient MCAO MHC II positive cells were detectable in this brain area (unpublished observation).

3.6 Chemokines

Chemokines are a subclass of cytokines, which are involved in controlling inflammation response, leukocytes recruitment to damaged tissue, angiogenesis and cellular differentiation. They are divided in subfamilies CC, CXC, XC, and CX3C, depending on the position of cysteine residues (Mirabelli-Badenier et al., 2011).

Related to stroke, the role of chemokines in the stroke pathophysiology is focus of current research. It is impossible to simply assign a positive or negative role to particular cytokines after stroke, since their role is dependent on the chemokine by itself but also on the spatiotemporal pattern after stroke (Mirabelli-Badenier et al., 2011). An example of this is the chemokine CXCL12 and its receptors CXCR4 and CXCR7. This pathway seems to be involved in the recruitment of protective endothelial cell progenitors, on the other hand, its inhibition during the first week after stroke it is beneficial, reducing detrimental inflammatory processes (Fan, Shen, Frenzel, Zhu, & Ye, 2009; Ruscher et al., 2013). This chemokine is also capable of triggering other chemokine pathways, as the chemokine CX3CL1 or fractalkine (Walter, van der Maten, Antunes, Wieloch, & Ruscher, 2015).

Importantly, the CXCR4 pathway can be antagonized pharmacologically by 1-[4-(1,4,8,11-tetrazacyclotetradec-1-ylmethyl)phenyl]methyl]-1,4,8,11-tetrazacyclo-tetradecan (AMD3100). Treatment with AMD3100 reduces expression level of proinflammatory cytokines and protects blood–brain barrier integrity and reduces infarct volumes in the acute phase after pMCAO in mouse (Huang et al., 2012). Prolonged treatment after tMCAO for 5 days significantly reduced the number of CD3/CD4 positive cells in the postischemic brain and reduced microglia activation after permanent focal ischemia induced by photothrombosis (Ruscher et al., 2013; Walter et al., 2015).

Fractalkine (FKN), also known as Neurotactin (NTN), is a chemokine with a CX3C domain. It can be found anchored to the membrane, as a transmembrane glicoprotein, or in a soluble form if proteolytically released (Bazan et al., 1997; Pan et al., 1997). It is expressed in active endotelial cells, dendritic cells and neurons (S. Jung et al., 2000) and establish high affinity interactions with the receptor CX3CR1, being its exclusive ligand (Cardona et al., 2006). CX3CR1 is a seven-transmembrane G-protein-coupled receptor expressed in monocytes, dendritic cells, sub

populations of T cells and Natural killers and in microglia cells (Cardona et al., 2006; Harrison et al., 1998; S. Jung et al., 2000). FKN in its soluble isoform, and through its receptor, acts as a chemoattractant of microglia cells (Hatori, Nagai, Heisel, Ryu, & Kim, 2002). This finding together with the fact that FKN is released by neurons establish that this pathway may be involved in the cross-talk between neurons and microglia.

4. Conclusion

Dependent on the severity and location of the insult in the brain ischemic stroke causes loss of neurological functions and represents a dramatic change in the personal life of patients. Despite early treatment options have been developed and successfully implemented in the management of stroke only a minority of stroke patients is eligible to either thrombolysis or mechanical interventions to remove the clot. This even more emphasizes the quest first for better primary prevention strategies and therapeutic options within an extended time window after stroke to enhance recovery of lost neurological functions in order to provide a more independent life for the majority stroke victims.

Preclinical research has identified that poststroke inflammation and immunodepression (a phenomenon which was not dealt with in the present overview) significantly affect the outcome after stroke. More in detail, several studies have shown that the reduction of the number of different immune cell populations accumulating in the postischemic brain significantly improves recovery after experimental stroke. Interstingly, an accumulation of MHC II positive cells in white matter tracts, namely the corpus callosum and the spinal cord, after stroke, has been observed, hence the function of these cells has not been determined.

Objectives

The objective of the present study was to investigate the role of MHC II positive cells in the corpus callosum contralateral to the lesioned hemisphere of mice subjected to permanent focal ischemia.

In order to reach this aim, heterozygous transgenic mice ($CX3CR1^{GFP/+}$) were subjected to photothrombosis, a model of permanent focal ischemia. From day 2 onwards, mice were treated with AMD3100, a specific CXCR4 antagonist, for 5 days. Neurological functions were assessed by a composite neuroscore. On day 7, animals were sacrificed and the corpus callosum and adjacent neocortical regions of the nonlesioned hemisphere were dissected. After RNA extraction, gene array analysis has been performed. Through this array, it will be possible to characterize and suggest candidate genes important for the function of MHCII positive cells upon a specific anti-inflammatory treatment in remote white matter tracts following experimental stroke.

Chapter 2 - Materials and Methods

1. Reagents

The reagents Rose Bengal, 2-methylbutane, tris base, boric acid, diethylpyrocarbonate (DEPC), ethylenediaminetetraacetic acid (EDTA), isopropanol and chloroform:isoamylalcohol (49:1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Saline solution (NaCl 0,9%) was purchased from B. Braun (Melsungen, Germany). AMD 3100 octahydrochloride (AMD) was obtained from Tocris Bioscience (Bristol, UK). The KAPA Mouse Genotyping Kit was provided by KAPA Biosystems (Wilmington, MA, USA). The primers CX3CR1_3945 [TTCACGTTCGGTCTGGTGGG], CX3CR1_39456 [GGTTCCTAGTGGAGCTAGGG] and CX3CR1_3947 [GATCACTCTCGGCATGGACG] were received from eurofins genomics (Ebersberg, Germany). The Kit RNeasy® MinElute® Cleanup was acquired from Qiagen (Hilden, Germany). Agarose was obtained from Thermo Scientific and TRIzol reagent was provided by Life Technologies, both companies part of Thermo Fisher Scientific (Waltham, MA, USA). All solutions were prepared with ultrapure water filtered by a Milli Q Millipore system.

2. Animals

The study was performed with 23 C57BL/6 male mice, carrying green fluorescent protein (GFP) under the control of the CX3CR1 promotor.(S. Jung et al., 2000). Three animals were excluded due to insufficient neurological deficits after stroke induction. One animal died and three mice were sacrificed due to infections.

Animals were obtained by own breeding. The breeding pairs (B6.129P(Cg)-Ptprca Cx3cr1tm1Litt/LittJ) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) . We used mice heterozygous (hz) for CX3CR1 ($CX3CR1^{GFP/+}$) allowing the visualization of microglia through imaging techniques and keeping the expression of a functional CX3CR1 allele.

3. Genotyping

As result from the breeding three different genotypes are expected, wild-type (wt), heterozygous (hz) and knockout (ko) mice. Mice were genotyped through PCR using the primers CX3CR1_3945 [TTCACGTTCGGTCTGGTGGG], CX3CR1_3946 [GGTTCCTAGTGGAGCTAGGG] and CX3CR1_3947 [GATCACTCTCGGCATGGACG].

Genomic DNA was extracted from ear punches. Extraction and PCR were performed using the KAPA Mouse Genotyping Kit with some alterations concerning the PCR machine program. For

DNA extraction tissue was incubated in extraction buffer at 25°C for 15 minutes. Thereafter, tissues were lysed at 75°C for 11 minutes and heat inactivated (95°C) for 6 minutes. Template DNA was mixed with 2x KAPA2G Fast Genotyping mix and the primers CX3CR1_3945 (0.5 µM), CX3CR1_3946 (0.5 µM) and CX3CR1_3947 (0.5 µM). Amplification was carried out in a MJ Mini Personal thermal cycler (BioRad, Hercules, USA). The PCR program is shown in Table 1.

PCR – DNA amplification	
Initial denaturation	
94 °C	3 min
Number of cycles – 1	
Denaturation	
94 °C	30 s
Annealing	
60 °C	30 s
Extension	
72 °C	2 min
Number of cycles – 35	
Final extension	
72 °C	10 min
Number of cycles – 1	
5 ℃	8

Table 1. PCR program for genotyping.

4. Experimental design

The animals were divided into 4 groups: sham/vh (n = 3), sham/AMD (n = 4), PT/vh (n = 5) and PT/AMD (n = 6). The mice from the sham/vh group were subjected to sham surgery and treated with saline solution during the experiment. Sham/AMD animals were subjected to sham surgery but instead of saline were injected with AMD. The groups PT/vh and PT/AMD were subjected to photothrombosis (PT), the first group received saline injections and the later an AMD injection.

For the RNA extraction and purification and for the gene array we used 3 animals Sham/vh, 3 animals Sham/AMD, 5 animals PT/vh and 5 animals PT/AMD.

Before PT (day -1), neurological function was tested in all the animals by a composite neuroscore. Two days after surgery, all mice were tested by the composite neuroscore and only animals subjected to PT with a significant neurological deficit (neuroscore lower than 11) were included in the injection study. Conversely, only sham operated mice without neurological deficit were included in the study (neuroscore equal to or higher than 16).

On day 2, drug or vehicle treatment was started and continued for in total 6 days (Figure 3). On day 7, animals were sacrificed and the brains were frozen and stored at -80°C.

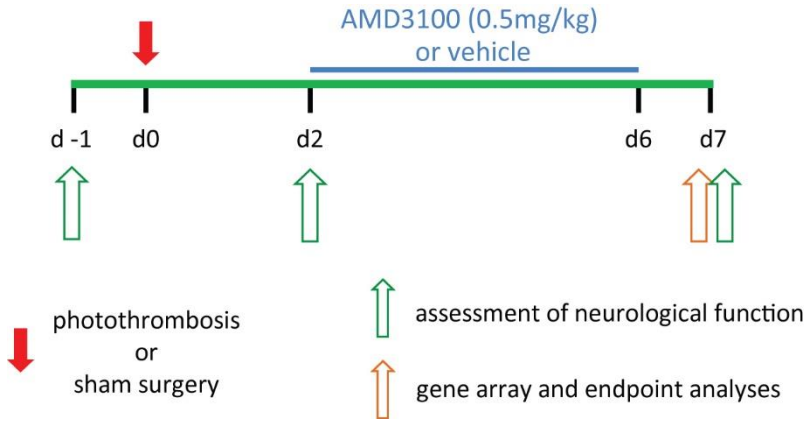


Figure 3. Experimental design.

5. Behavioral test analysis

To test neurological deficits we performed a composite neuroscore including 4 behavioural tests: paw-placement test, whisker-reflex, tail suspension test and a modified foot-fault test (L. Zhang et al., 2002) (Figure 4). The neuroscore was evaluated as described previously (Walter et al., 2015) (Table 11, Annex I). The maximum score an animal could obtain was 17 points. Animals, which underwent PT, were excluded from the study if they did not show sufficient neurological deficits (neuroscore > 11) on day 2 after surgery.

The paw placement test gives information about the tactile/proprioceptive response to limb stimulation (Madinier, Quattromani, Sjölund, Ruscher, & Wieloch, 2014). For the paw placement test the animals were placed on the edge of a table with the left side more closely to the table. Then the animals were moved along the edge so that the limbs loose contact with the table. The ability of the animal to move the limb back in contact with the table was scored according to the table 11 in Annex I.

The whisker-reflex test evaluates the mouse sensibility to stimulation of the whiskers. The animal left whiskers were stimulated with a stick. This stimulation was done from the back of the animal, preventing the animal to see the stick approaching. The response was analysed and scored (Table 11, Annex I).

The tail suspension test assesses the symmetry and laterality of the mouse. The mice were suspended upside down and the left forelimb flexion was analysed and scored (Table 11, Annex I).

The foot-fault test allows the detection of deficits of sensorimotor functions. The animals were placed on a grid with the following characteristics: length 46.5 cm, diameter of bars 2 mm, interspace 1.5 cm. Mice were allowed to walk on the grid 27 steps. If the paw felt or slipped in

between the bars, it has been counted as fault. Based on the number of faults mice obtained a test score between 0 and 6, as indicated in table 11, Annex I. Before surgery, the animals were trained 2 to 3 times until they crossed the grid without faults.

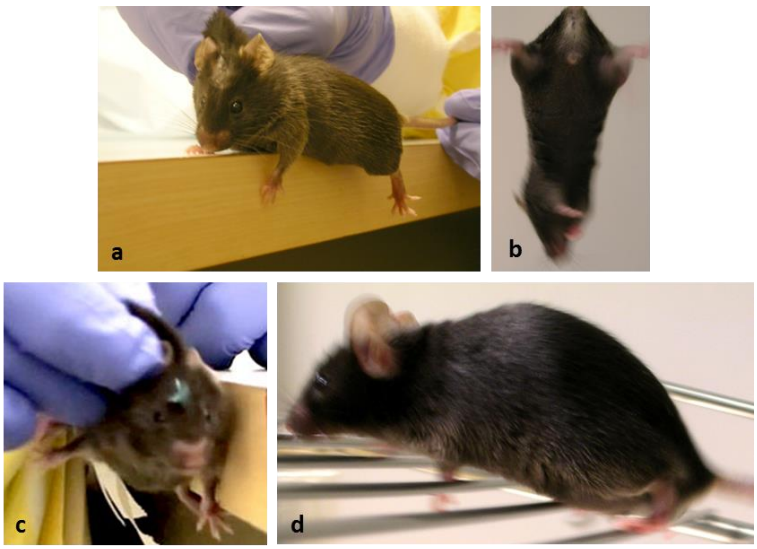


Figure 4. Composite neuroscore: a - paw placement, b - tail suspension test, c - whisker-reflex, d - foot-fault test.

6. Photothrombosis

Photothrombosis was performed as described previously (Walter et al., 2015). In brief, animals were initially anesthetized with isoflurane (4 l/min) and placed in a stereotactic frame. During surgery, the animals were spontaneously breathing through a face mask delivering 1.7 L/min of isoflurane in a gas mixture of 30% O_2 and 70% N_2O. The surgery started with a sagittal skin incision made on the scalp to expose the skull. Subcutaneous connective tissue was removed and the skullbone was dried. Thereafter, 0.1 ml of the photosensitizer dye Rose Bengal (concentration 10mg/ml) was injected intraperitoneally. Five minutes after injection, cold light was applied through a round aperture with a diameter of 2.5 mm through the intact skull at the following coordinates (related to bregma): anterior-posterior 1.5 mm and lateral 1 mm. Illumination was carried out for 20 minutes at an intensity of 3050 K/4D using a Schott cold light source (Schott KL 1500lcd, Mainz, Germany) (Figure 5). Following illumination, the scalp incision was sutured and the mice were transferred to their home cages. Animals subjected to sham surgery were injected intraperitoneally with saline solution. During surgery, the body temperature was monitored through a rectal thermistor probe connected to a heating pad

maintaining body temperature between 36.3 to 37.2°C. The breathing of the animal was also monitored to adjust anesthesia.

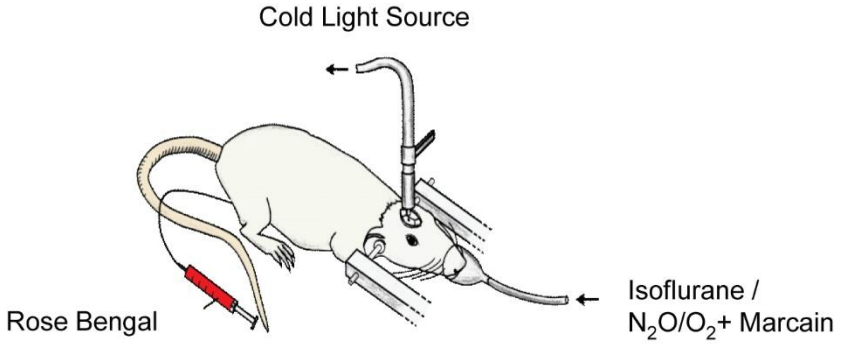


Figure 5. Representative picture of a mouse in the stereotactic frame, while being subjected to PT.

7. Treatment with AMD3100

Animals included in the drug treatment study have been randomized into treatment groups; every other animal received the same treatment. Administration of AMD 3100 or vehicle (vh) started 2 days after performing the surgery and was finished on day 6 poststroke, the day before the animals were sacrificed (day 6). AMD 3100 was administered at a dosage of 0,5mg/ml intraperitoneally (ip); saline solution was used as vehicle treatment. The volume of the drug or the saline solution administrated to the animals was adjusted to their weights: up to 20 g of BW 0.1 ml were injected, between 20 and 30 g BW 0.15 ml were injected and between 30 and 40 g BW 0.2 ml were injected.

8. Tissue dissection for gene array

Frozen brains stored at –80^{o}C were warmed up to a temperature of –20^{o}C in a Glove Box. Thereafter, brains were placed in a brain matrix and a 4 mm coronal section was cut including the infarct core in the ipsilateral hemisphere and corresponding contralateral hemisphere, respectively (Figure 6). After separation of the ipsilateral hemisphere, the corpus callosum and adjacent cortex of contralateral hemisphere was dissected and transferred into an Eppendorf tube. One milliliter of TRIzol was added and tissues were stored at -80^{o}C until further processing.

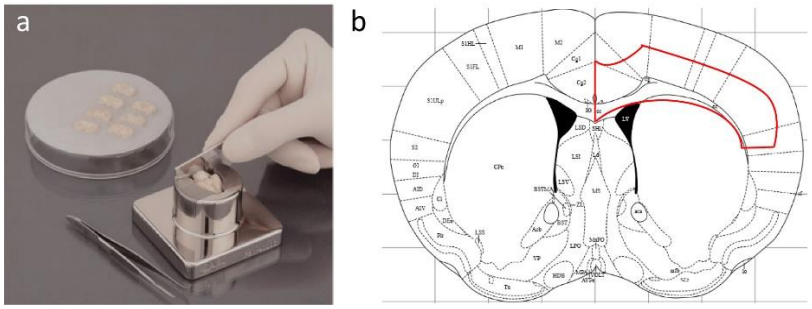


Figure 6. Corpus callosum and cortex dissection: a – brain matrix to cut 4 mm thick coronal sections of the brain (adapted from https://asi-instruments.com/rodent-brain-matrices/). b – representation of the area of dissection (adapted from (Paxinos & Franklin, 2004)).

9. RNA extraction and purification

All tissue samples were extracted at the same time to avoid systematic errors due to the extraction procedure. Brain tissue samples were homogenized with a Dounce homogenizer in TRIzol reagent and incubated for 10 min at room temperature (RT). Following, 250 µl of Chloroform:Isoamylalcohol (49:1) were added to the samples, then they were vortexed and incubated for 10 min at RT. Thereafter, it was possible to see two phases. The samples were centrifuged for 30 min, at 14000 rpm and 4^{o}C. After centrifugation, 3 phases were observed: aqueous (RNA), interphase (DNA) and organic (proteins). The aqueous phase was transferred to a new Eppendorf tube and placed on ice. The same volume of isopropanol was added and the sample was vortexed for 30 s. The mix was incubated on ice for 10 min and thereafter, for 1 hr at -80°C. Subsequently, the mix was centrifuged and a pellet was obtained containing RNA. The supernatant was discarded. The RNA was cleaned with 1 ml ethanol (70%). Thereafter, the sample was centrifuged for 60 min, at 14000 rpm, 4^{o}C. The supernatant was discarded and the pellet was air dried. Finally, the pellet was resuspended in DEPC-water (0.1% DEPC).

Following extraction, the RNA was purified using the RNeasy® MinElute® Cleanup kit from Qiagen. The protocol was done according to the manufacturer's instructions. In brief, the RNA was run through a column with RTL solution and absolute ethanol resulting in binding of the RNA to the column. Subsequently, RPE buffer and 80% ethanol were added to the column to eliminate all the contaminants. Finally, RNA was eluted with RNase-free water.

10. RNA quality analysis

RNA samples were further analyzed for their purity and integrity. These parameters are evaluated through the NanoDrop (ThermoScientific, Wilmington, USA) and the Agilent 2100

Bioanalyser (Agilent Technologies, Santa Clara, USA). These procedures allow to determine if the extraction and purification of the RNA was successful.

The NanoDrop measures absorbances, which enables to acquire the RNA concentrations and the wavelength ratios 260 nm/280 nm and 260 nm/230 nm. These ratios show how pure the RNA is since nucleotides absorb at 260 nm and contaminants, such as, proteins, phenols and chaotropic salts, absorb at 280 or 230. A pure RNA is considered at a 260 nm/280 nm ratio of around 2.0, the ratio 260 nm/230 nm should range between 2.0 to 2.2.

The results given by the NanoDrop are a good indication of the purity of the RNA but they need to be complemented with the analyses by the Agilent 2100 Bioanalyser. The bioanalyzer uses a lab on a chip approach to perform capillary electrophoresis and uses a fluorescent dye that binds to the RNA to determine RNA integrity and concentration.

The type of RNA analysed is the ribosomal RNA (rRNA) once it constitutes 80% of the total RNA, as opposite, to the messenger RNA (mRNA) which comprises only 1-3%, making it difficult to detect. The rRNA is mainly divided into two types, the 28S and 18S, and they exist in the proportion of 2:1, respectively. In case this proportion does not occur it indicates that the total RNA might be degraded or contaminated.

Another useful information given by the bioanalyzer technique is the RNA Integrity Number (RIN). The RIN is given by a software algorithm and constitutes a number from 1 to 10, where 1 indicates very degraded RNA and 10 highly intact RNA. In this project the NanoDrop was used between the RNA extraction and purification, after purification and after dilution before the samples have been forwarded to the next step, the microarrays. The bioanalyzer was performed after RNA purification in collaboration with Swegene Center for Integrative Biology at Lund University (SCIBLU).

11. Genetic Array

The geneChip® mouse gene 2.0 ST array (Affymetrix Inc., High Wycombe, UK) has been performed by SCIBLU (Lund University). Analysis of data included quality assurance of the experimental work, data filtration and normalization as well as data clustering and differentially expressed genes analysis.

12. Western Blot

12.1 Preparation of gels

Gels were prepared with a 5% polyacrylamide stacking gel (PAGE, 330 µL acrylamide 30% (bis), 400 µL 0.625 M Tris pH 6.8, 1.25 mL H2O, 20 µL SDS 10%, 10 µL of a 10% ammoniumpersulfate (APS) solution and 2 µL TEMED per gel) overlaid on a 10% PAGE separation gel containing 2 mL acrylamide 30% (bis), 1.2 mL 1.88 M Tris pH 8.8, 2.75 mL H_2O, 60 µL SDS 10%, 30 µL APS 10% and 5 µL TEMED per gel).

12.2 Sample preparation

Samples were prepared, having a final volume of either 10 or 20 µL, containing 10 or 20 µg of protein. Sample buffer (125 mM Tris-HCl pH6.8, 4% SDS, 20% glycerol, 100 mM DTT, 0.2% bromphenol blue) and lysis buffer were added to the samples according to calculations. The samples were mixed, spun down, denatured by boiling at 94˚C for 5 minutes and condensed liquids were collected by centrifugation.

12.3 Separation of proteins

Polymerized gels were placed in a tank filled with running buffer (Tris base, glycine, 1% SDS, pH 8.3). Samples were loaded in the slots, 6 µL biotinylated marker was added to the first lane. The amount of sample was either 9.5 µL. Stacking of proteins was achieved under low running conditions (12.5 mA/gel) until the gel front had reached the border of the separation gel. Subsequent separation was carried out at 50 mA/2 gels until the bromphenol front reached the bottom of the gel.

12.4 Transfer, blocking and incubation with primary antibody

For transfer membranes were cut, wetted in methanol and incubated in transfer buffer (25 mM Tris base, 0.2 M glycine, 20% methanol pH 8.5) for 20 minutes. Gels were incubated in transfer buffer for 5 minutes. Thereafter, a sandwich was prepared made of 2 pieces of Whatman paper, the membrane, the gel and 2 pieces of Whatman paper (direction: anode (-) to cathode (+)). Transfer was performed at 15V for 1 hour.

After transfer, membranes were blocked in 5% non-fat dry milk in TBS-T (blocking solution; 100 mL tris buffered saline 10fold buffer, 900 mL H_2O, 1 ml Tween 20) for one hour at RT. After, membranes were then rinsed in TBST for 3 times 5 minutes. Subsequently, membranes were incubated in a primary antibody solution (5% BSA at 4˚C over night). The primary antibody

used was a mouse monoclonal anti glutamic acid decarboxylase 67 (GAD67) diluted at 1:5000 (Merck Millipore, Temecula, USA).

12.5 Incubation with secondary antibody and exposure of membranes

The next day, membranes were rinsed in TBST for 3 x 1, 1 x 15 and 3 x 5 minutes. After membranes were incubated in secondary antibody solution containing an anti-mouse HRP (horse radish peroxidase) antibody (diluted at 1:10.000; Sigma-Aldrich, Deisenhofen, Germany) in blocking solution. After subsequent rinsing in TBST for 3 x 1, 1 x 15 and 3 x 5 minutes, membranes were exposed using a CCD camera system (LAS-1000, Fujifilm, Tokyo, Japan). Membranes were put in a plastic sheet, covered in detection solution. Using Image reader LAS1000 pro exposure was carried out for 30 seconds and 2 minutes. Pictures were exported as 16-bit grey Tagged Image File Format (TIFF) files.

12.6 Reprobing of membranes for β-actin

Membranes were rinsed for 1 minute in H_2O and stripped in respective buffer (SDS 10%, Tris-HCl pH 6.8) at 70°C for 30 minutes in order to remove antibodies. After blocking for one hour membranes were then rinsed in TBST for 3 times 5 minutes and incubated with a HRP conjugated anti β-actin (diluted at 1:75.000, Sigma-Aldrich) for 45 minutes. After rinsing 3 times for 5 minutes, the membranes were exposed as described before. Expression of fractalkine was calculated using β-actin as a loading control.

13. Immunofluorescence and confocal microscopy

Brain sections (thickness 30 µm) from 4% paraformaldehyde-perfused animals were washed in PBS (3x10min), blocking was achieved by 5% normal donkey serum in PBS supplemented with 0.25% Triton X-100 for 60 minutes. The sections were then incubated with an anti Iba1 (diluted at 1:1000; Wako Chemicals, Neuss, Germany) or anti MHC II (diluted at 1:100; AbD Serotec Duesseldorf, Germany) in Tx/PBS in 5% NDS at 4°C overnight. The next day, the sections were rinsed with 1% NDS in Tx/PBS 3 times 10 minutes. All subsequent steps have been performed in darkness in order to preserve the fluorophores conjugated to the secondary antibodies. Sections were incubated with a Cy3-conjugated secondary antibody in 2% NDS in Tx/PBS for 90 minutes at room temperature and rinsed in PBS 3 times for 10 minutes each. Finally, the sections were rinsed in PBS 3 times 10 minutes and mounted on supercharged glass slides, allowed to dry, cover-slipped using PVA-DABCO and subsequently analysed using a LSM 510

confocal microscopy. Unstained coronal sections from CX3CR1$^{GFP/+}$ were washed in PBS and mounted directly on supercharged glass slides, coverslipped and analysed by microscopy.

14. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6. Statistical analysis of body weight was performed by one-way ANOVA. Results from behavioral testings were evaluated by the Mann-Whitney-U-Test, (significance level 0.05, confidence interval 0.95). Expression levels of genes and Western blot results were evaluated by Students t-test (significance level 0.05, confidence interval 0.95).

Chapter 3 - Results

1. Accumulation of GFP cells in the corpus callosum

Previous investigations have shown an accumulation of MHC II positive cells in the corpus callosum contralateral to the lesioned hemisphere in rats after transient occlusion of the middle cerebral artery (Kuric & Ruscher, 2014). One week after PT, we studied GFP positive cells in the postischemic brain of CX3CR1$^{+/GFP}$ mice. An accumulation of cells was found in the ischemic infarct core and adjacient peri-infarct area (Figure 7a). In addition, we observed an accumulation of cells in the corpus callosum in both hemispheres. These cells appeared with a stellate morphology corroborating an accumulation of microglia cells. To further investigate the significance of these cells the corpus callosum and adjacent neocortex of the contralateral hemisphere were dissected for gene array analysis (Figure 7b).

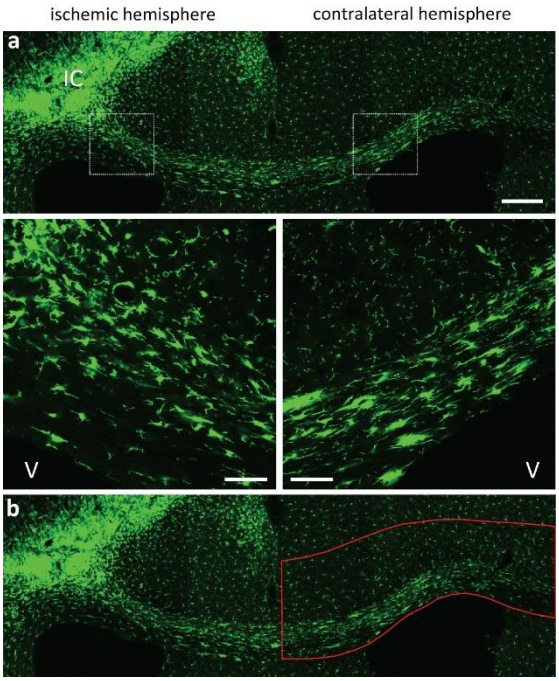


Figure 7. Presence GFP positive cells (green) in a brain section of a CX3CR1$^{GFP/+}$ animal. (a) Top: upper area of the brain section showing the infarct core (IC), the corpus callosum along the section and part of the cortex. Bottom: Higher magnification of the dotted squares in top picture to show the accumulation of the CX_3CR1 cells in the corpus callosum. V – lateral ventricle. (b) red line highlighting the area to be dissected for microarrays technique. Scale bars: higher magnification – 50 µM; lower magnification – 200 µM.

Further phenotyping of GFP positive cells in the contralateral corpus callosum by immunofluorescence analysis revealed that the majority of cells expressed Ionized Calcium-Binding Adapter Molecule 1 (Iba1) (Ito, Tanaka, Suzuki, Dembo, & Fukuuchi, 2001) (Figure 8). This protein is considered as a marker for activated brain resident microglial cells in

experimental stroke and further corroborates that activated microglia accumulate in the corpus callosum seven days after PT.

Moreover and as shown by Kuric and colleagues (Kuric & Ruscher, 2014) in transient rat middle cerebral artery occlusion (tMCAO) model we show an accumulation of MHCII positive cells in the corpus callosum, seven days after stroke. MHCII positive cells co stain with GFP demonstrating microglial cells express the MHC II antigen (Figure 9).

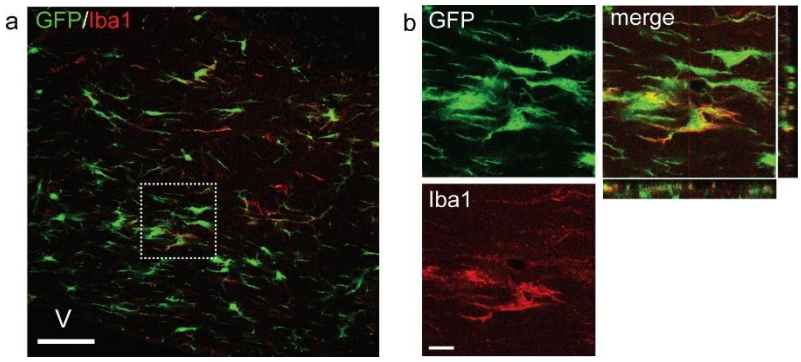


Figure 8. Expression of Ionized Calcium-Binding Adapter Molecule 1 (Iba1) (Cy3, red) in the corpus callosum contralateral to the lesioned hemisphere in a $CX_3CR1^{+/GFP}$ mouse. (a) Merged overview on the corpus callosum, abbreviation: V – lateral ventricle; scale bar – 200 µm. (b) Higher magnification of the area (white square) shown in (a). Scale bar – 50 µm.

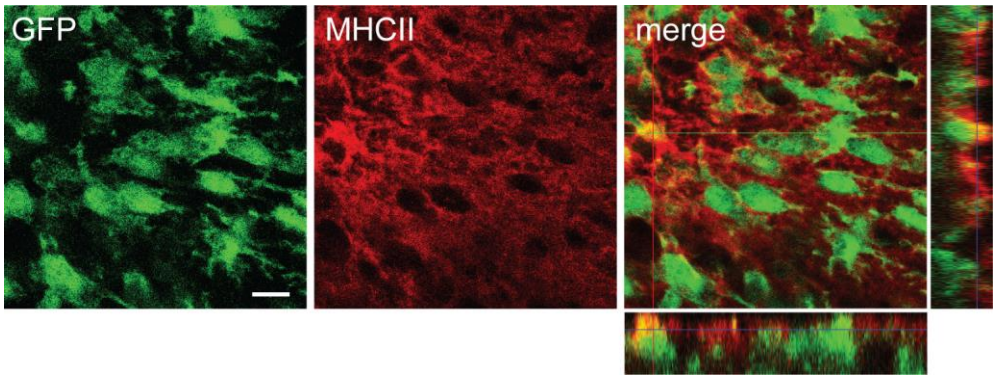


Figure 9. Expression of MHC class II (MHCII) (Cy3, red) in the corpus callosum contralateral to the lesioned hemisphere in a $CX_3CR1^{+/GFP}$ mouse. Scale bar - 10 µm

2. Neurological function after photothrombosis

We, previously, have shown that treatment with AMD3100 improves recovery of neurological deficits following PT (Walter et al., 2015). The present study was carried out according the previous study design using the same dosage of AMD3100 (0.5 mg/kg body weight) and a study endpoint showing the accumulation of microglia in the corpus callosum allowing

genomic profiling. To correlate the expression of genes to neurological function, a composite neuroscore has been performed at before PT (pre) and 1, 2 and 7 days after PT. As shown in Figure 10, it is possible to observe that all the animals subjected to PT suffer on a significant neurological deficit shown by a statistically significant decrease in the neuroscore. Animals subjected to sham surgery did not show any deficits after the intervention. As observed in previous studies (personal communication Helene L Walter, data not shown) treatment with AMD3100 did not improve the neuroscore compared to mice treated with saline for the same interval after PT. Of note is that some of the animals in the AMD3100 treatment group showed a certain degree of functional recovery 7 days after PT (Figure 10).

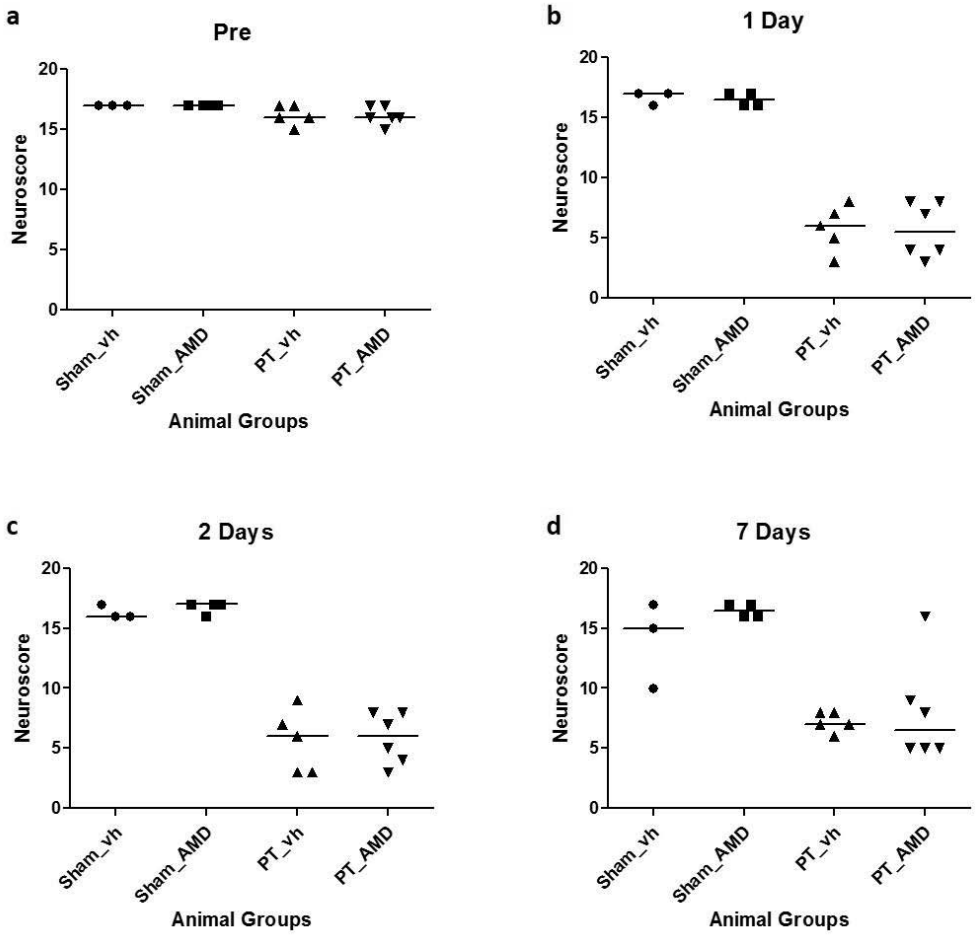


Figure 10. Impact of PT and administration of AMD3100 on functional recovery. Neurological function was analysed by a composite neuroscore before (pre) PT or sham surgery and at 1, 2 and 7 days after PT or sham surgery, respectively. The following numbers of animals were included: Sham/vehicle n = 3; Sham/AMD n = 4; PT/AMD n = 5; PT/vehicle n = 6. (a) Neurological outcome assessed pre surgery. (b) Neurological outcome assessed 1 day after surgery. (c) Neurological outcome assessed 2 day after surgery. (d) Neurological outcome assessed 7 day after surgery. Statistical analysis was performed by Mann-Whitney test.

3. Physiological data

During the surgery and treatment study physiological data of the animals were monitored. The temperature was acquired at different time points before and after the surgery, 1 h after and 1 and 2 days after and then organized by mean per experimental group, as shown in table 2. The mean of the temperatures, at the different time points, show that the animals kept their temperature between 35.5 and 39. Increased temperatures are an indication for stress and possible infections.

	Pre surgery	Post surgery	1h	Day 1	Day 2
Sham_vh	36.5 ± 0.40	36.2 ± 0.90	37.7 ± 1.07	37.7 ± 0.76	37.5 ± 0.96
Sham_AMD	37.5 ± 0.92	35.5 ± 0.84	35.7 ± 1.03	36.0 ± 1.49	39.0 ± 0.17
PT_vh	36.8 ± 0.62	36.5 ± 0.57	36.3 ± 0.48	37.3 ± 1.01	37.9 ± 0.31
PT_AMD	36,8 ± 0.60	37.1 ± 0.88	36.5 ± 0.70	37.1 ± 0.76	37.9 ± 0.46

Table 2. Animal temperatures. Temperatures are presented by experimental groups as mean± SD at the different time points: pre surgery, post surgery, 1 hr after the surgery and 1 and 2 days after the surgery. The following numbers of animals were included: Sham/vehicle n = 3; Sham/AMD n = 4; PT/AMD n = 5; PT/vehicle n = 6.

The body weight was also monitored starting before the surgery (Pre) until the day the animals were sacrificed (day 7). There are no significant changes comparing the different time points within each experimental group (Figure 11).

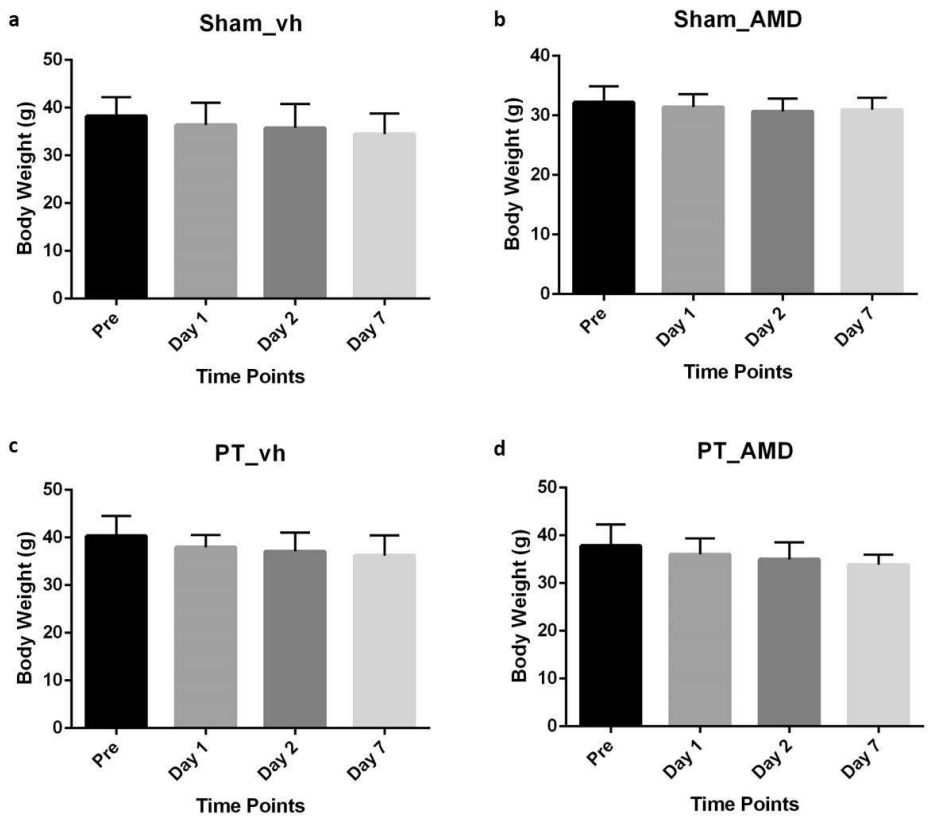


Figure 11. Body weight during the animal experiment. Body weight was measured pre PT or sham surgery and 1, 2 and 7 days after PT or sham surgery. The following numbers of animals were included: Sham/vehicle n = 3; Sham/AMD n = 4; PT/AMD n = 5; PT/vehicle n = 6. (a) Sham animals treated with saline. (b) Sham animals treated with AMD. (c) PT animals treated with saline. (d) PT animals treated with AMD. Statistical analysis was performed by One-way ANOVA.

4. RNA quality analysis

To assess the RNA quality the NanoDrop and the Agilent 2100 Bioanalyser were used. The NanoDrop analysis was done at three different time points of the experiment: after the extraction with TRIzol, after purification with the Kit RNeasy® MinElute® Cleanup and after dilution before the samples were sent to SCIBLU to perform the microarrays (Table 3, 4, 5). As mention in the Materials and Methods section a RNA sample is considered highly pure when the ratio 260/280 is approximately equal to 2 and the ratio 260/230 between 2 to 2.2. After extraction with TRIzol the samples showed a 260/280 ratio of approximately 2. Concerning the ratio 260/230 the majority of the samples had values within the acceptable range or very close to it, only samples 723 and 747 had a ratio too low. After purification, the 260/280 ratio remained at approximately 2 and the 260/230 ratio was within the range (2 to 2.2) for all the samples. Diluted samples showed a decrease in the 260/280 ratio to approximately 1.8 and an

increase in the ratio 260/230 to approximately 2.4. Together, these analyses as first step of quality control showed that the RNA in all samples had an acceptable quality for microarray analysis.

Samples	[RNA] (ng/µl)	A260	A280	260/280	260/230
644	1465.2	36.6	18.4	1.99	2.19
749	1275.7	31.9	16.0	1.99	2.10
723	945.7	23.6	11.9	1.98	1.53
747	434.6	10.8	5.6	1.93	1.02
642	1251.9	31.3	15.8	1.98	2.17
636	1148.3	28.7	14.7	1.95	2.18
676	1160.8	29.0	14.7	1.97	2.25
665	1111.5	27.8	14.1	1.97	2.21
632	1051.5	26.3	13.5	1.95	2.26
708	1431.0	35.8	17.8	2.00	2.18
727	662.4	16.6	8.5	1.96	2.20
706	688.4	17.2	8.8	1.97	2.26
807	598.0	15.0	7.6	1.96	2.04
637	681.5	17.0	8.7	1.96	2.18
755	764.3	19.1	9.6	1.99	2.25
633	1257.0	31.4	15.8	1.98	2.20

Table 3. Nanodrop measurements after RNA extraction with TRIzol but before purification with the Kit RNeasy® MinElute® Cleanup. The following numbers of animals were included: Sham/vehicle, n=3; Sham/AMD, n=3; PT/AMD, n=5; PT/vehicle, n=5.

Samples	[RNA] (ng/µl)	A260	A280	260/280	260/230
644	1934.8	55.7	26.8	2.08	2.21
749	3675.8	48.3	23.0	2.10	2.25
723	2227.8	55.7	26.8	2.08	2.21
747	1026.7	25.7	12.3	2.08	2.20
642	3154.4	78.9	37.6	2.10	2.26
636	3017.1	75.4	35.7	2.11	2.26
676	3589.7	89.7	42.7	2.10	2.26
665	2782.9	69.6	33.1	2.10	2.26
632	3629.8	90.7	43.3	2.09	2.26
708	3917.1	97.9	46.8	2.09	2.23
727	1950.8	48.8	23.0	2.12	2.19
706	3214.6	80.4	38.2	2.11	2.23
807	2927.6	73.2	34.9	2.10	2.26
637	4118.7	103.0	48.8	2.11	2.01
755	4455.8	111.4	53.8	2.07	2.19
633	3150.0	78.8	37.5	2.10	2.26

Table 4. Nanodrop measurements after purification with the Kit RNeasy® MinElute® Cleanup. The following numbers of animals were included: Sham/vehicle n = 3; Sham/AMD n = 3; PT/AMD n = 5; PT/vehicle n = 5.

Samples	[RNA] (ng/µl)	A260	A280	260/280	260/230
644	79.4	1.98	1.07	1.86	2.40
749	92.0	2.30	1.27	1.81	2.42
723	89.6	2.24	1.20	1.86	2.44
747	77.5	1.94	1.02	1.90	2.32
642	81.0	2.03	1.21	1.81	2.45
636	80.4	2.01	1.12	1.80	2.45
676	80.4	2.01	1.09	1.84	2.43
665	85.5	2.14	1.16	1.84	2.43
632	74.8	1.87	1.02	1.83	2.43
708	63.9	1.60	0.90	1.78	2.49
727	81.1	2.03	1.07	1.89	2.37
706	71.2	1.78	0.97	1.83	2.41
807	76.1	1.90	1.04	1.83	2.43
637	59.2	1.48	0.80	1.85	2.00
755	98.5	2.46	1.35	1.83	2.43
633	124.6	3.12	1.65	1.89	2.39

Table 5. Nanodrop measurements after dilution to be delivered to SCIBLU. The following numbers of animals were included: Sham/vehicle n = 3; Sham/AMD n = 3; PT/AMD n = 5; PT/vehicle n = 5.

The results of the bioanalyser were provided by the Swegene Center for Integrative Biology at Lund University (SCIBLU).

Through the blot view (Figure 12) two bands were observed, the top one being the 28S and the bottom one the 18S. The 28S band, as expected, show a higher intensity comparing to the 18S. Bands were quantified and the RNA integrity number (RIN) has been calculated. Concerning the RIN, the samples show a value within the range from 8.3 to 9.4 (corresponding 10 to highly intact RNA).

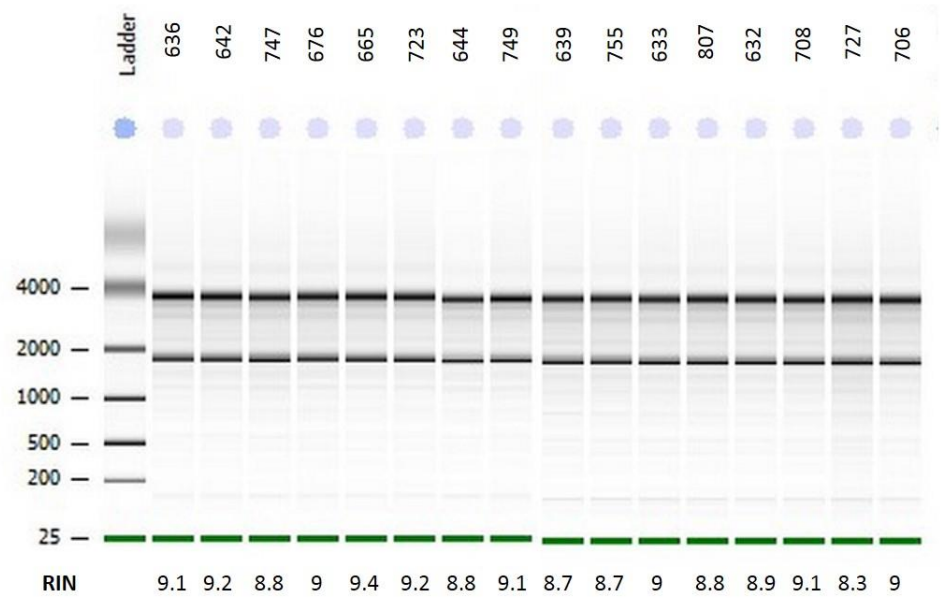


Figure 12. Quality control of RNA using the Agilent 2100 Bioanalyser. The following numbers of animals were included: Sham/vehicle n = 3; Sham/AMD n = 3; PT/AMD n = 5; PT/vehicle n = 5. Top: blot showing the bands 28S and 18S. Bottom: RNA Integrity Number (RIN) of each sample.

5. Genetic array analysis

The results of the microarrays, provided by SCIBLU, presented the genes up or downregulated within 3 comparison groups: stroke animals treated with saline comparing with stroke animals treated with AMD (Stroke_vh vs Stroke_AMD), Sham animals treated with saline comparing with stroke animals treated with saline (Sham_vh vs Stroke_vh) and Sham animals treated with saline comparing with sham animals treated with AMD (Sham_vh vs Sham_AMD). In total, 34390 genes/transcripts have been identified and dependent on comparisons between groups genes have been regulated. Regarding the analysis of each condition comparisons, we selected only genes significantly regulated with a p-value below to 0.05 (Table 6). Within these groups we picked out the genes which show an up or downregulation above to 1.45 fold changes (Table 7). From this last group of genes we sorted out candidate genes and categorized them

into three different groups of interest: immune response, inflammation and neuronal plasticity.

comparison	regulated genes	upregulated genes	downregulated genes
sham_vh versus sham_AMD	1715	546	1169
sham_vh versus PT_vh	2067	809	1258
PT_vh versus PT_AMD	1134	658	476

Table 6. Regulated genes on the level of p < 0.05 after treatment with vehicle or AMD3100 (0.5 mg/kg), respectively.

comparison	regulated genes	upregulated genes	downregulated genes
sham_vh versus sham_AMD	158	62	96
sham_vh versus PT_vh	94	59	35
PT_vh versus PT_AMD	45	35	10

Table 7. Regulated genes higher than 1.45fold (on the log2 level) after treatment with vehicle or AMD3100 (0.5 mg/kg), respectively.

5.1 Genes related to the poststroke immune response

Base on the criteria described above we found two genes regulated between the experimental conditions, T-cell receptor alpha joining 7 (TRAJ7) and T-cell receptor alpha joining 6 (TRAJ6) (Figure 13). TRAJ7 shows an upregulation in all comparisions, however, a significant upregulation was only observed comparing Stroke_vh with Stroke_AMD. In contrast, TRAJ6 showed a downregulation in all comparisons with statistical significance in two comparisons, Sham_vh vs Stroke_AMD and Sham_vh vs Sham_AMD.

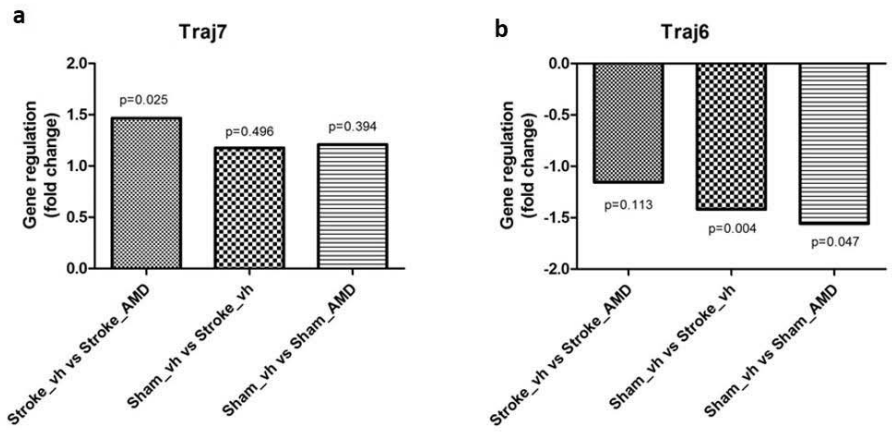
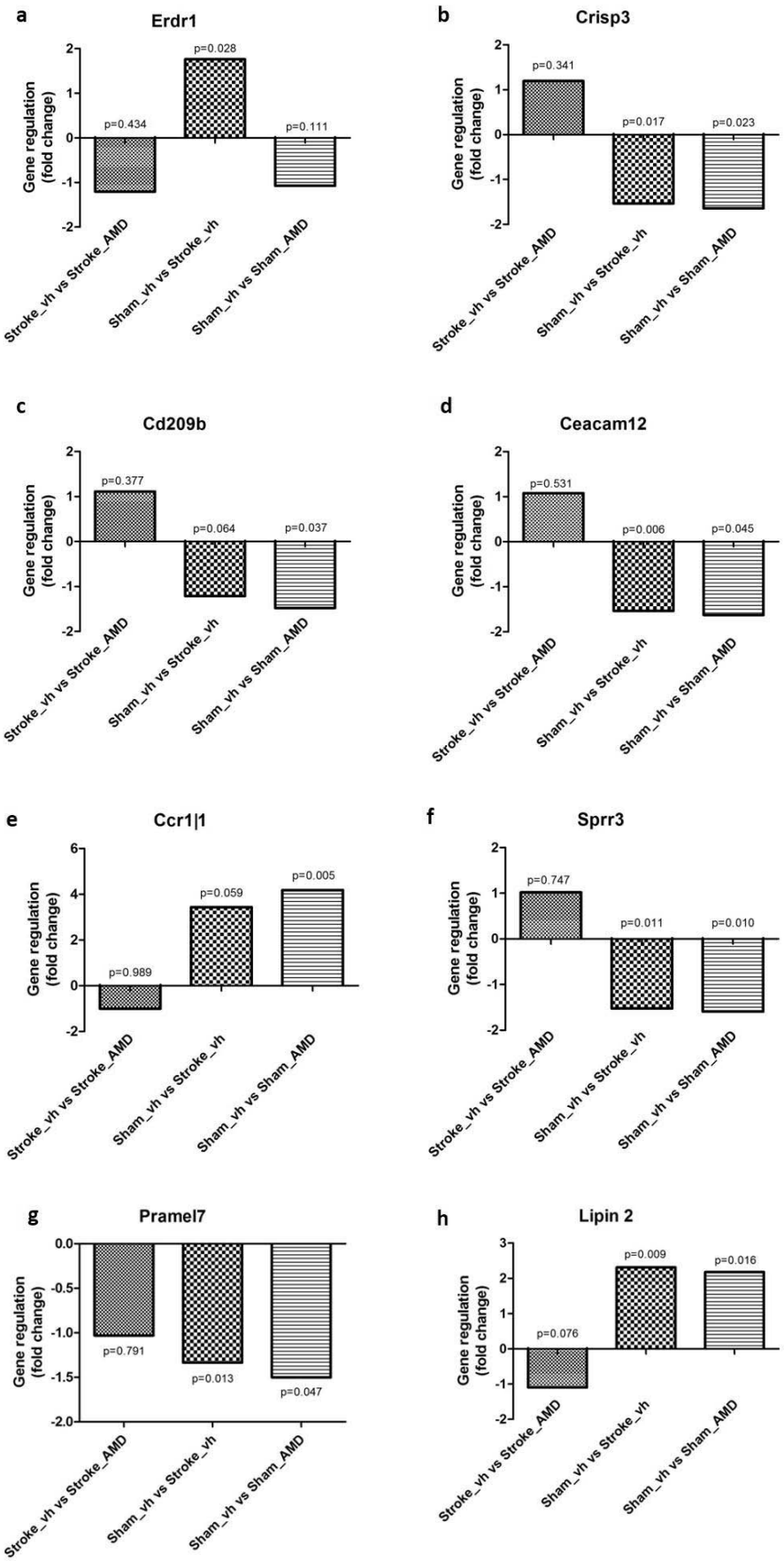


Figure 13. Regulated genes part of the immune response category. The graph shows the fold changes per condition comparison and respective p-values. The following numbers of animals were included: Sham/vehicle, n=3; Sham/AMD, n=3; PT/AMD, n=5; PT/vehicle, n=5. (a) Regulation of the gene TRAJ7 between different experimental groups. (b) Regulation of the gene TRAJ6 between different experimental groups. Statistical analysis has been performed by student´s t-test.

5.2 Inflammation related genes

Regulated genes from this category are presented in Figure 14 . In particular, the comparison of group Sham_vh vs Stroke_vh, the genes lymphocyte antigen 6 complex, locus C1 (Ly6c1), lymphocyte antigen 6 complex, locus C2 (Ly6c2), erythroid differentiation regulator 1 (Erdr1), small proline-rich protein 3 (Sprr3) are regulated with statistical significance (Figure 14 a, i, j, f). The genes chemokine (C-C motif) receptor 1-like 1 (Ccr1|1), preferentially expressed antigen in melanoma like 7 (Pramel7) and CD209b antigen (Cd209b) are regulated with statistical sgnificance in the comparison group Sham_vh vs Sham_AMD. Finally, lipin 2 (Lipin 2), carcinoembryonic antigen-related cell adhesion molecule 12 (Ceacam12) and cysteine-rich secretory protein 3 (Crisp3) are regulated with statistical significance in the comparison groups Sham_vh vs Stroke_vh and Sham_vh vs Sham_AMD. The gene killer cell lectin-like receptor subfamily B member 1 (Klrb1) is upregulated with statistical significance in the comparison group Stroke_vh vs Stroke_AMD (Figure 14 (k)). In summary, this preliminary evaluation indicates that both the induction of stroke and treatment with AMD3100 have effects on expression patterns of inflammatory genes in the corpus callosum and adjacent cortex of the non-lesioned contralateral hemsiphere and in sham-operated animals, respectively.


a
Erdr1
Gene regulation (fold change)
p=0.434
p=0.028
p=0.111
Stroke_vh vs Stroke_AMD
Sham_vh vs Stroke_vh
Sham_vh vs Sham_AMD
b
Crisp3
p=0.341
p=0.017
p=0.023
c
Cd209b
p=0.377
p=0.064
p=0.037
d
Ceacam12
p=0.531
p=0.006
p=0.045
e
Ccr1|1
p=0.989
p=0.059
p=0.005
f
Sprr3
p=0.747
p=0.011
p=0.010
g
Pramel7
p=0.791
p=0.013
p=0.047
h
Lipin 2
p=0.076
p=0.009
p=0.016

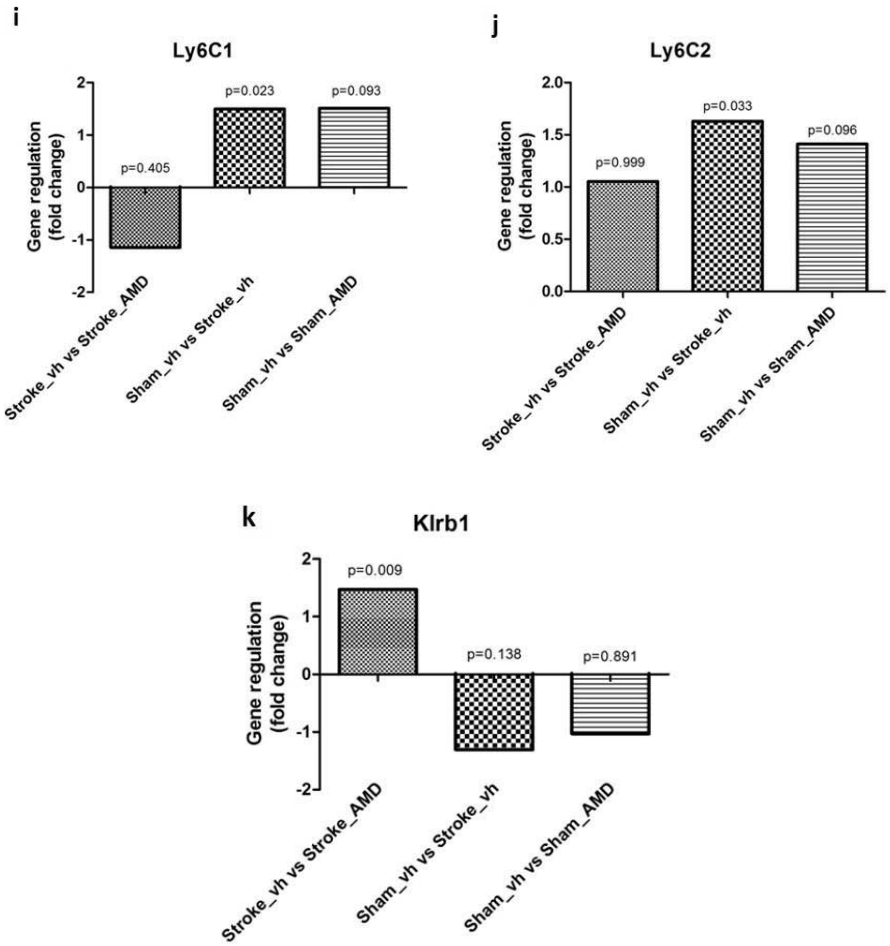


Figure 14. Regulation of inflammation associated genes. The graphs show the fold changes per condition comparison and its p value. The following numbers of animals were included: Sham/vehicle, n=3; Sham/AMD, n=3; PT/AMD, n=5; PT/vehicle, n=5. (a) regulation of the gene Erdr1 .(b) Genetic regulation of the gene Crisp3. (c) Genetic regulation of the gene Cd209b. (d) Genetic regulation of the gene Ceacam12. (e) Genetic regulation of the gene Ccr1|1. (f) Genetic regulation of the gene Sprr3. (g) Genetic regulation of the gene Pramel7. (h) Genetic regulation of the gene Lipin 2. (i) Genetic regulation of the gene Ly6c1. (j) Genetic regulation of the gene Ly6c2. (k) Genetic regulation of the gene Klrb1. Statistical analysis has been performed by student´s t-test.

5.3 Genes related to synaptic plasticity

Concerning the category synaptic plasticity, the genes paired Ig-like receptor B (PirB) and gamma-aminobutyric acid (GABA) receptor, rho 3 (Gabrr3) were regulated with statistical significance in the comparison groups Sham_vh vs Stroke_vh and Sham_vh vs Sham_AMD, respectively (Figure 15).

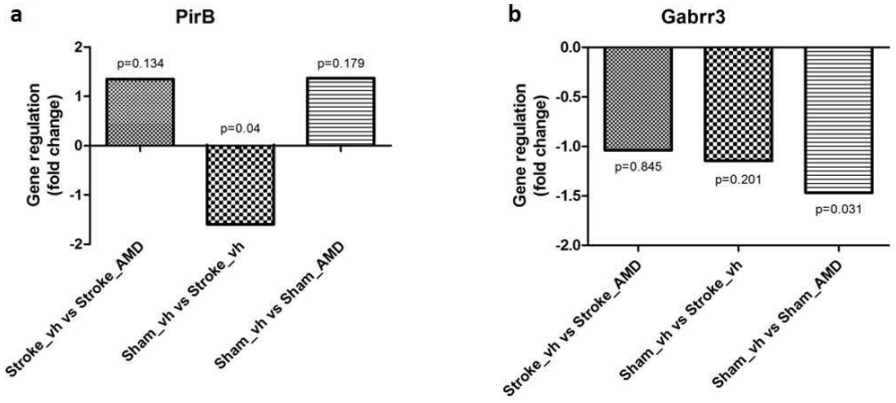


Figure 15. Regulated genes part of the synaptic plasticity category. The graphs show the fold changes per condition comparison with respective p-values. The following numbers of animals were included: Sham/vehicle n = 3; Sham/AMD n = 3; PT/AMD n = 5; PT/vehicle n = 5. (a) Genetic regulation of the gene PirB between different experimental groups. (b) Genetic regulation of the gene Gabrr3 between different experimental groups.

5.3.1 GABA synaptic transmission

Due to the results obtained for the gene Gabrr3, we started to perform a more in depth analysis of the GABA transmission system. The relevance of GABA transmission for functional recovery after stroke has been recognized in previous studies (Clarkson et al., 2010). We picked out all genes which express a GABA receptor subunit and their regulation with p-value, summarized in Tables 8, 9, 10. In addition, we selected all genes transcribing GABA transmission related proteins (Table 12 in Annex II, Table 13 in Annex III, and Table 14 in Annex IV).

Sham_vh vs Sham_AMD			
	GABA Receptor	p value	Regulation
Gabra1	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 1	0.704	↑
Gabra2	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 2	0.593	↓
Gabra3	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 3	0.209	↑
Gabra4	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 4	0.915	↑
Gabra5	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 5	0.752	↑
Gabra6	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 6	0.662	↑
Gabrb1	gamma-aminobutyric acid (GABA) A receptor, subunit beta 1	0.477	↓
Gabrb2	gamma-aminobutyric acid (GABA) A receptor, subunit beta 2	0.987	↑
Gabrb3	gamma-aminobutyric acid (GABA) A receptor, subunit beta 3	0.905	↓
Gabrg1	gamma-aminobutyric acid (GABA) A receptor, subunit gamma 1	0.579	↑
Gabrg2	gamma-aminobutyric acid (GABA) A receptor, subunit gamma 2	0.286	↑
Gabrg3	gamma-aminobutyric acid (GABA) A receptor, subunit gamma 3	0.672	↑
Gabrq	gamma-aminobutyric acid (GABA) A receptor, subunit theta	0.179	↑
Gabrp	gamma-aminobutyric acid (GABA) A receptor, subunit pi	0.839	↑
Gabrd	gamma-aminobutyric acid (GABA) A receptor, subunit delta	0.584	↑
Gabre	gamma-aminobutyric acid (GABA) A receptor, subunit epsilon	0.090	↓
Gabbr1	gamma-aminobutyric acid (GABA) B receptor, 1	0.874	↑
Gabbr2	gamma-aminobutyric acid (GABA) B receptor, 2	0.154	↓
Gabrr1	gamma-aminobutyric acid (GABA) C receptor, subunit rho 1	0.822	↑
Gabrr2	gamma-aminobutyric acid (GABA) C receptor, subunit rho 2	0.245	↓
Gabrr3	gamma-aminobutyric acid (GABA) C receptor, subunit rho 3	0.030	↓

Table 8. Regulation of GABA receptors subunits comparing the experimental groups Sham_vh with Sham_AMD.

Sham_vh vs Stroke_Vh			
	GABA Receptor	p value	Regulation
Gabra1	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 1	0.324	↑
Gabra2	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 2	0.359	↓
Gabra3	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 3	0.525	↑
Gabra4	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 4	0.259	↓
Gabra5	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 5	0.803	↓
Gabra6	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 6	0.155	↓
Gabrb1	gamma-aminobutyric acid (GABA) A receptor, subunit beta 1	0.821	↓
Gabrb2	gamma-aminobutyric acid (GABA) A receptor, subunit beta 2	0.423	↓
Gabrb3	gamma-aminobutyric acid (GABA) A receptor, subunit beta 3	0.283	↓
Gabrg1	gamma-aminobutyric acid (GABA) A receptor, subunit gamma 1	0.041	↓
Gabrg2	gamma-aminobutyric acid (GABA) A receptor, subunit gamma 2	0.690	↑
Gabrg3	gamma-aminobutyric acid (GABA) A receptor, subunit gamma 3	0.299	↓
Gabrq	gamma-aminobutyric acid (GABA) A receptor, subunit theta	0.294	↓
Gabrp	gamma-aminobutyric acid (GABA) A receptor, subunit pi	0.205	↑
Gabrd	gamma-aminobutyric acid (GABA) A receptor, subunit delta	0.917	↓
Gabre	gamma-aminobutyric acid (GABA) A receptor, subunit epsilon	0.313	↓
Gabbr1	gamma-aminobutyric acid (GABA) B receptor, 1	0.532	↓
Gabbr2	gamma-aminobutyric acid (GABA) B receptor, 2	0.463	↓
Gabrr1	gamma-aminobutyric acid (GABA) C receptor, subunit rho 1	0.571	↓
Gabrr2	gamma-aminobutyric acid (GABA) C receptor, subunit rho 2	0.063	↓
Gabrr3	gamma-aminobutyric acid (GABA) C receptor, subunit rho 3	0.201	↓

Table 9.Regulation of the GABA receptors subunits comparing the experimental groups Sham_vh with Stroke_vh.

Stroke_vh vs Stroke_AMD			
	GABA Receptor	p value	Regulation
Gabra1	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 1	0.535	↑
Gabra2	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 2	0.612	↓
Gabra3	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 3	0.957	↑
Gabra4	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 4	0.114	↑
Gabra5	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 5	0.648	↓
Gabra6	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 6	0.334	↑
Gabrb1	gamma-aminobutyric acid (GABA) A receptor, subunit beta 1	0.856	↓
Gabrb2	gamma-aminobutyric acid (GABA) A receptor, subunit beta 2	0.108	↑
Gabrb3	gamma-aminobutyric acid (GABA) A receptor, subunit beta 3	0.543	↓
Gabrg1	gamma-aminobutyric acid (GABA) A receptor, subunit gamma 1	0.071	↑
Gabrg2	gamma-aminobutyric acid (GABA) A receptor, subunit gamma 2	0.371	↑
Gabrg3	gamma-aminobutyric acid (GABA) A receptor, subunit gamma 3	0.492	↑
Gabrq	gamma-aminobutyric acid (GABA) A receptor, subunit theta	0.631	↑
Gabrp	gamma-aminobutyric acid (GABA) A receptor, subunit pi	0.388	↓
Gabrd	gamma-aminobutyric acid (GABA) A receptor, subunit delta	0.713	↑
Gabre	gamma-aminobutyric acid (GABA) A receptor, subunit epsilon	0.484	↑
Gabbr1	gamma-aminobutyric acid (GABA) B receptor, 1	0.496	↓
Gabbr2	gamma-aminobutyric acid (GABA) B receptor, 2	0.927	↓
Gabrr1	gamma-aminobutyric acid (GABA) C receptor, subunit rho 1	0.505	↓
Gabrr2	gamma-aminobutyric acid (GABA) C receptor, subunit rho 2	0.437	↑
Gabrr3	gamma-aminobutyric acid (GABA) C receptor, subunit rho 3	0.845	↓

Table 10. Regulation of the GABA receptors subunits comparing the experimental groups Stroke_vh with Stroke_AMD.

5.3.2 Levels of glutamic acid decarboxylase isoform 67 (GAD67) after treatment with AMD3100

Based on the results from the array study and hypothesizing an increased inhibitory tonus in the nonlesioned contralateral hemisphere upon treatment with AMD3100 we further analyzed the level of GAD67 in the nonlesioned hemisphere of mice subjected to PT and treated either with vehicle or AMD3100 (0.5 mg/kg) according to the experimental design. As shown in Figure 16, mice treated with AMD3100 for 5 days showed higher levels of GAD67 although statistical significance has not been reached. These preliminary results suggest that the treatment can affect GABA neurotransmission and thereby increase the inhibitory tonus in the hemisphere contralateral to the ischemic lesion.

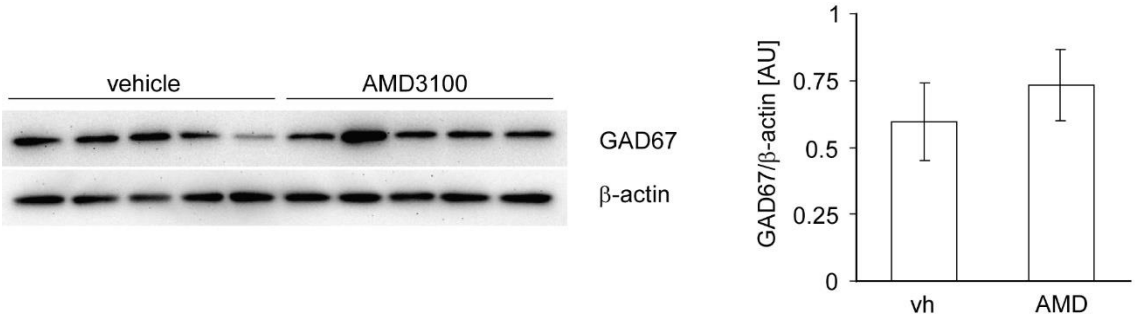


Figure 16. GAD67 levels in the non-lesioned hemisphere from mice subjected to PT and treated either with vehicle (n = 5) or AMD3100 (0.5 mg/kg) (n = 5) for 5 consecutive days. Semiquantitative analysis of GAD67 presented as a GAD67/β-actin ratio as means ± standard error of the mean (SEM), students t-test: p = 0.47.

Chapter 4 - Discussion

The present study has been conducted to further investigate microglia cells accumulating in white matter tracts i.e. in the corpus callosum in experimental stroke models. This is a continuation of our previous studies showing that MHC class II positive microglia accumulate in the corpus callosum within the first days after tMCAO (Kuric & Ruscher, 2014). Moreover, this study has been performed to evaluate if specific anti-inflammatory treatment with AMD3100 affects this cell population and if the treatment leads to changes in the genetic profile in the corpus callosum and adjacent cortex of hemisphere contralateral to the ischemic lesion.

1. Accumulation of GFP/CX3CR1 positive cells in the corpus callosum after PT

As mentioned above we previously have demonstrated that MHC class II positive microglia accumulate in the corpus callosum after tMCAO. In the present study we could verify that microglia cells accumulate in the corpus callosum if mice are subjected to permanent focal ischemia i.e. photothrombosis. Based on these results we speculate that this kind of cellular immune response might be a general response to an acute ischemic insult in the CNS since an immune cell accumulation has also been reported in rats after tMCAO (Morioka, Kalehua, & Streit, 1993) and global ischemia (Wakita et al., 1994). Although it was not focus of the present study to phenotype these cells, immunofluorescence analysis showed that most of the cells expressed Iba1 considered as a marker protein for activated microglia in the postischemic brain (Ito et al., 2001). Further studies, therefore, are needed to exactly phenotype these cells i.e. by FACS analysis and describe the dynamics of their accumulation in white matter tracts following stroke.

2. Effect of AMD3100 treatment on functional recovery after PT

The rationale to perform a study in CX3CR1$^{+/GFP}$ transgenic mice was to easily approach accumulating microglia cells in the corpus callosum after PT. Moreover, we previously have shown that treatment with the CXCR4 antagonist AMD3100 significantly improved functional recovery in mice and rats and affected the microglial response (Ruscher et al., 2013; Walter et al., 2015). We, therefore, hypothesized that treatment with AMD3100 affects the gene expression profile during the first week after stroke. From our previous study we were also aware that the treatment interval of 5 days might be to short to achieve a significant recovery of neurological function 7 days after PT.

As expected, PT induced a significant decrease in the animals neuroscore and AMD3100 treatment did not afford a clear improvement in the neuroscore 7 days after PT. Hence, a more thorough analysis of the experimental group of PT animals treated with AMD3100 showed a slight increase in the median of the neuroscore from day 1 to day 7 which also could be observed on the individual level. A slight increase in the neuroscore has also been observed in animals treated with vehicle. Therefore, this slight improvement is more likely to be due to spontaneous recovery after stroke than a treatment effect of AMD3100. These results are in opposition to what has been shown by Walter and colleagues (Walter et al., 2015) and Ruscher and colleagues (Ruscher et al., 2013). Both studies showed that AMD3100 effected an improvement in the functional outcome after stroke. This might be explained by the use of a different experimental design, animal model and stroke model. Walter and colleagues, in their study, compared day 1 with day 14 and, in the case of Ruscher and colleagues, they used the transient middle cerebral artery occlusion model in rats. Together, in present study AMD3100 might have an effect on animals, a treatment interval of 5 days, however, might be too short to show behavioral improvements. Moreover, the number of animals might have been too small to show differences.

Due to the experimental design of the present study, brains were snap frozen for genomic profiling, infarct volumes have not been determined. Hence, it would have been important to do this in parallel animals. Such data would be used to verify that AMD does not affect infarct volume and that the treatment interferes with neuroprotective or detrimental cascades. To avoid the interference with acute events after stroke, treatment was initiated on day 2, a time point when cell death essentially has been finished and the infarct volume has been subsided. Indeed, it is supported by our previous study (Walter et al., 2015) that AMD does not change infarct volumes when comparing PT animals treated with saline or AMD. Also in a rat tMCAO model, Ruscher and colleagues already showed no effect of AMD3100 on infarct volume (Ruscher et al., 2013).

During animal studies, it remains essential to monitor physiological parameters, thereof the body weight and temperature are very important. Monitoring body temperature and the body weight is an important issue since it is firstly an indication of the animal health. Any animal that lost a lot of weight or shows temperatures out of the normal range should be observed and if needed sacrificed. Experiments that involve the use of animals are accompanied by a ethical permit, including defined study endpoints. This determined when the animal can or should be sacrificed.

Concerning the temperature, several groups have been studying the relationship between the body temperature and the severity and development of the stroke (Boysen & Christensen, 2001; Karaszewski et al., 2013; Kvistad, Thomassen, Waje-Andreassen, & Naess, 2012). Nevertheless, it is not certain how they influence each other or even if their is a relationship. Our data show that there is an increase in the body temperature comparing the temperatures before the surgery (pre-surgery) and the temperatures presented at day 2 (day in each we consider that the stroke lesion is completed). Nonetheless, this increase is present in the animals subjected to PT but also in sham animals. It is, therefore, likely that this increase is mainly due to the fact that the animals were subjected to a surgery and not specifically because they suffered a stroke. On the other hand, body temperature may increase after stroke due to poststroke infections associated to a depression of the immune system affecting mortality.

Regarding the body weight, our data show that there is a slight decrease in the body weight after the surgery and until day 7, in all condition groups, yet not statistically significant. This decrease might be due to stress caused by the surgery. Although, others have shown that weight loss can be a factor in post stroke complications (Jöonsson, Lindgren, Norrving, & Lindgren, 2008; Scherbakov, Dirnagl, & Doehner, 2011), the weight loss in our study is very moderate and does not influence the animal recuperation. In general, permanenet focal ischemia induced by photothrombosis is a tecnique with a high reproducibility of lesion size (Bacigaluppi, Comi, & Hermann, 2010; Braeuninger & Kleinschnitz, 2009; Sicard & Fisher, 2009; Watson, Dietrich, Busto, Wachtel, & Ginsberg, 1985). This is extremely important since it withdraws the variability due to the differences in infarct size and allows to do comparisons with more confidence.

3. Impact of AMD3100 treatment on gene expression

Initially, we aimed at isolating exclusively GFP positive cells from coronal sections from the region of interest. Due to technical problems and lack of feasibility of the laser capture microdissection procedure we isolated the entire corpus callosum and adjacent cortex of the contralatal hemisphere under microscopic control. After optimization, this tissue separation technique and the RNA extraction procedure high quality RNA have been submitted for the genetic array study.

Due to the complexicity of genetic array studies we first focussed on analyzing regulated genes first by stroke and secondly by treatment with AMD3100 compared with sham operated and

vehicle treated mice, respectively. Out of regulated genes we did a first approach to cluster the genes into the following categories: 1. genes related to the poststroke immune response; 2. inflammatory genes; and 3. genes related to neuronal plasticity. Due to time restrictions this first approach seemed feasible, a systematic analysis of data is ongoing. These categories have been chosen due to the treatment and relevance for recovery of function after stroke (Wieloch & Nikolich, 2006).

4. Poststroke immune response

In the category of genes related to the poststroke immune response we found the regulation of T-cell receptor alpha joining 6 (TRAJ6) and T-cell receptor alpha joining 7 (TRAJ7) which are joining regions in the alpha and beta receptors expressed in T-cells, usually during T-cell activation and antigen presentation (Smith-Garvin & Koretzky, 2009; Turner, Doherty, McCluskey, & Rossjohn, 2006). Solely, the regulation of these molecules, mainly the upregulation of TRAJ 7 after stroke, may indicate the presence of T-cells in the ischemic brain in the contralateral hemisphere 7 days after stroke. Moreover, as shown in figure 9 in the same region there is the presence of microglia cells, which express MHC II. Together, these data support the notion that populations of T-cells are present in the contralateral hemisphere and populations of microglia cells expressing MHC class II putatively serve as antigen presenting cells.

The upregulation of TRAJ 7 is higher in animals subjected to PT and treated with AMD comparing to the upregulation in other groups. TRAJ 6, on the other hand, is downregulated in all comparison conditions but looking deeply it is possible to observe that the presence of the drug in animals subjected to PT seems to antagonize this downregulation. T-cell infiltration, activation and effects after stroke are controversial (Iadecola & Anrather, 2011) but the data above and knowing that the AMD treatment improve recovery may indicate the need for the infiltration of T-cell populations and antigen presentation.

5. Poststroke inflammatory response

We found several regulated genes which can be related to the postischemic inflammatory response. However, not all of them have been studied in experimental stroke and here some of them are discussed and brought into the context of stroke. Among them Erdr1 is a stress-related survival factor which enhances cell survival, primarily related with cancer disease

(Dörmer, Spitzer, & Möller, 2004; M. K. Jung et al., 2011). Lee and colleagues demonstrated its expression in the mouse brain of the tubby mice in each Erdr1 seems to exert protective functions. (Lee, Kim, Kim, & Ahn, 2009). Moreover, a structural homologue of erythroid differentiation regulator-1 was identified in the mouse brain, having possibly a positive role in axonal regulation and neuron degeneration (Gillingwater et al., 2006).

As shown in Figure 14, the upregulation of this factor in ischemic conditions draws attention because it supports the idea that under ischemic conditions the brain upregulates this factor possibly involved in axonal regeneration. On the other hand, AMD3100 treatment seems to downregulate the expression of Erdr1, neverthless, this regulation seems to be independent of the stroke conditions since the sham animals treated with the drug comparing to sham treated with vehicle show the same changes.

The protein Crisp3 seems to be involved in inflammation mechanisms through its expression in neutrophils (Udby, Calafat, Sørensen, Borregaard, & Kjeldsen, 2002). It is linked to diseases like chronic pancreatitis (CP) and prostate cancer (Kosari, Asmann, & Cheville, 2002; Liao et al., 2003). Regarding stroke, it is known that neutrophils respond rapidly promoting blood–brain barrier disruption, cerebral edema, and brain injury (Jickling et al., 2015). It would be expected, considering the information above, that in the presence of the AMD3100, an anti-inflammatory drug, downregulates levels of crisp3. This is not supported by our gene array data, being the crisp3 upregulated in ischemic animals treated with the drug. This might be justified by the action of the neutrophils as antigen-presenting cells and the need of the activation of the adapative immune reponse involved in mechanisms of functional recovery after stroke (Iadecola & Anrather, 2011). Neutrophils seem to have deleterious actions in the acute phase but in the delayed phase after stroke they might be important for recovery, AMD3100 seems to anticipate this role.

CD209b antigen is a C-type leptin receptor, expressed in dendritic cells and macrophages, which plays a role in apoptosis/phagocytosis in the spleen and in HIV disease (Dennis et al., 2012; Prabagar et al., 2013). In the brain, Park and colleagues showed that CD209b is expressed in microglial cells (Park et al., 2009). Analysing our results, it seems that AMD3100 normalizes the expression of the receptor, which may lead to the clearance of the debris,

creating an environment supportive for to neuronal remodelling and finally for recovery of lost neuronal function.

CEACAM12 is part of the CEACAMs family which seem to be involved in cell to cell recognition, neovascularization and T cell proliferation. It is not directly related to any brain diseases but is involved in cancer progression and metastasis (Beauchemin & Arabzadeh, 2013). Based on the array data, AMD3100 seems to upregulate the levels of this adhesion molecule in the ischemic conditions and reverses the downregulation found in mice subjected to PT and treated with vehicle. Taking in account the possible roles of CEACAMs and our results, we may suggest that the AMD might promote neovascularization and the proliferation of T-cell populations, possibly important for recovery.

Ccr1|1 is not being extensively studied but this is a chemokine receptor with C-C motif. In general, chemokines play a role in the inflammatory response in stroke but also neuronal survival and neo-angiogenesis. It is commonly known that chemokines can be pro-inflammatory but also anti-inflammatory, depending on the chemokine and on the time point of action. Receptors are important to activate the downstream events in target cells (Mirabelli-Badenier et al., 2011). Through the regulation of this chemokine receptor, it seems to be possible to unravel the role of the chemokine ligand of this receptor. AMD3100 apparently slightly downregulates the expression of the receptor in ischemic conditions, which is upregulated in stroke animals treated with vehicle. The downregulation of the receptor may avoid the effect of the chemokine ligand and taking the anti-inflammatory role of AMD3100 into account this chemokine/chemokine receptor system might have a pro-inflammatory effect during stroke.

Sprr3 is related to neoplastic and inflammatory skin diseases (Huber, Panizzon, & Hohl, 2000). Concerning the brain sprr3 was not connected to the same but sprr1A appears to be expressed in axotomized neurons and has a role in promoting axonal outgrowth (Bonilla, Tanabe, & Strittmatter, 2002). Due to lack of studies, however, it is difficult to understand the possible role of Sprr3 in the brain and the stroke context. Nevertheless, the results show that in the presence of the drug there is an upregulation of the gene under ischemic conditions. Moreover, knowing that a protein from the same family, sprr1A, has a role in promoting

axonal outgrowth, we may propose that sprr3 is implicated in the axonal re-organization after stroke. This also shows that genes related to inflammatory conditions are not necessary related to inflammatory processes in the brain, but might be involved in processes such as neuronal plasticity.

Lipin 2 is a member of the lipin family enzymes, which are key factors in the lipid biosynthesis.(Reue & Dwyer, 2009; Valdearcos et al., 2012) Valdearcos and colleagues attribute the role of an anti-inflammatory enzyme to Lipin 2, since it blocks the inflammatory action of saturated fatty acids in both monocyte-derived human macrophages and the murine macrophage cell line RAW264.7. They proposed that fatty acids activate JNK pathway, that induces the expression of pro-inflammatory genes. Lipin 2 controls the synthesis of the fatty acids inhibiting the JNK pathway (Valdearcos et al., 2012). In view of these results, we propose that lipin 2 may have an analogous role in the brain and acts as an anti-inflammatory enzyme. The stroke animals comparing to sham show an upregulation of lipin 2, each may indicate a defense of the brain to diminish the harmful effects of inflammation. In contrast, the use of the drug seems to downregulate the expression of lipin 2 in the stroke animals. Hence, further studies on the protein levels are required to confirm these data to provide informations on the functional relevance of lipin 2 on poststroke inflammation.

Functions of various immune cells accumulating in the postischemic brain (Gelderblom et al., 2009) have not been unravelled. Here, we found several genes which are expressed in different immune cells. One of those is Klrb1 expressed in Natural killer cells. These cells are lymphocytes that mediate cytotoxicity and secrete cytokines after immune stimulation. Natural killer cells seem to have a role in stroke damage, however, the time point of action after stroke and their effects to harm is still controversial and focus of ongoing studies (Gan et al., 2014; Y. Zhang et al., 2014). AMD3100 seems to upregulate this receptor in animals subjected to PT, which may indicate a presence of natural killer cells in the contralateral side of the postischemic brain. An upregulation of the protein by AMD3100 suggests beneficial actions of these cells in the contralateral side of the infarcted brain seven days after stroke.

6. Neuronal plasticity

Interestingly, we also observed the regulation of genes related to neuronal plasticity corroborating the idea of a crosstalk between poststroke inflammation and plasticity mechanisms.

PirB is type I transmembrane glycoprotein, involved in the inhibition of axonal regeneration after stroke through different mechanism (Adelson et al., 2012). Its location in the ischemic brain so far is confined to the peri-infarct area during the frist seven days (Gou et al., 2013). Downregulation of PirB in the contralateral hemisphere of the ischemic brain, shown in figure 15 may indicate that the brain by itself is promoting an environment permissive to initiate axonal regeneration and organization also in brain areas remote to the ischemic territory. The results, regarding treatment with AMD3100, seem to be a controversial since the treatment upregulates the expression of the glycoprotein. As mentioned before for other genes, further investigations will be needed to elaborate on the function of PirB upon AMD3100 treatment. It can be speculated, however, that AMD3100 leads to a normalization of PirB expression to prevent abberant sprouting in the contralateral hemisphere.

Of particular interest is the regulation of genes related to neurotransmission. A fine tuned balance between different transmitter systems is required to promote the reorganization of neuronal circuits after acute brain injury (Dhawan et al., 2011; Clarkson et al., 2010). After stroke, it has been recognized that an imbalance between excitatory and inhibitory inputs exists with a dominance of inhibition preventing mechanisms of neuronal plasticity (Nudo, 2013; Schmidt, Bruehl, Frahm, Redecker, & Witte, 2012).

Interstingly, we found a downregulation of the gamma-aminobutyric acid (GABA) receptor, rho 3 (Gabrr3) in mice subjected to PT and treated with vehicle compared with respective sham operated mice. Moreover, our array data suggest that AMD3100 treatment further downregulated Gabrr3 indicating that AMD3100 affects the expression of GABA receptors and thus GABAergic transmission in the contralateral hemisphere. This prompted us to evaluate all GABA receptor subunits on the gene level and data show that some of those were regulated. Future more in depth analyses need to evaluate if AMD3100 treatment affects GABA receptor composition with relevance for neurotranmission. An increased expression of GAD67 suggests an increased inhibitory tonus in the contralateral hemisphere on day 7 after stroke upon treatment with AMD3100. This is required since an increased excitatory input of the

contralateral hemisphere towards the lesioned hemisphere inhibits plasticity processes and ultimately recovery of lost neurological function (Jones et al., 2013)

Chapter 5 - Final Remarks

1. Conclusions and new prespectives

The present study shows that the accumulation of brain resident microglia cells in white matter tracts i.e. the corpus callosum is not dependent on the species and the experimental stroke model. Here, we found an accumulation of microglia in the corpus callosum in mice subjected to permanent focal ischemia induced by photothrombosis one week after the insult. In those mice, we found the regulation of genes related to inflammation and immune response but also genes related to neuronal plasticity have been affected by the treatment with the CXCR4 antagonist AMD3100. Results presented here can be seen as new perspective to overcome the traditional view on neuronal plasticity and inflammation as isolated processes in the postischemic brain. Further in depth investigations will be required to elucidate the mechanisms of the inflammation-neuronal plasticity crosstalk after stroke.

Chapter 6 - References

(1995) Stroke study group Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. N Engl J Med 333:1581-1587.

(2009)The penumbra pivotal stroke trial: safety and effectiveness of a new generation of mechanical devices for clot removal in intracranial large vessel occlusive disease. Stroke 40:2761-2768.

Adelson, J. D., Barreto, G. E., Xu, L., Kim, T., Brott, B. K., Ouyang, Y. B., … Giffard, R. G. (2012). Neuroprotection from Stroke in the Absence of MHCI or PirB. *Neuron*, *73*(6), 1100–1107. http://doi.org/10.1016/j.neuron.2012.01.020

Akiyoshi, K., Ren, X., Dziennis, S., Vandenbark, A. a, Herson, P. S., Hurn, P. D., & Offner, H. (2011). Regulatory B cells limit CNS inflammation and neurologic deficits in murine experimental stroke. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(23), 8556–8563. http://doi.org/10.1523/JNEUROSCI.1623-11.2011

American Heart Association.
http://www.strokeassociation.org/STROKEORG/AboutStroke/AboutStroke_UCM_308529_SubHomePage.jsp.
http://www.strokeassociation.org/STROKEORG/AboutStroke/EffectsofStroke/Effects-ofStroke_UCM_308534_SubHomePage.jsp.
http://www.strokeassociation.org/STROKEORG/AboutStroke/TypesofStroke/IschemicClots/Ischemic-Strokes-Clots_UCM_310939_Article.jsp.
http://www.strokeassociation.org/STROKEORG/AboutStroke/TypesofStroke/TIA/TIATransient-Ischemic-Attack_UCM_310942_Article.jsp.
http://www.strokeassociation.org/STROKEORG/AboutStroke/UnderstandingRisk/Understanding-Stroke-Risk_UCM_308539_SubHomePage.jsp.
http://www.strokeassociation.org/STROKEORG/LifeAfterStroke/RegainingIndependence/PhysicalChallenges/Post-Stroke-Rehabilitation_UCM_310447_Article.js

American stroke Association. (2004). A Rehab Revolution. *Stroke Connection Magazine*, 20–24.

Anderson, M. F., Blomstrand, F., Blomstrand, C., Eriksson, P. S., & Nilsson, M. (2003). Astrocytes and stroke: networking for survival? *Neurochemical Research*, *28*(2), 293–305. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12608702

Appelros, P., Stegmayr, B., & Terent, a. (2009). Sex Differences in Stroke Epidemiology: A Systematic Review. *Stroke*, *40*(4), 1082–1090. http://doi.org/10.1161/STROKEAHA.108.540781

Arumugam, T., Granger, D. N., & Mattson, M. (2005). Stroke and T-cells. *NeuroMolecular Medicine*, *7*(3), 229–242. http://doi.org/10.1385/NMM:7:3:229

Bacigaluppi, M., Comi, G., & Hermann, D. M. (2010). Animal models of ischemic stroke. Part two: modeling cerebral ischemia. *The Open Neurology Journal*, *4*, 34–38. http://doi.org/10.2174/1874205X01004020034

Bazan, J. F., Bacon, K. B., Hardiman, G., Wang, W., Soo, K., Rossi, D., … Schall, T. J. (1997). A new class of membrane-bound chemokine with a CX3C motif. *Nature*, *385*(6617), 640–644. Retrieved from http://dx.doi.org/10.1038/385640a0

Beauchemin, N., & Arabzadeh, A. (2013). Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) in cancer progression and metastasis. *Cancer and Metastasis Reviews*, *32*(3-4), 643–671. http://doi.org/10.1007/s10555-013-9444-6

Becker, K. J., McCarron, R. M., Ruetzler, C., Laban, O., Sternberg, E., Flanders, K. C., & Hallenbeck, J. M. (1997). Immunologic tolerance to myelin basic protein decreases stroke size after transient focal cerebral ischemia. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(20), 10873–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=23514&tool=pmcentrez&rendertype=abstract

Biddison, W. E., & Martin, R. (2000). Peptide Binding Motifs for MHC Class I and II Molecules INTERACTION WITH A SPECIFIC, 1–7.

Bonilla, I. E., Tanabe, K., & Strittmatter, S. M. (2002). Small proline-rich repeat protein 1A is expressed by axotomized neurons and promotes axonal outgrowth. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(4), 1303–1315. http://doi.org/22/4/1303 [pii]

Boysen, G., & Christensen, H. (2001). Stroke severity determines body temperature in acute stroke. *Stroke; a Journal of Cerebral Circulation*, *32*(2), 413–417. http://doi.org/10.1161/01.STR.32.7.1697

Braeuninger, S., & Kleinschnitz, C. (2009). Rodent models of focal cerebral ischemia: procedural pitfalls and translational problems. *Experimental & Translational Stroke Medicine*, *1*, 8. http://doi.org/10.1186/2040-7378-1-8

Broderick, J. P. (2002). Treatment of Acute Ischemic Stroke: Part II: Neuroprotection and Medical Management. *Circulation*, *106*(13), 1736–1740. http://doi.org/10.1161/01.CIR.0000030407.10591.35

Burda, J. E., & Sofroniew, M. V. (2014). Reactive Gliosis and the Multicellular Response to CNS Damage and Disease. *Neuron*, *81*(2), 229–248. http://doi.org/10.1016/j.neuron.2013.12.034

Cardona, A. E., Pioro, E. P., Sasse, M. E., Kostenko, V., Cardona, S. M., Dijkstra, I. M., … Ransohoff, R. M. (2006). Control of microglial neurotoxicity by the fractalkine receptor. *Nature Neuroscience*, *9*(7), 917–924. http://doi.org/10.1038/nn1715

Chen, J., Venkat, P., Zacharek, A., & Chopp, M. (2014). Neurorestorative therapy for stroke. *Frontiers in Human Neuroscience*, *8*(June), 382. http://doi.org/10.3389/fnhum.2014.00382

Chinnery, P. F., Elliott, H. R., Syed, A., & Rothwell, P. M. (2010). Mitochondrial DNA haplogroups and risk of transient ischaemic attack and ischaemic stroke: a genetic association study. *Lancet Neurology*, *9*(5), 498–503. http://doi.org/10.1016/S1474-4422(10)70083-1

Citri, A., & Malenka, R. C. (2008). Synaptic plasticity: multiple forms, functions, and mechanisms. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *33*(1), 18–41. http://doi.org/10.1038/sj.npp.1301559

Clarkson, N. A., Huang, B., MacIsaac, S., Mody, I., & Carmichael, S. T. (2010). Reducing excessive GABAergic tonic inhibition promotes poststroke functional recovery. *Nature*, *468*(7321), 305–309. http://doi.org/10.1038/nature09511.Reducing

Cramer, S. C. (2008). Repairing the human brain after stroke. II. Restorative therapies. *Annals of Neurology*, *63*(5), 549–60. http://doi.org/10.1002/ana.21412

Cramer, S. C., & Nudo, R. J. (2010). *Brain repair after stroke*.

Davignon, J., & Ganz, P. (2004). Role of endothelial dysfunction in atherosclerosis. *Circulation*, *109*(23 Suppl 1), III27–32. http://doi.org/10.1161/01.CIR.0000131515.03336.f8

Del Zoppo, G. J., Higashida, R. T., Furlan, a. J., Pessin, M. S., Rowley, H. a., & Gent, M. (1998). PROACT: A Phase II Randomized Trial of Recombinant Pro-Urokinase by Direct Arterial Delivery in Acute Middle Cerebral Artery Stroke. *Stroke*, *29*(1), 4–11. http://doi.org/10.1161/01.STR.29.1.4

Dennis, M. K., Field, A. S., Burai, R., Ramesh, C., Whitney, K., Bologa, C. G., … Prossnitz, E. R. (2012). Dendritic Cell-Specific Intercellular Adhesion Molecule 3Grabbing Nonintegrin/CD209 Is Abundant on Macrophages in the Normal Human Lymph Node and Is Not Required for Dendritic Cell Stimulation of the Mixed Leukocyte Reaction, *127*(7), 358–366. http://doi.org/10.1016/j.jsbmb.2011.07.002.Identification

Dhawan, J., Benveniste, H., Luo, Z., Nawrocky, M., Smith, S. D., & Biegon, A. (2011). A new look at glutamate and ischemia: NMDA agonist improves long-term functional outcome in a rat model of stroke. *Future Neurology*, *6*(6), 823–834. doi:10.2217/fnl.11.55

Di Filippo, M., Tozzi, A., Costa, C., Belcastro, V., Tantucci, M., Picconi, B., & Calabresi, P. (2008). Plasticity and repair in the post-ischemic brain. *Neuropharmacology*, *55*(3), 353–62. http://doi.org/10.1016/j.neuropharm.2008.01.012

Dirnagl, U., Iadecola, C., & Moskowitz, M. a. (1999). Pathobiology of ischaemic stroke: an integrated view. *Trends in Neurosciences*, *22*(9), 391–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10441299

Dörmer, P., Spitzer, E., & Möller, W. (2004). EDR is a stress-related survival factor from stroma and other tissues acting on early haematopoietic progenitors (E-Mix). *Cytokine*, *27*(2-3), 47–57. http://doi.org/10.1016/j.cyto.2004.03.014

Doyle, K. P., Simon, R. P., & Stenzel-Poore, M. P. (2008). Mechanisms of ischemic brain damage. *Neuropharmacology*, *55*(3), 310–8. http://doi.org/10.1016/j.neuropharm.2008.01.005

EuroHYP-1. http://www.eurohyp1.eu/.

Fan, Y., Shen, F., Frenzel, T., Zhu, W., & Ye, J. (2009). Endothelial progenitor cell transplantation improves longâ€term stroke outcome in mice. *Annals of …*, *67*(4), 488–497. http://doi.org/10.1002/ana.21919.Endothelial

Feigin, V. L., Lawes, C. M. M., Bennett, D. a, & Anderson, C. S. (2003). Stroke epidemiology: a review of population-based studies of incidence, prevalence, and case-fatality in the late 20th

century. *Lancet Neurology*, *2*(1), 43–53. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12849300

Francis, J., Raghunathan, S., & Khanna, P. (2007). The role of genetics in stroke. *Postgraduate Medical Journal*, *83*(983), 590–5. http://doi.org/10.1136/pgmj.2007.060319

Gan, Y., Liu, Q., Wu, W., Yin, J.-X., Bai, X.-F., Shen, R., … Shi, F.-D. (2014). Ischemic neurons recruit natural killer cells that accelerate brain infarction. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(7), 2704–9. http://doi.org/10.1073/pnas.1315943111

Gelderblom, M., Leypoldt, F., Steinbach, K., Behrens, D., Choe, C. U., Siler, D. a., … Magnus, T. (2009). Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. *Stroke*, *40*(5), 1849–1857. http://doi.org/10.1161/STROKEAHA.108.534503

Gillingwater, T. H., Wishart, T. M., Chen, P. E., Haley, J. E., Robertson, K., MacDonald, S. H. F., … Ribchester, R. R. (2006). The neuroprotective WldS gene regulates expression of PTTG1 and erythroid differentiation regulator 1-like gene in mice and human cells. *Human Molecular Genetics*, *15*(4), 625–635. http://doi.org/10.1093/hmg/ddi478

Go, A. S., Mozaffarian, D., Roger, V. L., Benjamin, E. J., Berry, J. D., Blaha, M. J., … Turner, M. B. (2014). *Heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation* (Vol. 129). http://doi.org/10.1161/01.cir.0000441139.02102.80

Gou, X., Zhang, Q., Xu, N., Deng, B., Wang, H., Xu, L., & Wang, Q. (2013). Spatio-temporal expression of paired immunoglobulin-like receptor-B in the adult mouse brain after focal cerebral ischaemia. *Brain Injury*, *27*(11), 1311–1315. http://doi.org/10.3109/02699052.2013.812241

Hacke, W., Kaste, M., Bluhmki, E., Brozman, M., Dávalos, A., Guidetti, D., … Investigators, E. (2008). Thrombolysis with Alteplase 3 to 4.5 Hours after Acute Ischemic Stroke. *The New England Journal of Medicine*, *359*(13), 1317–1329.

Harrison, J. K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R. K., … Feng, L. (1998). Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(18), 10896–10901. http://doi.org/10.1073/pnas.95.18.10896

Hassan, a, & Markus, H. S. (2000). Genetics and ischaemic stroke. *Brain : A Journal of Neurology*, *123 (Pt 9*, 1784–812. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10960044

Hatori, K., Nagai, A., Heisel, R., Ryu, J. K., & Kim, S. U. (2002). Fractalkine and fractalkine receptors in human neurons and glial cells. *Journal of Neuroscience Research*, *69*(3), 418–426. http://doi.org/10.1002/jnr.10304

Heiss, W.-D. (2010). The concept of the penumbra: can it be translated to stroke management? *International Journal of Stroke : Official Journal of the International Stroke Society*, *5*(4), 290–5. http://doi.org/10.1111/j.1747-4949.2010.00444.x

Heiss, W.-D. (2012). The ischemic penumbra: how does tissue injury evolve? *Annals of the New York Academy of Sciences*, *1268*, 26–34. http://doi.org/10.1111/j.1749-6632.2012.06668.x

Hermann, D. M., & Chopp, M. (2012). Promoting brain remodelling and plasticity for stroke recovery: therapeutic promise and potential pitfalls of clinical translation. *Lancet Neurology*, *11*(4), 369–80. http://doi.org/10.1016/S1474-4422(12)70039-X

Ho, V. M., Lee, J.-A., & Martin, K. C. (2011). The cell biology of synaptic plasticity. *Science (New York, N.Y.)*, *334*(6056), 623–8. http://doi.org/10.1126/science.1209236

Huang, J., Li, Y., Tang, Y., Tang, G., Yang, G. Y., & Wang, Y. (2012). CXCR4 antagonist AMD3100 protects blood-brain barrier integrity and reduces inflammatory response after focal ischemia in Mice. *Stroke*, *44*(1), 190–197. http://doi.org/10.1161/STROKEAHA.112.670299

Huang, J., Upadhyay, U. M., & Tamargo, R. J. (2006, September 1). Inflammation in stroke and focal cerebral ischemia. *Surgical Neurology*. Elsevier Science Publishing. Retrieved from http://linkinghub.elsevier.com/retrieve/pii/S0090301906001017?showall=true

Huber, M., Panizzon, R., & Hohl, D. (2000). Expression of small proline rich proteins in neoplastic and.pdf, 733–740.

Hurn, P. D., Subramanian, S., Parker, S. M., Afentoulis, M. E., Kaler, J., Vandenbark, A. A., & Offner, H. (2007). T- and B-cell-deficient mice with experimental stroke have reduced lesion size and inflammation, *27*(11), 1798–1805. http://doi.org/10.1038/sj.jcbfm.9600482.T-

Iadecola, C., & Anrather, J. (2011). The immunology of stroke: from mechanisms to translation. *Nature Medicine*, *17*(7), 796–808. http://doi.org/10.1038/nm.2399

Ito, D., Tanaka, K., Suzuki, S., Dembo, T., & Fukuuchi, Y. (2001). Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. *Stroke; a Journal of Cerebral Circulation*, *32*(5), 1208–1215. http://doi.org/10.1161/01.STR.32.5.1208

Jander, S., Kraemer, M., Schroeter, M., Witte, O. W., & Stoll, G. (1995). Lymphocytic infiltration and expression of intercellular adhesion molecule-1 in photochemically induced ischemia of the rat cortex. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *15*(1), 42–51. http://doi.org/10.1038/jcbfm.1995.5

Jickling, G. C., Liu, D., Ander, B. P., Stamova, B., Zhan, X., & Sharp, F. R. (2015). Targeting neutrophils in ischemic stroke: translational insights from experimental studies. *J Cereb Blood Flow Metab*, *35*(6), 888–901. Retrieved from http://dx.doi.org/10.1038/jcbfm.2015.45

Jin, R., Yang, G., & Li, G. (2010). Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. *Journal of Leukocyte Biology*, *87*(5), 779–89. http://doi.org/10.1189/jlb.1109766

Johnston, S. C. (2002). TRANSIENT ISCHEMIC ATTACK. *The New England Journal of Medicine*, *347*(21), 1687–1692.

Jöonsson, A. C., Lindgren, I., Norrving, B., & Lindgren, A. (2008). Weight loss after stroke: A population-based study from the Lund Stroke Register. *Stroke*, *39*(3), 918–923. http://doi.org/10.1161/STROKEAHA.107.497602

Jung, M. K., Park, Y., Song, S. B., Cheon, S. Y., Park, S., Houh, Y., … Cho, D. (2011). Erythroid differentiation regulator 1, an interleukin 18-regulated gene, acts as a metastasis suppressor in melanoma. *The Journal of Investigative Dermatology*, *131*(10), 2096–2104. http://doi.org/10.1038/jid.2011.170

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M. J., Kreutzberg, G. W., Sher, a, & Littman, D. R. (2000). Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Molecular and Cellular Biology*, *20*(11), 4106–4114. http://doi.org/10.1128/MCB.20.11.4106-4114.2000

Karaszewski, B., Carpenter, T. K., Thomas, R. G. R., Armitage, P. a, Lymer, G. K. S., Marshall, I., … Wardlaw, J. M. (2013). Relationships between brain and body temperature, clinical and imaging outcomes after ischemic stroke. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *33*(7), 1083–9. http://doi.org/10.1038/jcbfm.2013.52

Khaja, A. M., & Grotta, J. C. (2007). Established treatments for acute ischaemic stroke. *Lancet*, *369*(9558), 319–30. http://doi.org/10.1016/S0140-6736(07)60154-8

Kim, A. S., & Johnston, S. C. (2013). Temporal and geographic trends in the global stroke epidemic. *Stroke; a Journal of Cerebral Circulation*, *44*(6 Suppl 1), S123–5. http://doi.org/10.1161/STROKEAHA.111.000067

Kleinschnitz, C., Kraft, P., Dreykluft, A., Hagedorn, I., Göbel, K., Schuhmann, M. K., … Wiendl, H. (2013). Regulatory T cells are strong promoters of acute ischemic stroke in mice by inducing dysfunction of the cerebral microvasculature. *Blood*, *121*(4), 679–91. http://doi.org/10.1182/blood-2012-04-426734

Kleinschnitz, C., Schwab, N., Kraft, P., Hagedorn, I., Dreykluft, A., Schwarz, T., … Stoll, G. (2010). Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. *Blood*, *115*(18), 3835–42. http://doi.org/10.1182/blood-2009-10-249078

Koenig-bruhin, M., Kolonko, B., At, A., Annoni, J., & Hunziker, E. (2013). Aphasia following a stroke : recovery and recommendations for rehabilitation. *Swiss Arquives of Neurology and Psychiatry*, *164*(8), 292–298.

Kornblum, H. I. (2007). Introduction to neural stem cells. *Stroke; a Journal of Cerebral Circulation*, *38*(2 Suppl), 810–6. http://doi.org/10.1161/01.STR.0000255757.12198.0f

Kosari, F., Asmann, Y. W., & Cheville, J. C. (2002). Cysteine-rich Secretory Protein-3 : A Potential Biomarker for Prostate Cancer Cysteine-rich Secretory Protein-3 : A Potential Biomarker for Prostate Cancer, *11*(November), 1419–1426.

Kubes, P., Suzuki, M., & Granger, D. N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(11), 4651–5. Retrieved from

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=51723&tool=pmcentrez&rendertype=abstract

Kunz, A., Dirnagl, U., & Mergenthaler, P. (2010). Acute pathophysiological processes after ischaemic and traumatic brain injury. *Best Practice & Research. Clinical Anaesthesiology*, *24*(4), 495–509. http://doi.org/10.1016/j.bpa.2010.10.001

Kuric, E., & Ruscher, K. (2014). Dynamics of major histocompatibility complex class II-positive cells in the postischemic brain - influence of levodopa treatment. *Journal of Neuroinflammation*, *11*(1), 145. http://doi.org/10.1186/s12974-014-0145-z

Kuric, E., Wieloch, T., & Ruscher, K. (2013). Dopamine receptor activation increases glial cell line-derived neurotrophic factor in experimental stroke. *Experimental Neurology*, *247*, 202–8. http://doi.org/10.1016/j.expneurol.2013.04.016

Kvistad, C. E., Thomassen, L., Waje-Andreassen, U., & Naess, H. (2012). Low body temperature associated with severe ischemic stroke within 6 hours of onset: The Bergen NORSTROKE Study. *Vascular Health and Risk Management*, *8*(1), 333–338. http://doi.org/10.2147/VHRM.S31614

Lakhan, S. E., Kirchgessner, A., & Hofer, M. (2009). Inflammatory mechanisms in ischemic stroke: therapeutic approaches. *Journal of Translational Medicine*, *7*, 97. http://doi.org/10.1186/1479-5876-7-97

Lee, J. H., Kim, C. H., Kim, D. G., & Ahn, Y. S. (2009). Microarray analysis of differentially expressed genes in the brains of tubby mice. *The Korean Journal of Physiology & Pharmacology : Official Journal of the Korean Physiological Society and the Korean Society of Pharmacology*, *13*(2), 91–97. http://doi.org/10.4196/kjpp.2009.13.2.91

Liao, Q., Kleeff, J., Xiao, Y., Guweidhi, a., Schambony, a., Töpfer-Petersen, E., … Friess, H. (2003). Preferential expression of cystein-rich secretory protein-3 (CRISP-3) in chronic pancreatitis. *Histology and Histopathology*, *18*(2), 425–433.

Liesz, A., Suri-Payer, E., Veltkamp, C., Doerr, H., Sommer, C., Rivest, S., … Veltkamp, R. (2009). Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nature Medicine*, *15*(2), 192–9. http://doi.org/10.1038/nm.1927

Liesz, A., Zhou, W., Mracskó, É., Karcher, S., Bauer, H., Schwarting, S., … Veltkamp, R. (2011). Inhibition of lymphocyte trafficking shields the brain against deleterious neuroinflammation after stroke. *Brain : A Journal of Neurology*, *134*(Pt 3), 704–20. http://doi.org/10.1093/brain/awr008

Lin, Z., & Yan, T. (2011). Long-term effectiveness of neuromuscular electrical stimulation for promoting motor recovery of the upper extremity after stroke. *Journal of Rehabilitation Medicine*, *43*(6), 506–10. http://doi.org/10.2340/16501977-0807

Maas, M. B., & Safdieh, J. E. (2009). Ischemic Stroke : Pathophysiology and Principles of Localization. *Neurology Board Review Manual*, *13*.

Madinier, A., Quattromani, M. J., Sjölund, C., Ruscher, K., & Wieloch, T. (2014). Enriched housing enhances recovery of limb placement ability and reduces aggrecan-containing

perineuronal nets in the rat somatosensory cortex after experimental stroke. *PLoS ONE*, *9*(3), 1–15. http://doi.org/10.1371/journal.pone.0093121

Manoonkitiwongsa, P. S., Jackson-Friedman, C., McMillan, P. J., Schultz, R. L., & Lyden, P. D. (2001). 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*, *21*(10), 1223–1231. http://doi.org/10.1097/00004647-200110000-00011

Meschia, J. F., Worrall, B. B., & Rich, S. S. (2014). Genetic susceptibility to ischemic stroke, *7*(7), 369–378. http://doi.org/10.1038/nrneurol.2011.80.Genetic

Ming, G.-L., & Song, H. (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron*, *70*(4), 687–702. http://doi.org/10.1016/j.neuron.2011.05.001

Mirabelli-Badenier, M., Braunersreuther, V., Viviani, G. L., Dallegri, F., Quercioli, A., Veneselli, E., … Montecucco, F. (2011). CC and CXC chemokines are pivotal mediators of cerebral injury in ischaemic stroke. *Thrombosis and Haemostasis*, *105*(3), 409–420. http://doi.org/10.1160/TH10-10-0662

Morioka, T., Kalehua, a N., & Streit, W. J. (1993). Characterization of microglial reaction after middle cerebral artery occlusion in rat brain. *The Journal of Comparative Neurology*, *327*(1), 123–132. http://doi.org/10.1002/cne.903270110

Morris, D. L., & Schroeder, E. B. (n.d.). Stroke Epidemiology. *Ferne*.

Mukherjee, D., & Patil, C. G. (2011). Epidemiology and the global burden of stroke. *World Neurosurgery*, *76*(6 Suppl), S85–90. http://doi.org/10.1016/j.wneu.2011.07.023

Nakajima, K., & Kohsaka, S. (2001). Microglia : System Activation and Their Significance in the Central Nervous. *The Journal of Biochemistry*, *130*(2), 169–175.

National Stroke Association. http://www.stroke.org/understand-stroke/preventing-stroke.

Nudo, R. J. (2013). Recovery after brain injury: mechanisms and principles. *Frontiers in Human Neuroscience*, *7*(December), 887. http://doi.org/10.3389/fnhum.2013.00887

Pan, Y., Lloyd, C., Zhou, H., Dolich, S., Deeds, J., Gonzalo, J.-A., … Gearing, D. (1997). Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature*, *387*(6633), 611–617. Retrieved from http://dx.doi.org/10.1038/42491

Panel, Sacco, R. L., Benjamin, E. J., Broderick, J. P., Dyken, M., Easton, J. D., … Wolf, P. A. (1997). Risk Factors. *Stroke* , *28* (7), 1507–1517. http://doi.org/10.1161/01.STR.28.7.1507

Park, J. Y., Choi, H. J., Prabagar, M. G. V, Choi, W. S., Kim, S. J., Cheong, C., … Kang, Y. S. (2009). The C-type lectin CD209b is expressed on microglia and it mediates the uptake of capsular polysaccharides of Streptococcus pneumoniae. *Neuroscience Letters*, *450*(3), 246–251. http://doi.org/10.1016/j.neulet.2008.11.070

Parkin, J., & Cohen, B. (2001). An overview of the immune system. *Lancet*, *357*(9270), 1777–89. http://doi.org/10.1016/S0140-6736(00)04904-7

Paxinos, G., & Franklin, K. B. J. (2004). *The mouse brain in stereotaxic coordinates*. *Academic Press* (Vol. 2nd). http://doi.org/10.1016/S0306-4530(03)00088-X

Prabagar, M. G., Do, Y., Ryu, S., Park, J.-Y., Choi, H.-J., Choi, W.-S., … Kang, Y.-S. (2013). SIGN-R1, a C-type lectin, enhances apoptotic cell clearance through the complement deposition pathway by interacting with C1q in the spleen. *Cell Death and Differentiation*, *20*(4), 535–45. http://doi.org/10.1038/cdd.2012.160

Ren, X., Akiyoshi, K., Vandenbark, A., Hurn, P., & Offner, H. (2011). CD4+FoxP3+ regulatory T-cells in cerebral ischemic stroke. *Metab Brain Dis.*, *26*(1), 87–90. http://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted

Reue, K., & Dwyer, J. R. (2009). Lipin proteins and metabolic homeostasis. *Journal of Lipid Research*, *50 Suppl*, S109–S114. http://doi.org/10.1194/jlr.R800052-JLR200

Ruscher, K., Kuric, E., Liu, Y., Walter, H. L., Issazadeh-Navikas, S., Englund, E., & Wieloch, T. (2013). Inhibition of CXCL12 signaling attenuates the postischemic immune response and improves functional recovery after stroke. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *33*(8), 1225–34. http://doi.org/10.1038/jcbfm.2013.71

Ruscher, K., Kuric, E., & Wieloch, T. (2012). Levodopa treatment improves functional recovery after experimental stroke. *Stroke*, *43*(2), 507–513. http://doi.org/10.1161/STROKEAHA.111.638767

Sá, M. J. (2009). Avc – primeira causa de morte em portugal 1, *6*, 12–19.

Schäbitz, W.-R., Steigleder, T., Cooper-Kuhn, C. M., Schwab, S., Sommer, C., Schneider, A., & Kuhn, H. G. (2007). Intravenous brain-derived neurotrophic factor enhances poststroke sensorimotor recovery and stimulates neurogenesis. *Stroke; a Journal of Cerebral Circulation*, *38*(7), 2165–72. http://doi.org/10.1161/STROKEAHA.106.477331

Scherbakov, N., Dirnagl, U., & Doehner, W. (2011). Body weight after stroke lessons from the obesity paradox. *Stroke*, *42*(12), 3646–3650. http://doi.org/10.1161/STROKEAHA.111.619163

Schilling, M., Besselmann, M., Müller, M., Strecker, J. K., Ringelstein, E. B., & Kiefer, R. (2005). Predominant phagocytic activity of resident microglia over hematogenous macrophages following transient focal cerebral ischemia: An investigation using green fluorescent protein transgenic bone marrow chimeric mice. *Experimental Neurology*, *196*(2), 290–297. http://doi.org/http://dx.doi.org/10.1016/j.expneurol.2005.08.004

Schlaug, G., & Renga, V. (2008). Transcranial direct current stimulation: a noninvasive tool to facilitate stroke recovery, *5*(6), 759–768. http://doi.org/10.1586/17434440.5.6.759.Transcranial

Schmandke, A., Schmandke, A., & Schwab, M. E. (2014). Nogo-A: Multiple Roles in CNS Development, Maintenance, and Disease . *The Neuroscientist* , *20* (4), 372–386. http://doi.org/10.1177/1073858413516800

Schmidt, S., Bruehl, C., Frahm, C., Redecker, C., & Witte, O. W. (2012). Age dependence of excitatory-inhibitory balance following stroke. *Neurobiology of Aging*, *33*(7), 1356–1363. http://doi.org/10.1016/j.neurobiolaging.2010.11.019

Schmitt, a B., Brook, G. a, Buss, a, Nacimiento, W., Noth, J., & Kreutzberg, G. W. (1998). Dynamics of microglial activation in the spinal cord after cerebral infarction are revealed by expression of MHC class II antigen. *Neuropathology and Applied Neurobiology*, *24*(3), 167–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9717181

Schmitt, a B., Buss, a, Breuer, S., Brook, G. a, Pech, K., Martin, D., … Nacimiento, W. (2000). Major histocompatibility complex class II expression by activated microglia caudal to lesions of descending tracts in the human spinal cord is not associated with a T cell response. *Acta Neuropathologica*, *100*(5), 528–36. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11045675

Shah, S. (n.d.). Stroke Pathophysiology. *Ferne*.

Shichita, T., Sugiyama, Y., Ooboshi, H., Sugimori, H., Nakagawa, R., Takada, I., … Yoshimura, A. (2009). Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury. *Nature Medicine*, *15*(8), 946–50. http://doi.org/10.1038/nm.1999

Sicard, K. M., & Fisher, M. (2009). Animal models of focal brain ischemia. *Experimental & Translational Stroke Medicine*, *1*, 7. http://doi.org/10.1186/2040-7378-1-7

Simpson, J. E., Ince, P. G., Higham, C. E., Gelsthorpe, C. H., Fernando, M. S., Matthews, F., … Group, the M. R. C. C. F. and A. N. S. (2007). Microglial activation in white matter lesions and nonlesional white matter of ageing brains. *Neuropathology and Applied Neurobiology*, *33*(6), 670–683. http://doi.org/10.1111/j.1365-2990.2007.00890.x

Siniscalchi, A., Gallelli, L., Malferrari, G., Pirritano, D., Serra, R., Santangelo, E., & De Sarro, G. (2014). Cerebral stroke injury: the role of cytokines and brain inflammation. *Journal of Basic and Clinical Physiology and Pharmacology*, *25*(2), 131–7. http://doi.org/10.1515/jbcpp-2013-0121

Smith, W. S., Sung, G., Saver, J., Budzik, R., Duckwiler, G., Liebeskind, D. S., … Silverman, I. E. (2008). Mechanical thrombectomy for acute ischemic stroke: final results of the Multi MERCI trial. *Stroke; a Journal of Cerebral Circulation*, *39*(4), 1205–12. http://doi.org/10.1161/STROKEAHA.107.497115

Smith-Garvin, J., & Koretzky, G. (2009). T cell activation. *Annual Review of Immunology*, *27*, 591–619. http://doi.org/10.1146/annurev.immunol.021908.132706.T

Sofroniew, M. V. (2009). Molecular dissection of reactive astrogliosis and glial scar formation. *Trends Neurosci*, *32*(12), 638–647. http://doi.org/10.1016/j.tins.2009.08.002.Molecular

Sofroniew, M. V, & Vinters, H. V. (2010). Astrocytes: biology and pathology. *Acta Neuropathologica*, *119*(1), 7–35. http://doi.org/10.1007/s00401-009-0619-8

Strbian, D., Kovanen, P. T., Karjalainen-Lindsberg, M.-L., Tatlisumak, T., & Lindsberg, P. J. (2009). An emerging role of mast cells in cerebral ischemia and hemorrhage. *Annals of Medicine*, *41*(6), 438–450. http://doi.org/10.1080/07853890902887303

Subramanian, S., Zhang, B., Kosaka, Y., Gregory, G., Grafe, M. R., Vandenbark, A. A., … Offner, H. (2009). Recombinant T Cell Receptor Ligand (RTL) Treats Experimental Stroke. *Stroke*, *40*(7), 2539–2545. http://doi.org/10.1161/STROKEAHA.108.543991.Recombinant

Taussky, P., Tawk, R. G., Daugherty, W. P., & Hanel, R. a. (2011). Medical therapy for ischemic stroke: review of intravenous and intra-arterial treatment options. *World Neurosurgery*, *76*(6 Suppl), S9–15. http://doi.org/10.1016/j.wneu.2011.05.048

Tenser, M. (2012). Endovascular Management of ischemic Stroke. *Neurotransmitter*, 10.

The Penumbra Pivotal Stroke Trial Investigators. (2009). The penumbra pivotal stroke trial: safety and effectiveness of a new generation of mechanical devices for clot removal in intracranial large vessel occlusive disease. *Stroke; a Journal of Cerebral Circulation*, *40*(8), 2761–8. http://doi.org/10.1161/STROKEAHA.108.544957

Turner, S. J., Doherty, P. C., McCluskey, J., & Rossjohn, J. (2006). Structural determinants of T-cell receptor bias in immunity. *Nat Rev Immunol*, *6*(12), 883–894. Retrieved from http://dx.doi.org/10.1038/nri1977

Udby, L., Calafat, J., Sørensen, O. E., Borregaard, N., & Kjeldsen, L. (2002). Identification of human cysteine-rich secretory protein 3 (CRISP-.pdf, *3*.

Valdearcos, M., Esquinas, E., Meana, C., Peña, L., Gil-de-Gómez, L., Balsinde, J., & Balboa, M. a. (2012). Lipin-2 reduces proinflammatory signaling induced by saturated fatty acids in macrophages. *Journal of Biological Chemistry*, *287*(14), 10894–10904. http://doi.org/10.1074/jbc.M112.342915

Wakita, H., Tomimoto, H., Akiguchi, I., & Kimura, J. (1994). Glial activation and white matter changes in the rat brain induced by chronic cerebral hypoperfusion: an immunohistochemical study. *Acta Neuropathologica*, *87*(5), 484–92. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8059601

Walter, H. L., van der Maten, G., Antunes, A. R., Wieloch, T., & Ruscher, K. (2015). Treatment with AMD3100 attenuates the microglial response and improves outcome after experimental stroke. *Journal of Neuroinflammation*, *12*(1), 1–15. http://doi.org/10.1186/s12974-014-0232-1

Wang, Q., Tang, X. N., & Yenari, M. A. (2007). The inflammatory Response in Stroke. *Journal of Neuroimmunology*, *184*(1-2), 53–68.

Watson, B. D., Dietrich, W. D., Busto, R., Wachtel, M. S., & Ginsberg, M. D. (1985). Induction of reproducible brain infarction by photochemically initiated thrombosis. *Annals of Neurology*, *17*(5), 497–504. http://doi.org/10.1002/ana.410170513

Weston, R. M., Jones, N. M., Jarrott, B., & Callaway, J. K. (2007). Inflammatory cell infiltration after endothelin-1-induced cerebral ischemia: histochemical and myeloperoxidase correlation with temporal changes in brain injury. *Journal of Cerebral Blood Flow and Metabolism : Official*

Journal of the International Society of Cerebral Blood Flow and Metabolism, *27*(1), 100–14. http://doi.org/10.1038/sj.jcbfm.9600324

WHO. (2002). *The World Health Report 2002*.

Wieloch, T., & Nikolich, K. (2006). Mechanisms of neural plasticity following brain injury. *Current Opinion in Neurobiology*, *16*(3), 258–264. http://doi.org/10.1016/j.conb.2006.05.011

Wilhelmsson, U., Bushong, E. a, Price, D. L., Smarr, B. L., Phung, V., Terada, M., … Pekny, M. (2006). Redefining the concept of reactive astrocytes as cells that remain within their unique domains upon reaction to injury. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(46), 17513–17518. http://doi.org/10.1073/pnas.0602841103

Winship, I. R., & Murphy, T. H. (2008). In vivo calcium imaging reveals functional rewiring of single somatosensory neurons after stroke. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *28*(26), 6592–6606. http://doi.org/10.1523/JNEUROSCI.0622-08.2008

Yilmaz, G., Arumugam, T. V, Stokes, K. Y., & Granger, D. N. (2006). Role of T lymphocytes and interferon-gamma in ischemic stroke. *Circulation*, *113*(17), 2105–12. http://doi.org/10.1161/CIRCULATIONAHA.105.593046

Yilmaz, G., & Granger, D. N. (2010). Leukocyte Recruitment and Ischemic Brain Injury, *12*(2), 193–204. http://doi.org/10.1007/s12017-009-8074-1.Leukocyte

Zemmar, A., Weinmann, O., Kellner, Y., Yu, X., Vicente, R., Gullo, M., … Schwab, M. E. (2014). Neutralization of nogo-a enhances synaptic plasticity in the rodent motor cortex and improves motor learning in vivo. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *34*(26), 8685–98. http://doi.org/10.1523/JNEUROSCI.3817-13.2014

Zhang, L., Schallert, T., Zhang, Z. G., Jiang, Q., Arniego, P., Li, Q., … Chopp, M. (2002). A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. *Journal of Neuroscience Methods*, *117*(2), 207–214. http://doi.org/10.1016/S0165-0270(02)00114-0

Zhang, R., Chopp, M., & Zhang, Z. G. (2013). Oligodendrogenesis after cerebral ischemia. *Frontiers in Cellular Neuroscience*, *7*(October), 201. http://doi.org/10.3389/fncel.2013.00201

Zhang, Y., Gao, Z., Wang, D., Zhang, T., Sun, B., Mu, L., … Wang, G. (2014). Accumulation of natural killer cells in ischemic brain tissues and the chemotactic effect of IP-10. *Journal of Neuroinflammation*, *11*(1), 79. http://doi.org/10.1186/1742-2094-11-79

Annex I

Test 1	
Left forelimb flexion in suspension	0 - impaired paw is not moving and tightly held to the trunk
	1 - impaired paw mostly held to the trunk, slightly movable
	2 - impaired paw moves up and down, asymmetry to other paw
	3 - slight asymmetry between the paws, both paws well movable
	4 - normal, symmetric
Test 2	
Paw Placement of the left side	0 - paw totally immobilized, hanging down, no movement
	1 - paw hanging, but little movement back and forth (horizontal plane)
	2 - paw hanging, horizontal and vertical movement below table's level
	3 - paw hanging horizontal and vertical movement up to table's surface level
	4 - paw hanging, mobile in all directions, reaching the table's surface
	5 - normal, paw is immediately taken up to the table's surface
Test 3	
Whisker-Reflex, left side	0 - not present
	2 - present
Test 4	
Faults of the left forelimb walking 27 steps on a grid, 46,5 cm long, step size 2 mm, interspace 1,5 cm (2-3 training sessions)	0 - 6 faults and more
	1 - 5 faults
	2 - 4 faults
	3 - 3 faults
	4 - 2 faults
	5 - 1 fault
	6 - no fault step

Table 11. Composite neuroscore for quantification of neurological deficits before and after photothrombotic stroke at days 2 and 7.

Annex II

Sham_vh vs Sham_AMD			
	GABA related proteins	p value	Regulation
Tacr1	tachykinin receptor 1	0.025	↓
Nlgn2	neuroligin 2	0.054	↓
Cckbr	cholecystokinin B receptor	0.108	↓
Nps	neuropeptide S	0.151	↓
Nlgn1	neuroligin 1	0.313	↑
Prkce	protein kinase C, epsilon	0.359	↑
Car7	carbonic anhydrase 7	0.467	↑
Adra1a	adrenergic receptor, alpha 1a	0.478	↑
Grik1	glutamate receptor, ionotropic, kainate 1	0.552	↑
Tac1	tachykinin 1	0.636	↑
Adora2a	adenosine A2a receptor	0.676	↓
Erbb4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	0.871	↑
Car2	carbonic anhydrase 2	0.906	↑
Oxtr	oxytocin receptor	0.918	↓
Stxbp1	syntaxin binding protein 1	0.052	↓
Cnr2	cannabinoid receptor 2 (macrophage)	0.070	↓
Htr1b	5-hydroxytryptamine (serotonin) receptor 1B	0.225	↓
Npy5r	neuropeptide Y receptor Y5	0.397	↓
Bdnf	brain derived neurotrophic factor	0.441	↑
Adora1	adenosine A1 receptor	0.953	↑
Kras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	0.019	↑
Clstn3	calsyntenin 3	0.315	↑
Cnr1	cannabinoid receptor 1 (brain)	0.316	↑
Dbi	diazepam binding inhibitor	0.358	↓
Usp46	ubiquitin specific peptidase 46	0.695	↓
Nisch	nischarin	0.743	↓
Nf1	neurofibromatosis 1	0.880	↓
Plcl1	phospholipase C-like 1	0.898	↑
Plcl2	phospholipase C-like 2	0.924	↑
Cacnb4	calcium channel, voltage-dependent, beta 4 subunit	0.983	↓
Cntnap4	contactin associated protein-like 4	0.985	↓
Jakmip1	janus kinase and microtubule interacting protein 1	0.143	↓
Arfgef2	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited)	0.207	↓
Ppp2ca	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	0.210	↑
Akap5	A kinase (PRKA) anchor protein 5	0.367	↓
Gabarapl2	gamma-aminobutyric acid (GABA) A receptor-associated	0.417	↑

	protein-like 2		
Gpr156	G protein-coupled receptor 156	0.684	↓
Gabarap	gamma-aminobutyric acid receptor associated protein	0.698	↓
Trak2	trafficking protein, kinesin binding 2	0.776	↑
Trak1	trafficking protein, kinesin binding 1	0.940	↑
Gabarapl1	gamma-aminobutyric acid (GABA) A receptor-associated protein-like 1	0.099	↑
Gls	glutaminase	0.524	↑
Gad1	glutamate decarboxylase 1	0.691	↑
Gad2	glutamic acid decarboxylase 2	0.734	↑
Gata2	GATA binding protein 2	0.588	↑
Helt	helt bHLH transcription factor	0.589	↓
Arx	aristaless related homeobox	0.739	↑
Cntn2	contactin 2	0.770	↑
Drd1a	dopamine receptor D1A	0.808	↑
Drd2	dopamine receptor D2	0.914	↑
Fezf2	Fez family zinc finger 2	0.962	↓

Table 12. Regulation of GABA related proteins comparing the experimental groups Sham_vh with Sham_AMD.

Annex III

Sham_vh vs Stroke_vh			
	GABA related proteins	p value	Regulation
Tacr1	tachykinin receptor 1	0.023	↓
Cckbr	cholecystokinin B receptor	0.014	↑
Nps	neuropeptide S	0.596	↑
Nlgn1	neuroligin 1	0.472	↑
Prkce	protein kinase C, epsilon	0.506	↑
Adra1a	adrenergic receptor, alpha 1a	0.652	↑
Tac1	tachykinin 1	0.822	↓
Adora2a	adenosine A2a receptor	0.629	↓
Erbb4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	0.312	↓
Car2	carbonic anhydrase 2	0.738	↑
Oxtr	oxytocin receptor	0.667	↓
Stxbp1	syntaxin binding protein 1	0.392	↓
Cnr2	cannabinoid receptor 2 (macrophage)	0.073	↓
Htr1b	5-hydroxytryptamine (serotonin) receptor 1B	0.751	↓
Npy5r	neuropeptide Y receptor Y5	0.089	↓
Bdnf	brain derived neurotrophic factor	0.624	↑
Adora1	adenosine A1 receptor	0.448	↑
Kras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	0.038	↑
Clstn3	calsyntenin 3	0.206	↓
Cnr1	cannabinoid receptor 1 (brain)	0.402	↑
Usp46	ubiquitin specific peptidase 46	0.603	↓
Nisch	nischarin	0.368	↓
Nf1	neurofibromatosis 1	0.087	↓
Plcl1	phospholipase C-like 1	0.114	↓
Plcl2	phospholipase C-like 2	0.542	↑
Cntnap4	contactin associated protein-like 4	0.427	↓
Jakmip1	janus kinase and microtubule interacting protein 1	0.265	↓
Arfgef2	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited)	0.602	↓
Ppp2ca	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	0.539	↓
Akap5	A kinase (PRKA) anchor protein 5	0.534	↓
Gabarapl2	gamma-aminobutyric acid (GABA) A receptor-associated protein-like 2	0.950	↑
Gpr156	G protein-coupled receptor 156	0.352	↓
Gabarap	gamma-aminobutyric acid receptor associated protein	0.955	↑
Trak2	trafficking protein, kinesin binding 2	0.537	↑
Trak1	trafficking protein, kinesin binding 1	0.983	↑

Gabarapl1	gamma-aminobutyric acid (GABA) A receptor-associated protein-like 1	0.060	↑
Gls	glutaminase	0.282	↑
Gad1	glutamate decarboxylase 1	0.811	↑
Gad2	glutamic acid decarboxylase 2	0.178	↓
Gata2	GATA binding protein 2	0.106	↑
Helt	helt bHLH transcription factor	0.968	↑
Cntn2	contactin 2	0.498	↑
Drd1a	dopamine receptor D1A	0.960	↓
Drd2	dopamine receptor D2	0.562	↓

Table 13. Regulation of GABA related proteins comparing the experimental groups Sham_vh with Stroke_vh.

Annex IV

Stroke_vh vs Stroke_AMD			
	GABA related proteins	p value	Regulation
Cckbr	cholecystokinin B receptor	0.523	↓
Nlgn1	neuroligin 1	0.854	↓
Adra1a	adrenergic receptor, alpha 1a	0.808	↓
Tac1	tachykinin 1	0.690	↑
Car2	carbonic anhydrase 2	0.545	↓
Oxtr	oxytocin receptor	0.481	↑
Stxbp1	syntaxin binding protein 1	0.903	↑
Cnr2	cannabinoid receptor 2 (macrophage)	0.793	↑
Htr1b	5-hydroxytryptamine (serotonin) receptor 1B	0.258	↓
Npy5r	neuropeptide Y receptor Y5	0.416	↓
Bdnf	brain derived neurotrophic factor	0.266	↓
Adora1	adenosine A1 receptor	0.335	↓
Kras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	0.657	↓
Clstn3	calsyntenin 3	0.322	↑
Cnr1	cannabinoid receptor 1 (brain)	0.283	↓
Usp46	ubiquitin specific peptidase 46	0.647	↑
Nisch	nischarin	0.838	↓
Nf1	neurofibromatosis 1	0.456	↑
Plcl1	phospholipase C-like 1	0.631	↑
Plcl2	phospholipase C-like 2	0.531	↑
Cntnap4	contactin associated protein-like 4	0.683	↓
Arfgef2	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited)	0.603	↓
Ppp2ca	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	0.552	↓
Gabarapl2	gamma-aminobutyric acid (GABA) A receptor-associated protein-like 2	0.271	↑
Gpr156	G protein-coupled receptor 156	0.742	↓
Gabarap	gamma-aminobutyric acid receptor associated protein	0.673	↓
Trak2	trafficking protein, kinesin binding 2	0.061	↓
Trak1	trafficking protein, kinesin binding 1	0.698	↓
Gabarapl1	gamma-aminobutyric acid (GABA) A receptor-associated protein-like 1	0.236	↓
Gls	glutaminase	0.311	↓
Gad1	glutamate decarboxylase 1	0.314	↑
Gad2	glutamic acid decarboxylase 2	0.732	↑
Gata2	GATA binding protein 2	0.336	↓
Helt	helt bHLH transcription factor	0.341	↑
Cntn2	contactin 2	0.852	↑

Drd2	dopamine receptor D2	0.658	↑
Fezf2	Fez family zinc finger 2	0.649	↓
Erbb4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	0.502	↑
Npsr1	neuropeptide S receptor 1	0.479	↓

Table 14. Regulation of GABA related proteins comparing the experimental groups Stroke_vh with Stroke_AMD.