Neuroprotective role of gonadal steroid hormones in a rat stroke model

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1 Introduction

1.1 Types of stroke, incidence rates and pathology

Stroke is classified by the World Health Organisation (WHO) as a pathological acute brain disease of cerebrovascular origin and can lead to long-lasting neurological impairment or even death. Deprivation or interruption of oxygen and nutrients by undersupply of blood through intracranial vessels can cause heavily and irreparably damage in distinct brain regions, which results in functional deficits such as aphasia, apraxia, attenuation in concentration and memory, hemiparesis or neglect. (Hartmann, A., 2001). Stroke is the third leading cause of mortality and first listed for severe disability and intensive aftercare in the United States and Federal Republic of Germany (Hinkle, J.L., 2007, Thom, T., 2006, Eschenfelder, C.C., 2006, Statistisches Jahrbuch: Statistisches Bundesamt, Wiesbaden 2003).

1.1.1 Types of stroke of arterial origin

Evaluation of annual stroke registration (Eschenweiler, C.C., 2006, ESPro - The Erlangen Stroke Project) reveals an incidence of approximately 79% for ischemic stroke and 18% for cerebral hemorrhages of arterial origin. About 3% had stroke of unspecified type (Kolominsky-Rabas, PL, 1998, 2002). Within these two different types of arterial stroke, detailed classifications are defined to describe the course of pathology.

1.1.1.1 Ischemic stroke

The clinical course of ischemic stroke is delicately subdivided into different episodes.

A transient ischemic attack (TIA) shows described neurological symptoms after onset of stroke, which declines and vanish within 24 h or less. Sustained neurological disability more than 24 h, but less than a week with minor to full functional recovery is defined as reversible ischemic neurological deficit or “RIND”.

Moreover, neurological dysfunction after onset of stroke can maintain for hours until days without remission of symptoms. This progressive form of stroke is severe due to long-lasting moto- and senso-functional disability, but can also induce speech disorder.

A major stroke or complete stroke results in clinical deficits with partial or no remission of the neurological symptoms and lead to irreparable moto-function. Thus, it is diagnostically
awkward to differentiate with these terms of definition between RIND, progressive or progredient and major stroke (Caplan, L.R., 1983). Occlusions of large intracranial arteries account for 79% of ischemic stroke cases. Insults of the *A. cerebri media* or middle cerebral artery (MCA) occur 50-60%, whereas the *A. carotis interna* is affected in 15-25% (Xavier, A.R., 2003).

1.1.1.2 Intracerebral haemorrhages

Intracerebral haemorrhages are spontaneous bleedings into the brain parenchyma. This type of bleeding can be subclasified into supratentorial or infratentorial bleedings depending on their anatomical location. Supratentorial bleeding is usually localized within the basal ganglia (Solomon, D.H., 1994), whereas infratentorial haematoma is constricted to *Pons* and *Cerebellum* (Eschenfelder, C.C., 2006). In most cases, hypertonia is responsible for spontaneous supratentorial bleeding, which affects predominantly the basal ganglia penetrating branches with percentage rates of 40% to 60% of all spontaneous intracerebral bleedings (Brott, T., 1986, Fisher, C.M., 2003). Furthermore, vascular abnormalities such as aneurysm are responsible for 6% of all intracerebral hemorrhages (Benoit, B.G., 1982). Aneurysms are blood-filled saccate or berry-like bulges of the arterial vascular wall, which extent the vessel diameter permanently, thus risk for rupturing of blood vessel increases with the rate of dilatation. Ehlers-Danlos-Syndrom and Marfan-Syndrom are innate diseases, which are also called “connective tissue weaknesses”, and frequently result in aneurysm formation (van den Berg, J.S.P., 1996). Moreover, environmental risk factors such as smoking or fatty food can abet vascular wall diseases such as artheriosclerosis (Nagai, Y., 2001).

With respect to latest aetiological studies, cerebral amyloid angiopathy (CAA) is the second most common underlying mechanism of intracerebral haemorrhages with 7-14% in the elderly (Vinters, H.V., 1983, Friedmann, E., 1991). The histopathological manifestation of CAA is an accumulation of β-amyloid protein in the arterial vascular wall within the *Tunica Adventia*, which leads to expulsion of the smooth muscle layer in the *Tunica Media* with demolishing consequences to the *Lamina externa* and *interna* by loosing their vessel wall integrity and flexibility. Due to vascular stenosis, formation of aneurysm and obliteration, affected vessels are highly vulnerable for rupturing in the close vicinity of these pathological vascular changes. In general, CAA is accompanied with hypertonic microangiopathy (Vinters, H.V., 1983). Interestingly, β-amyloid protein in CAA is identical to the plaque forming β-amyloid protein in Alzheimer’s disease (Masters, C.L., 1985).
1.1.1.3 Extracerebral hemorrhages

In the contrary to intracerebral hemorrhages, extracerebral bleeding affects upper vessels on the brain surface and especially vessels, which supply the meninges. The *A. meningea media* is at risk during a craniocerebral injury. Arterial blood streams with high pressure between the skull and the *Dura mater* (epidural hemorrhage), occurs fast and results in an elevated ICP. Thus, epidural hemorrhages can lead to tremendous life-threatening conditions. Another type of extracerebral arterial bleeding without traumatic background is the aneurysmal subarachnoid hemorrhage (SAH). Affected vessels are primarily arteries that form the so called “Circulus arteriosus Willisii”. Within this big anastomotic bloodcircuit, intracranial aneurysmal structures can be found in 30% of the cases at the *A. communicans anterior*, 25% at the *A. communicans posterior*, 20% at the *A. cerebri media*, 7.5% at the *A. carotis interna* and 7% at the basilar tip. 3% are miscellaneous aneurysms, which are located somewhere else (Brisman, L.J., 2006). Leading symptoms for SAH are sudden and severe painful headaches, so called “thunderclap headache”, reduction or even loss of consciousness, vomiting and meningism.

1.1.2 Types of stroke of venous origin

In contrast to arterial stroke, cerebral venous thrombosis (CVT) affects intracranial veins and sinuses. It is a very rare type of cerebrovascular disease with a prevalence of 5 cases among 1 million and accounts for 0.5 % for all strokes (Bousser, M.-G., 2007). Furthermore, rupturing of bridging veins- branches of the upper veins *Vv. superiores cerebri*, which cross the subarachnoid space and penetrate the *Arachnoidea mater* to end in the venous *Sinus sagittalis superior*- lead to the formation of an artificial blood-filled space between the *Dura mater* and *Arachnoidea mater*. This so called “subdural bleeding” results in space-occupying haematoma, which, depending on size and location, can compress important brain areas and elevate ICP (Trepel, M., 2008). In most cases, acute subdural hemorrhage (ASDH) emerges after marked external mechanical forces but also minor traumatic brain injuries (TBI) can underlie shearing forces between *Dura* and *Arachnoidea mater*. In general, ASDH occur in younger adults, after a major trauma accompanied with structural brain injury, and is clinically evident within 72h after disease onset (Adhiyaman, V., 2002). The neurological literature distinguishes acute from chronic subdural haematoma (CSDH). CSDH is a disease pattern predominantly found in the elderly after a trivial injury without directly harming the brain parenchymal structures. The course of this event is slow and consistent because of the
low blood pressure within the bridging veins. As a consequence, affected patients show signs of symptoms such as headache or dizziness one week after lesion onset. This is accompanied with neurological disturbed functions (Adhiyaman, V., 2002, Miranda, L.B., 2011). The incidence is estimated at 7.4/100.000 per year in the age of 70-79 groups (Bernard, K., 2004, Miranda, L.B., 2011). Figure 1 lists and categorizes the mentioned types of stroke.

1.1.3 Correlation between affected topographical brain regions and neurological dysfunction after infarct of the *A. cerebri media* territory

Occlusion of the *A. cerebri media* is one of the most frequently pathogenesis with incidences of 50-60% for all ischemic strokes (Xavier, A.R., 2003). The *A. cerebri media* is the biggest branch and a direct continuation of the *A. carotis interna*. It is most likely that aterio-arterial emboli originate from the left ventricle of the heart, passe the *A. carotis interna* and finally occlude the *A. cerebri media*. The vascular territory of the *A. cerebri media* includes the convex cortical hemispheres including the *Lobus insularis* (see figure 2).

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**Figure 1: Schematic illustration of different stroke types.** Strokes originate from a dysfunction of the vascular (arterial or venous) system. Further categorisation differentiates between acute and chronic events. CVT: cerebral venous thrombosis, ASDH: acute subdural hemorrhage, CSDH: chronic subdural hemorrhage.
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Figure 2: Vascular territory of the major intracerebral arteries.
a) Lateral view of the cerebrum, b) View of a medio-sagital brain section, c) View of a horizontal brain section; 1) Vascular territory of the A. cerebri anterior, 2) Vascular territory of the A. cerebri media, 3) Vascular territory of the A. cerebri posterior; I) Capsula interna, II) Gyrus precentralis, III) Gyrus postcentralis (Image from Trepel, M., 2008).

After an occlusion of the MCA, specific neurological dysfunctions manifest as motoric and sensoric disabilities. One-third of all infarct affected in the MCA territory are allocated to the lower segments of the A. cerebri media, where branching arteries called Aa. lenticulostriatae perforate into deeper brain structures and supply functional areas of the basal ganglia. Suppression of blood supply within this part of the A. cerebri media is constricted to the vascular territory of cortex and basal ganglia, concurrently (Trepel, M., 2008). Affected topological brain structures are located in the somatomotorical dorsal cortex of the frontal lobe, the Gyrus precentralis, but also the upper part of the back crural sections of the Capsula interna. Neurological deficiency comprises brachiofacial hemiparesis with paralisis of the legs or even the whole contralateral side.
Stroke-induced lesions compromising the *Gyrus postcentralis*, an area of the parietal lobe, cause dysfunction of somatosensorical qualities of the contralateral side (face, arms and legs). The extent of functional disability during MCA infarcts depend on the duration and localization of occlusion in the *A. cerebri media* as well as its aetiology.

### 1.1.4 Risk factors

In the last decades, clinical evidence and epidemiological research identify several risk factors for possible stroke events. Such factors include hypertonia, diabetes mellitus, different types of heart diseases and lifestyle habits, including smoking, overweight, alcohol abusus and gender (Bronner, L.L., 1995). One of the main risk factors for stroke, intracerebral hemorrhages, SAH as well as TIA is hypertonia. Level of blood pressure and rate of risk are linear correlated. It has been demonstrated that systolic blood pressure values between 120 and 129mmHg elevate the risk to suffer of stroke by 40% compared to participants with values bellow 120mmHg (O’Donnell, C.J., 1997).

#### 1.1.4.1 Smoking

Meta-analysis of 32 different single studies reveals a 50% higher probability to suffer from stroke in smokers (Shinton, R., 1989). Among smokers, heavy smokers (~730 packs/year) are 2-times more affected to sustain stroke compared to moderate smokers (~182 packs/year). Remarkably, the Framingham-Study demonstrates a remission of high risk for smokers to the level of non-smokers after 5 years of abdication. The risk declines already to the half after 1 year after cassation of smoking (Wolf, P.A., 1988). Therefore, reducing or stopping of smoking attenuates and decrease behaviour-depending risk factors for stroke.

#### 1.1.4.2 Heart diseases

Heart diseases represent an important risk factor for stroke. Risk for cardiac dysfunction, coronar heart diseases, cardiac insufficiency, leftventricular hypertrophy and atrial fibrillation elevates in the elderly simultaneously with the risk for stroke. Coronary heart disease and ventricular hypertrophy can fortify risk for ischemic insult. Moreover, cardiac insufficiency can increase the risk for stroke events by 3-times and arterial fibrillation even by 5-times (Wolf, P.A., 1991). Stroke patients with atrial fibrillation have a higher risk to suffer for
intracranial vessel occlusion by an embolus originated from the left ventricle (Bougousslavsky, J, 1990).

1.1.4.3 Diabetis mellitus

The metabolic disorder diabetes mellitus is another important risk factor for cerebral stroke. In many studies, diabetics have shown high prevalence for cardiovascular heart disease such as hypertonia, adiposity and dyslipoproteinaemia. The PROCAM-Study was clearly able to demonstrate a direct coherence between diabetes mellitus and stroke. The study involved men from 30-64 years of age. Beside a higher incidence of atherosclerosis, reduced fibronolytic activity, aggregation of thrombocytes together with high levels of fibrinogen factor 7 and 8 might play an important role (Berger, K., 1998, Mallinow, M. R., 1997, Manson, J. E., 1991).

1.1.4.4 Overweight

Another behavioural-dependent risk factor besides smoking is overweight, which, in the first place, can be the result of missing sporty activity and unhealthy food uptake. High blood pressure, dyslipoproteinaemy, hyperinsulinemia or disturbed glucose metabolism are additionally accompanied with overweight as independent risk factors. Studies identified overweight as an independent hazard factor for stroke. “Bodymass-Index” or “Waist-to-hip-Ratio” reveals a direct correlation between abdominal adiposity and stroke risk (Walker, S.P., 1996, Shinton, R. 1991, 1995). The insensitivity for insulin inhibition on lipophile reactions of intra-abdominal fatty tissue compared to skin fat might be one major reason. Due to the increase of intra-abdominal fatty tissue, abnormal levels of fatty acid within the liver can be measured. Thus, high concentrations of free available fatty acids results in dyslipoproteinemia, high blood pressure etc. which can provoke in final stages atherosclerotic plaques within the arterial vessel wall (Bjorntop, P., 1990). Extensive clinical data analysis such as the Framingham, Physician Health or the Honolulu Heart Study are suggesting physical or regular sport activities as preventing and stroke-risk-factor reducing parameters. Risk factors decreased about 40-50% in women as well in men, respectively (Abbott, R.D., 1994, Lee, I.M., 1999, Wolf, P.A., 1988).
1.1.4.5 Gender-specific and age-related differences are risk factors for stroke

Incidence and outcome of ischemic and traumatic brain injury alters between sexes and with ages. Moreover, it is characterized by a significantly different male-to-female ratio being higher in male (Herson, P.S., 2009). Epidemiological studies reveal male sex as a risk factor for stroke (Giroud, M., 1991, Bogousslavsky, J. 1988, Sacco, R.L., 1998). This gender-dependent dimorphic disease pattern maintains apparent until ages before menopause (Giroud, M. 1991, Appelros, P. 2008, Bushnell, C.D., 2007). Furthermore, stroke risk increases with age in both genders, and there are broad evidences that outcome from stroke is worse in reproductively senescent women and women within the menopause than in their male counterparts (Appelros, P. 2008, Di Carlo, A., 2003). In the elderly, the risk factor profile for stroke differs between women and men. For instance, women are more vulnerable to have atrial fibrillation and hypertension, whereas men are more likely to suffer from coronary artery disease (Holroyd-Leduc, J.M., 2000, Roquer, J., 2003, Kapral, M.K., 2003). Recently published studies showed sex-specific responses to acute stroke thrombolysis. Women who were treated with tissue plasminogen activator (t-PA) had a better clinical outcome after 90 days compared to their placebo-group. On the contrary, outcome of t-PA-treated men revealed a slightly improvement after lysis compared to men without t-PA medication. Interestingly, placebo-treated women displayed a significantly worse outcome than placebo-treated men (Kent, D.M., 2005, Hill, M.D., 2006). Additionally, women, who received t-PA, showed highly improved outcome already after 24 h compared to men (Saposnik, G., 2006).

1.1.5 Therapy and prevention

Hitherto, there is less common acceptable therapy for acute stroke. Specialized “stroke units”, which are affiliated to neurological departments, ensure clinical diagnosis for instance by magnetic resonance imaging and provide the appropriate therapy including medicamentous lysis of blood clots or surgical intervention. Members of stroke units usually include neurologists, internists, neuroradiologists, and experienced nursing staff. Directly after therapy, rehabilitation programs to set up early recovery procedure for the patient. Therefore, special skilled physiotherapists, speech therapists and occupational therapist are locally resident.

The correct interpretation of a computer tomograph (CT) or magnetic resonance scanning to exclude hemorrhage is crucial within a time frame from 3-6 h after stroke onset. The
immediate thrombolytic recanalisation after diagnostic determination of stroke with ischemic origin results in considerable recovery, smaller infarcts and ameliorates the functional outcome (Wechsler, L., 2000, Young, A.R., 1997). Until now systemic intravenous or catheter-supported local intra-arterial thrombolysis with 0.9 mg recombinant tissue-plasminogen activator (rtPA)/kg BW is the only medicamentous way of treatment for acute ischemic stroke. Treatment should start within 3h after stroke onset after CT imaging excluded hemorrhage definitely (Hacke, W., Poeck, K., 2001, The NINDS rt-PA Stroke Study Group 1995). After lysis, antihypertensive (Angiotensin Converting Enzyme, ACE) and blood-thinning medicaments (acetylsalicylic acid, heparin or phenprocoumon) are subscribed as preventive prophylaxis (Hartmann, A, 2001).

Since several years, research aims to develop treatment strategies, e.g., against oxidative stress (ROS, free radicals etc.) or excitatory amino acid such as glutamate (see 1.2.2) to prolong neuronal cell survival after ischemia. A lot of promising substances were investigated in animal experiments with impressive effects on reducing the lesion volume and neurological deficits. Unfortunately, all clinical trials hitherto failed. Some reasons might be the timing of application in the animal experimental set up, which were before or during experimentally-induced stroke. Application after 24h or even 48h of stroke did not depict the clinical scenario. Moreover, some substances caused unexpected serious side effects such as psychosis, hypotonia and agitation in clinical trials (Wahlgren, N.G., 2004).

1.2 Pathobiological mechanism of ischemic progression

Brain tissue requires high level of glucose and oxygen to provide energy-consuming cellular mechanisms, which are responsible to maintain the intracellular homoeostasis. Metabolic pathways such as glycolysis or the citric acid cycle provide in several biochemical steps the energetic equivalent adenosintriphosphat (ATP) from adenosindiphosphat (ADP) by oxidative phosphorylation. Within seconds to minutes of inadequate blood supply provoked by ischemic events, crucial cascades of biochemical reactions take place to counteract deprivation of oxygen and glucose by switching to anaerobic metabolism. Especially in neurons, levels of ATP and glucose drop down close to the minimum, whereas in the same time lactate level accumulates (Lowry, O.H., 1964). Increase of lactate production and ATP consumption concurrently raises intracellular hydrogen ion concentration (H⁺). Counteractive stabilization of homeostatic conditions remain ineffective based on failure of ATP-depending membrane anchored H⁺-K⁺-pumps, which could transport H⁺ into the extracellular milieu. In
particular, malfunction of the Na-K-ATPase pump leads to imbalance of ionic homeostasis within the cell. Rebalancing of ionic distribution along the pre-synaptic membrane by ordinary non-selective ion- or water channels fail after a certain threshold. In normal circumstances, the resting membrane potential at the axon terminal shows intracellular negative polarisation compared to the extracellular space. Basically, to maintain the status quo the most important ions such as $K^+$, $Na^+$, $Ca^{2+}$ and $Cl^-$ are precisely distributed along the membrane. High concentration of potassium is evident within the cell, whereas sodium, calcium and chloride can be found in greater amounts extracellularly (Alberts, B., 2004). According to the law of diffusion, these ions cross the membrane through specific ion channels (e.g. potassium ion channel) to equalize the concentration gradient. In the cytosol, anionic proteins are present in high concentration, thus different membrane transport systems are required within the membrane to counteract the osmotic reactions to maintain the electrical gradient. ATP-depending shuttled transporter systems (e.g. Na-K-ATPase pump) in the plasma membrane actively pump the surplus of sodium and calcium into the extracellular space. In neurons, ischemic conditions cause depolarisation in the pre-synaptic terminal mainly due to lactate accumulation and ischemic-induced failure of ATPase pumps (Siesjö, B., 1988). As a consequence, the depolarization opens voltage-depending, calcium-selective ion channels located in the pre-synaptic membrane resulting in influx of calcium into the cell. Via calcium-binding mechanism on synaptic vesicle containing neurotransmitters, the release of these messengers is triggered into the synaptic cleft via exocytosis. Independent of neurotransmitters, the transmission occurs rapidly by ligand-receptor interaction on the post-synaptic side (Nicholls, D., 1999). For another signal incoming, removal of neurotransmitter in the synaptic cleft and especially on the receptor is either catalysed through enzymes (e.g. acetylcholin esterase) or is re-uptaked by the pre-synaptic membrane via specific symporters or ATP-consuming transporters. Restoration of the synaptic cleft is also enhanced by diffusion processes, which disperse the neurotransmitter into the adjacent border. Despite of degradation by enzymes, breakdown of ATP-gated reuptake mechanisms caused fatal high accumulation of neurotransmitters, which diffuse uncontrolled and react with receptors on other neurons.

1.2.1 Glutamate excitotoxicity

One of the most important and ubiquitous excitatory amino acid neurotransmitters in the central nervous system (CNS) is L-glutamate. Under above mentioned ischemic pathological conditions, the accumulation of high concentration of glutamate in the synaptic cleft results in
dispersion and causes non desirable continuous activation on neighboured neuronal ionotropic and metabotropic glutamate receptors located post-synaptically. Persistent binding on ionotrophic receptors such as NMDA (N-methyl D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) or kainite receptors evokes overload influx of calcium (Hazell, A.S., 2003, Hollmann, M., 1991). In contrast to AMPA and kainite receptors, which are permeable to K⁺ and Na⁺ as well as to Ca²⁺ to a lower level, NMDA receptors are voltage-dependent and ligand-gated receptors, which are highly permeable to Ca²⁺. The existing concentration gradient causes binding of glutamate to AMPA or kainite receptors and provokes high influx of Na⁺ and in smaller amounts of calcium into the postsynaptic neuron and a slowly K⁺ outflow into the synaptic cleft. Water streams passively intracellularly and causes cell swelling. As a result, the membrane depolarizes and leads to an active potential or excitatory post-synaptic potential (EPSP), which opens the glutamate-mediated NMDA receptor for calcium (Hossmann, K.-A., 1994a, Turski, L., 1998).

Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors that trigger upper- and downstream signalling regulation involving membrane-anchored protein kinase C, cyclic AMP or phosphoinositide-dependent mechanism (Choi, D., 1988). Activation of mGluRs increase intracellular Ca²⁺ concentration by second-messenger-mediation of inositol 1,4,5-trisphosphate (IP₃), which binds on ligand-gated IP₃-receptors on the endoplasmic reticulum (ER). Sustained overactivation of mGluRs by glutamate excitotoxicity mediates abnormal intracellular calcium release from the ER. Concomitantly, the decline of calcium in the ER evokes ER-stress, manifested by protein misfolding, disturbances in protein synthesis and expression of stress proteins. Free cytosolic Ca²⁺ is a key signalling agent and has versatile important regulatory mechanisms e.g. vehicle release via exocytosis, enzymatic regulation or controlling of channels to maintain cytosolic homeostasis (Lemasters, L.L. 2009). Uncontrolled rise of cytosolic Ca²⁺ upregulates calcium-dependent catabolic enzymes and provoke mitochondrial disturbances (Paschen, W.1996).

### 1.2.2 Calcium-mediated neurotoxicity

Under normal conditions, the intracellular concentration of Ca²⁺ ions is tightly controlled by highly efficient calcium transport systems to maintain the cellular homeostasis. Within the cell, gradients between the cytosol and the cell compartments such as ER or mitochondria are also strictly regulated. Following ischemic conditions, these cellular transport systems break down leading to high influx of calcium into the cell. The calcium ion activity and the release of endogenous calcium by the ER or mitochondrion elevate intracellular calcium levels to a
neurotoxic degree. The overload of calcium ions triggers intracellular second messenger signalling cascades, which are usually regulated and controlled under physiological conditions. Ca$^{2+}$-binding proteins such as calmodulin, which is a versatile, intracellular Ca$^{2+}$-receptor, can interact with Ca$^{2+}$/calmodulin-depending protein kinases (CaM-Kinases) but also the binding of free calcium to membrane-anchored proteinase c (PKC) can activate gene as well as protein regulation. The latter comprises regulation of the NF-κB pathway, CREP-induced signalling directly or indirectly activating proteases, kinases, phospholipases and endonucleotidases (Racioppi, L., 2008, Feske, S., 2007, Klein, J., 1997, Du, K., 1998, Berridge, M. J., 2003). Calcium-mediated neurotoxicity is a complex event and the impact of its fatal outcome is restricted to the degree of ischemia.

1.2.3 Oxidative stress and cellular defence mechanism

High surplus of oxidants, such as ROS, which consist of free radicals and reactive metabolites, can imbalance antioxidative defence mechanisms to an inappropriate degree, which results in oxidative stress. Inefficient elimination of oxidants leads to protein, membrane and RNA/DNA damaging that might cause the cell to undergo necrosis or apoptosis (Durackova, Z., 2010). ROS are generated during normal cellular metabolism and can also be involved in many signaling pathways (Jabs, T., 1999). The physiological aerobic process of molecular oxygen reduction occurs mainly at the mitochondrial respiratory chain and produce ROS such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH•), and organic peroxides (Fridovich, I., 1978, Poyton, R. O., 2009). To avoid toxic accumulation, antioxidants such as phase II enzymes conjugate oxidized proteins, biotransform these proteins in a water-soluble compound, which can than be excreted through urine or bile. A major role in the cellular defence against oxidative or electrophilic stress is the activation of the NF-E2-related factor 2 (Nrf2)-antioxidant response element (ARE) signaling pathway. This pathway regulates the expression of genes coding for proteins, which are crucial in the detoxification and elimination of reactive oxidants and electrophilic agents (Nguyen, T., 2009, Lee, J.-M., 2005). It is well known that the activation of Nrf2 is controlled by the cytosolic protein Kelch-like ECH-associated protein 1 (Keap1). The pathway and mechanism by which Keap 1 acts to inhibit Nrf2 activation is still not fully characterized. Upon activation, cytosolic Nrf2 is translocated to the nucleus and binds to the ARE sequence in the promoter regions of many detoxificativ and antioxidativ proteins and initiate the transcription for instance of glutathione S-transferase (GST) (Sakai, M., 1988), NAD(P)H quinone oxidoreductase-1 (NQO1) (Favreau, L.V., 1991) or haem oxygenase 1 (HO-1). GST
and NQO1 are two major detoxification enzymes and are directly activate by Nrf2, whereas HO-1 can also be regulated by other transcriptional factors such as hypoxic inducible-factor 1 alpha (Hif1a) (Ockaili, R., 2005, Kim, Y-M, 2011). In stroke pathology oxidative stress is profound, thus, Nrf2-ARE signalling pathway might be a major player to influence ischemia/reperfusion-provoked oxidative injury of brain tissue caused by ROS and could have an enormous impact in improving outcomes of ischemic brain disease.

**Figure 3: Overview of the ischemic cascade.** ATP = Adenosintriphat; Na$^+$ = Sodium-Cation; K$^+$ = Potassium-Cation; H$^+$ = Hydrogen-Cation; NMDA = N-methyl D-aspartate; AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ER = Endoplasmatic Reticulum; ROS = Reactive Oxygen Species; MPT = Mitochondrial Permeability Transition.
1.2.4 Neuroinflammation

After brain ischemia, microglia and astrocytes become activated following subsequent sequestering of a subset of inflammatory mediators, ROS or proteases, which elevates the vulnerability of neurons, disrupt the blood-brain barrier (BBB) and stimulate formation of gliosis (Silver, J. 2004). Cytokines abet expression of adhesion molecules on the endothelial cell surface mediating the infiltration of neutrophil granulocytes, monocytes and macrophages (Iadecola, C., 1997). Additionally, local secretion of chemokines attracts and activates microglia as well as astrocyte and extravasated leukocytes (Ritter, L.S., 2000, Wang, Q., 2007).

Ca\(^{2+}\)-induced activation of intracellular signalling pathways, the rise of ROS and the condition of hypoxia itself jointly trigger the expression of a number of pro-inflammatory genes by inducing the expression of several transcriptional factor such as nuclear factor-kB, hypoxia inducible factor 1 or interferon regulatory factor 1 (O’Neill, L.A. 1997, Ruscher, K. 1998). Tumour necrosis factor α (TNFα) or interleukin 1β (IL-1β) are examples of induced genes by injured brain cells (Hopkins, S.J., 1995). IL-6, IL-10 and IL-1β expression is massively upregulated after ischemic stroke. *In vivo* overexpression of the IL-1β receptor antagonist IL-1ra decrease lesion volume after tMCAO indicating a crucial role for this proinflammatory response (Yang, G.Y., 1999, Waje-Andreassen, U., 2005). IL-1β is closely involved with expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), E- and P-selectins (Lindsberg, P.J., 1990, Gong, C., 1998) after ischemia. Peak expression is evident between 6 and 12h after onset of stroke (Wang, X.K., Feuerstein, G.Z., 1995). Genomic deletion of ICAM-1 *in vivo* showed reduced infarct volume after tMCAO, proposing that activation of ICAM-1 by IL-1β plays an important role in IL-1β-induced injury (Soriano, S.G., 1996). Recanalisation of occluded brain vessels ameliorates brain damage but on the other hand leads to a secondary reperfusion injury (Hossmann, K.A., 2006, Simi, A. 2007).

The inflammatory response is more severe due to participation of blood-borne or peripheral immune cells. High expression of adhesion molecules predestined invasion of non-brain immune cells by interaction with their complementary cell surface receptors. Neutrophils followed by macrophages and monocytes adhere and cross the endovascular wall to enter the brain paryenchnyma. Chemokines such as monocyte chemoattractant protein 1, which is produced by damaged brain cells and is pivotal for microglias activation, becomes one of the main chemoattractant mediators for peripheral immune cells (Chen, Y., 2003). Macrophages and monocytes migrate to the lesion side and fortify immune response. They become the predominant immune cell type 5-7 days after stroke (Iadecola, C., 1997). Microglias activation
is evident already after 30 min of ischemia. The number of activated microglia progressively increases with time (Rupalla, K. 1998). Microglia might act and maintain following inflammatory cascade by an early-response and is a focus of stroke research for therapeutically intervention (Polazzi, E., 2010).

1.3 Progression of the ischemic insult

The progression can be characterized by three phases after onset of stroke. Once, the cerebral blood flow is interrupted by occlusion orrupturing of a vessel, lack of nutrients and oxygen induces ischemic cascades and initiates the acute phase within minutes to hours. As a result, tissue ph shifts towards to a homeostatic imbalance. Chain-linking deleterious ischemic mechanisms occur such as glutamate release or increase of intracellular calcium, which cause excito- and intoxicity. Water accumulation in the interstitial space provokes cell swelling and tissue edema and can elevate the ICP. Cell damage is exacerbated via ROS production by mitochondria dysfunction, as well as misfolding of proteins by ER stress. Influx of high concentration of calcium triggers activation of endonucleotidases, phospholipases and proteinases, which induces decomposition of membrane integrity, DNA degradation or protein malfunction. After few hours to a few days of ischemia, the second subacute phase develops and is characterized by an apoptotic and neuroinflammatory response as a consequence of the stimulatory induction of the acute phase (Hossmann K.-A., 2006, Dirnagl, U., 1999, Barone, F.C., 1999). Both, glutamate excitotoxicity and calcium neurotoxicity subsequently spread beyond the ischemic core region formed in the acute phase to the surrounded area and impair normal physiological homeostatic conditions leading to apoptosis, inflammatory reactions and overactivation of proteolytic enzyme systems. These processes are accompanied by deprived cerebral blood flow and allow the demarcation of a described pathological area, namely the penumbra. The last phase or the chronic phase is defined by a complete lesion and tissue repair and regeneration (Chu, L.S., 2006) (Fig. 4).
Progression of the structural lesion accompanied by decline of functional areas. Progression of stroke can be subclassified into an acute, subacute and chronic phase. Impairment of function is mostly prominent after minutes within the acute phase but can persist or even increase due to structural lesion grow in the subacute phase. Early in the course of stroke, clinical symptoms mostly reflect an impairment of function. Some functions recover either spontaneously, or because of therapy (modified from Dirnagl, U., 1999).

1.3.1 Concept of the peri-infarkt region, the penumbra

The conception of penumbra rose in 1977, in which Astrup and colleagues (Astrup, J. 1977) demonstrated that after onset of stroke in non-human primate, brain electric activity in some regions are disturbed. After return of normal blood flow, these regions restored and were able to maintain temporary action potentials. Astrup and colleagues named the refunctional brain area “penumbra”, a terminology from the astronomy indicating half-light and half-shadow. The original definition of the ischemic penumbra describes damaged but not yet dead brain areas around the unsalvageable, non-reperfused and dead ischemic core. Since the last decades, stroke research enlightened physiological parameters and contributed to the better understanding of penumbral tissue after ischemia. The latter contains mapping of cerebral blood flow, the quantification of oxygen and glucose rate and elucidation of the metabolic threshold (Baron, J.C., 2001). Based on these data, focus was shifted to the intricate cellular mechanism of neuronal cell death. Exitotoxicity, oxidative stress, inflammatory as well as apoptotic-like mediators were depicted. Thus, penumbral science covered an active field of molecular biology, which gathered multiple targets for neuroprotective therapeutic intervention, only with one goal: to rescue neurons in the tissue at-risk. It is possible in our days to image the penumbra region after stroke. Positron emission tomography (PET)
identifies brain areas suffering from reduced blood flow, although still metabolic active. Furthermore, magnetic resonance imaging (MRI) allows visualizing ischemic tissue that succumbed cell swelling and necrosis. With both methods, we are now able to “see” the penumbra in real-time. The problem still remains. Why is only one common acceptable therapy for acute stroke existing to date? This is an interesting matter and will be challenged in the “Discussion” section.

1.4 Animal models of cerebral ischemia

Animal models of cerebral ischemia already exist for over 160 years (Fluorens, M., 1847 cited by Durukan, A., 2008). Cerebral ischemia in humans can differ referring to localization, causation, degree of insult and reversibility of injury. To guarantee the appropriate transmission and interpretation of data to human, several different surgery methods to model ischemia were developed in animals. In general one can distinguish between focal and global models. Global models are often applied to investigate cardiac arrest-induced cerebral damages. In contrast, focal models are more relevant to simulate ischemia of focal origin (Mhairi Macrae, I., 1992, Hunter, A.J., 1995).

1.4.1 Models of global ischemia

1.4.1.1 Extravascular methods

Decapitation
Decapitation leads to immediate global ischemia. The head is stored at room temperature (37 degrees) for a defined period and can biochemically, pathophysiological and histologically analysed afterwards (Ginsberg, M.D., 1989).

Cardiac arrest
Heart beat can be chemically stopped, thus ischemia is evident in the whole body. After a predetermined time, the animal is reanimated. This model of global ischemia in animals evokes similar cerebral damage as can be observed in humans with cardiac arrest (Mhairi Macrae, I., 1992).
1.4.1.2 Intravascular methods

Model of 2-vessel occlusion

The model of 2-vessel occlusion is induced by bilateral common carotid artery occlusion, which is accompanied with systemic hypotension. Hereby, cerebral blood flow is significantly reduced in the forebrain (<5% of control in the cortex) (Kagstrom, E. 1983b). Oscillated changes between both the ischemia and reperfusion are almost immediate. This model of forebrain ischemia was designed to study characterized cerebral energy state following incomplete ischemia, especially in selective vulnerable brain regions such as the hippocampus, caudoputamen and neocortex (Eklof, B. 1972a, Smith, M.L., 1984b).

Model of 4-vessel occlusion

The model of 4-vessel occlusion is technically more difficult to perform. In contrast to the 2-vessel occlusion model, atraumatic claps are placed loosely around both common carotid arteries following electrocauterisation of the vertebral arteries, forebrain ischemia can be induced by occlusion of the common carotid arteries by tightening the claps, while the animal is awakened. Greater deviation in lesion size and mortality rate is a reason why the 2-vessel occlusion model is more commonly used. Pathohistological changes between these two models are comparable (Pulsinelli, W.A. 1988; Ginsberg, M.D. 1989, Smith M.L. 1984b).

1.4.2 Models of focal ischemia

Vessel occlusion by photochemistry

Vessel occlusion by photochemistry is mediated by intravenous injection of photosensitizing dyes such as rose bengal. The location of the desired infarct is craniotomied with the Dura mater intact. Vessel is occluded by expose to an argon dye laser beam. Hereby, rose bengal becomes activated, which leads to intraval aggregation of thrombocytes and in consequence in thrombotic formation. This model requires only a small craniotomy, and the Dura mater remains intact. The procedure exhibits consistent infarction in the frontoparietal neocortex. There are no thermal damages observed around the affected tissue (Watson, B.D. 1997, Hunter, A. J. 1995).

Vessel occlusion by embolisation

The model of vessel occlusion via embolization is based on the artificial formation of a thrombus within the cerebral arteries. Injection of coagulation factors such as thrombin
together with artificial macrospheres (300-400 µm diameters) lead to thrombus formation at the desired location, however there is the risk to cause undesirable multiple thrombi in other vessels. (Rosenblum, W.I., 1977, Orset, C., 2007).

Model of intraluminal occlusion
Developed by Tamura, A. in 1981, the occlusion of the MCA is achieved by subtemporal entrance with extraction of the Os Zygomaticus in rats. A less invasive modification was introduced by Koizumi, K in 1986 and was later modified from Longa, E.Z, 1989. This model became one of the most used models for focal ischemia and it is known as the intraluminal thread model to occlude the A. cerebri media. This model was adopted from Hara, H.in 1996 for mice. The intraluminal occlusion model transiently blocks the cerebral blood flow (CBF) in the MCA. In most cases, a silicon round-shape suture is introduced via extracranial arteries to the the origin of the MCA and remains for a defined period depending on species, age, weight etc. (Carmichael, S.T. 2005). Thread release induces recanalization and reperfusion of brain tissue within the blood supplying vascular territory of the MCA. This model mimics important apcets of human stroke realistically (Xavier, A.R., 2003). Moreover, the transient intraluminal thread model is predestinated to investigate the progress of ischemia including deprivation of nutrients and oxygen with following blood reperfusion. Thus, this model is closest to the human stroke pathology (Lo, E., 2008). CT and MRI determine ischemic degree of tissue damage between delineated penumbrial and core region in rodents and human congruently (Fabricius, M., 2006). Additionally, the MCAO model allows investigating pathophysiology of the penumbra region on the cellular and molecular level.

1.5 Role of reproductive female steroid hormones in stroke
The gonadal steroid hormones 17b-estradiol (E) and progesterone (P) have regulatory reproductive functions, but are also pivotal in stabilization of the bone mineral density and play a crucial role in prevention and protection against cardiovascular diseases (Brann, D.W., 1995, Teede, H.J., 2007, Turner, R.T., 1994). The risk for cardiovascular events increases in women after cessation of the final menstrual cycle. According to that, neurodegenerative disorders appear in the first instance more often in postmenopausal women than in premenopausal women (Wyller, T.B., 1999, Appelros, P., 2009). Epidemiological studies of the last few decades indicated a difference between the vulnerability to stroke in same-aged women and men. In general more men suffer from stroke than age-matched women
(Merchenthaler, I, 2003, Kipp, M., 2009). Thus, gender-specific characteristics in fertile women were thought to play an important role in the incidence of stroke as well as in its outcome. In 1998, Alkayed and colleagues reported that female rats showed better outcome than age-matched male or ovariectomized rats after tMCAO. Conclusively, clinical pictures and current research results indicate that endogeneous hormonal mechanisms play a tremendous role in this gender-specific aspect.

1.5.1 17ß-Estradiol

Studies in the last decades focused on the regulation of hormonal events in the CNS by E, which is one of the three endogenous forms of estrogens. Modulation of synaptic plasticity, cognition, memory, fine motor skills, neuroprotection and antioxidatv properties are regulatory mechanism of E (McEwen, B. 2002, Wise, P.M., 2000, Roof, R.L., Hall, E.D., 2000). Estrogen receptors are widely expressed throughout the brain with no restriction to a specific cell type (Kuiiper, G.G., 1997, Quadros, P.S., 2007).

Two classical nuclear estrogen receptors (ER) were found in glia and neurons (Chaban, V.V. 2004, Lebesgue, D., 2009, Wilson, M.E., 2009). ER-alpha (ERa) and ER-beta (ERb) are found around the cell nucleus in various cell types of the brain (Ronnekleiv, O.K., 2007). These receptors interact with the estrogen response elements (EREs) in order to modulate gene expression and cell function (Klinge, C.M., 1999, Lebesgue, D., 2009). However, the role for both receptor isoforms in neuroprotection after tMCAO seems to be delicately distributed in a cell-specific manner. Experiments using cell-specific ERb knockout mice revealed that neuronal ERb expression is critically involved in E-mediated neuroprotection (Elzer, J.G., 2010). ERb has also been described to be expressed by microglia and might assist the attenuation of neuroinflammation by suppressing microglia activation (Mor, G., 1999, Saijo, K., 2011).

ERs not only modulate gene regulation, they also mediate rapid changes in cell function. This fast mechanism of signal transduction by steroids is called membrane-initiated steroid signalling (MISS) and occurs in neurons as well as glia cells (Bondar, G., 2009). ERs are not restricted to the nucleus or perinuclear compartment, but are also found in the cytosol and can be attached to the plasma membrane (Bondar, G., 2009, Micevych, P., 2009). Specific novel membrane associated ERs (e.g. ER-X) which interact with different signalling cascades, in particular the MAPK pathway, are also discussed to facilitate neuroprotection. These ER-X can activate extracellular signal-regulated kinases (ERK1 and ERK2) by binding to 17β-estradiol, while ERa may be an inhibitory regulator of the same enzymes. However, ER-X
seems to be structurally closely related to the classical ERα (Toran-Allerand, C.D., 2002). ERα can also localize to the plasma membrane where it interacts with metabotropic glutamate receptors (mGluRs) and, thereby, activates cell signalling pathways (Bondar, G., 2009). Pawlak and colleagues (2005) showed the existence of ERα, but not ERβ, in plasma membranes of astrocytes. Treatment of astrocytes with E combined with surface biotinylation showed membrane trafficking of both ERα and mGluR1α in parallel which was followed by an increase of the intracellular calcium concentration (Bondar, G., 2009). Special membrane estrogen receptors, e.g. GPR30 are G-protein-coupled and thereby activate second messenger systems by MISS. These cell cascades imply the activation of MAPK, phospholipase C (PLC), protein kinase A (PKA) and protein kinase C (PKC) which in turn cause the phosphorylation of cAMP-responsive element binding protein (CREB) and an alteration in cell function (Singer, C.A., 1999, Wade, C.B., 2001, Ivanova, T., 2001; Pawlak, P., 2005, Ronneklev, O.K., 2007; Lebesgue, D., 2009). MISS not only takes place in astrocytes but has also been described in neurons. In isolated GABAergic and dopaminergic midbrain neurons, E could activate a signal transduction mechanism by binding to receptors in the membrane which lead to a rapid calcium release from intracellular calcium stores (Beyer, C., 1998). This signal transduction mechanism might be triggered by PLC. Altogether, estrogen is given an immensely variable role to play during neuroprotection.

1.5.2 Progesterone

Progesterone (P) has multiple functions in the CNS and regulates inflammation, neurogenesis, and regeneration. P often acts in concert with E to control non-reproductive brain functions such as cognition and neuroprotection (Kipp, M, 2009, Acs, P., 2009). ERs and progesterone receptors (PRs) are widely expressed throughout the brain with no restriction to a specific cell type (Kuiper, G.G., 1997; Quadros, P.S., 2007). P elicits its effect through PRs which similar to ERs have classically been described as nuclear transcription factors. After binding of the ligand to the native receptor and receptor homo-dimerization, the ligand-receptor complex translocates to the cell nucleus where it interacts with specific palindromic regions in the promoter region of different target genes. Besides that, P shows similarly to E other cell signaling mechanisms including interaction with membrane-associated receptors and stimulation of intracellular signaling cascades (Evans, R. M., 1988; Landers, J.P., 1992; Hurn, P.D, 2000).
Beside E, the sex hormone P came into focus for its neuroprotective property in the last decades but has not been investigated in that expanded range for pathological brain diseases such as E (Liu, R.L., 2010). Till now, it has been shown that P is a potent therapeutic agent for diverse brain injuries and diseases, including ischemic stroke (Stein, D.G., 2008; Wang, J., 2011). P improves the neurological long-term outcome after stroke (Chen, J., 1999). It can dampen inflammation (Gibson, C.L., 2005; Aggarwal, R., 2008), reduce oxidative stress (Ozacmak, V.H., 2009), decrease edema after cerebral ischemia (Gibson, C.L., 2005; Cai, W., 2008) and after traumatic injury (Roof, R.L., 1993) as well as activation of MAP-kinases and phosphoinositide-2-kinase (PI3K) (Kaur, P., 2007, Liu, R.L. 2010). A possible mechanism of P to facilitate neuroprotection is the inhibition of voltage-gated calcium channels which impedes neurotoxicity (Luoma, J.I., 2011). Furthermore, P exerts membrane stabilizing effect and can thereby prevent lipid peroxidation (Roof, R.L., 2000). Leukocytes have pro-inflammatory effects in the ischemic brain and can worsen the secondary damage in the penumbra (Wang, Q., 2007). After ischemia, P reduces the infiltration of leukocytes in the brain by inhibiting the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) (Wang, J., 2011). Further studies have to show whether this effect of P are secondary in nature.

1.6 17ß-Estradiol and progesterone action using in vivo models

In experimental models using either males or females, the administration of E and P improved the outcome after cerebral ischemia as well as after traumatic brain injury (Roof, R.L., 2000). E application before, during or after the surgery reduced the infarct volume after transient and permanent MCAO (Toung, T.J.K., 1998; Rau, S.W., 2003; Suzuki, S., 2009; Zhang, Z., 2010). E enhances post-stroke recovery and neurogenesis after tMCAO (Li, J., 2011). The anti-inflammatory effect of P has been validated after cerebral ischemia in mice (Gibson, G.L., 2005; Aggarwal, R., 2008). After permanent MCAO, P inhibits inflammatory processes by reducing the infiltration of leukocytes in the ischemic brain (Wang, J., 2011). P was tested in mice after MCAO which showed significant reduction of infarct areas as well as significant improvement of the survival rate, weight recovery and motor ability (Gibson, C.L., 2004). In the contrary to the pre-treatment paradigm with E, the pre-treatment with P did not affect the infarct area in mice after MCAO (Coomber, B., 2010). P seems to be most effective in co-treatment with E (Toung, T.J.K., 2004; Kipp, M., 2009). An up-regulation of ERa has been detected in the penumbra 23 hours after MCAO in rats. As there exist many promoters which
are able to regulate the expression of ERa mRNA (Kos, M. 2001), it is still uncertain why ERa is so highly expressed after MCAO and which mechanisms are responsible for that. So far, one can only consider a connection of the existence of ERa on astrocytes and the reduction of the proinflammatory function of astrocytes by activation of these receptors (Kipp, M. 2009). As mentioned before, neurotoxicity plays a major role in stroke. The MAP kinase pathway might, therefore, be involved by the trigger of neuroprotection by E. The neuroprotective effects of both E and P are dose-dependent (Liu et al., 2010). An overdose of E or P can abolish neuroprotective effects. The duration of infarct, exposure time of the hormones as well as age and sex of animals have also strong influence on hormonal-mediated neuroprotection in the ischemic brain (Singh, M., 2008; Carwile, E., 2009; Draca, S., 2009; Kramer, M., 2010; Strom, J.O., 2010).
2 Author’s contributions

Part of this work was published in peer-reviewed manuscripts. The Nrf2-study was accomplished in close collaboration with Dr. Christoph Jan Wruck from the Institute of Anatomy and Cell Biology of the RWTH Aachen. Name of articles and author’s contributions are mentioned below:


**Contributions:**

**Dang, J.** performance of all experiments [exception: hormone dose-dependency *in vivo* (0.25 and 2.5 µg E, 0.1 mg and 1.0 mg P); gene expression analysis of all chemokines], gene selection; primer design; manuscript preparation: references; conception of project; discussion of review.

**Mitkar, B.** gene expression of all chemokines, electrophoretical analysis of the RT-rtPCR products, morphometrical analysis of Iba-1 positive cells

**Kipp, M.** hormone dose-dependency *in vivo* (0.25 and 2.5 µg E, 0.1 mg and 1.0 mg P); conception of project: discussion of review

**Beyer, C.** conception of project; manuscript preparation; discussion of review; grant holder


**Contributions:**

**Dang, J.** performance of all *in vivo* experiments; fixation of brain tissue; cutting of paraffin-embedded brain slides; manuscript preparation; conception of project; discussion of review

**Brandenburg, L.O.** Nrf2 double-immunofluorescence study with astrocytes, microglia and neurons; manuscript preparation; conception of project

**Rosen, C.** morphometric staging of Nrf2-positive astrocytes and neurons; manuscript preparation; review discussion
Fragoulis, A.: morphometric staging of Nrf2-positive microglia; manuscript preparation; review discussion

Kipp, M.: review discussion

Pufe, T.: manuscript preparation; review discussion

Beyer, C.: manuscript preparation; review discussion

Wruck, J.C.: manuscript preparation; conception of project; evaluation of morphometric staging; review discussion; grant holder

I declare that the information above is correct,

Jon Marc Ngoc-Huy Dang

Signature_________________________________ Place, date_________________________________
3 Focus of thesis

In the last decades, stroke research discovered many potential pharmaceutical interventions being able to reduce infarct volume after experimental stroke. Candidate compounds include calcium channel blockers, glutamate antagonists and inhibitors for leukocyte adhesion. These factors were subsequently clinically tested, but failed to prove effectiveness. The negative outcome in humane stroke trials raises the criticism about the reliability of animal stroke models and the translation from “bench” to “bedside”.

There is basic evidence that 17ß-estradiol is neuroprotective after stroke in animal models. Furthermore, studies suggest, that progesterone might attenuate ischemic infarct volume. Studies were conducted so far to investigate the impact of a combined 17ß-estradiol/progesterone application on histological/functional outcome after ischemic stroke in animal models. These studies have been performed in senescent female rats and pre-treatment with both hormones before tMCAO has been elaborated.

Our first main issue was to analyse, whether 17ß-estradiol and progesterone in single or combinative application is neuroprotective in male rats and ovariectomized female rats in a survival window of 23 h after tMCAO. The effectiveness of protection was evaluated by measurement of the brain infarct volume which was determined by a viability dye, staining metabolic active brain tissue red, whereas apoptotic/necrotic tissue appears pale. Behavioural deficits were analyzed by means of senso-motoric testing paradigms to underline the effect of the steroid-induced neuroprotection on the functional level. Furthermore, to understand the protectiveness of steroids, we tried to unravel involved cellular and molecular mechanisms within the delineated penumbra. Since anti-inflammatory properties of 17ß-estradiol and progesterone are already known, we aimed to analyze whether pro-inflammatory marker genes, as well as inflammatory cells, which are typically active in the post-stroke period, are diminished in the penumbra of 17ß-estradiol/progesterone treated animals. In a parallel study, possible endogenous defence systems against oxidative stress, which is one major event during cerebral ischemia were investigated. In particular, the role of Nrf2, a major regulator against oxidative stress, and its interaction with hormones will be enlightened in my thesis.
4 Materials and methods

4.1 Materials

Materials, chemicals and buffers/solutions, which are not noted in the “Methods” part, are listed here. For all buffers and solutions: if not mentioned, *Aqua bidest* is used as diluting agent.

4.1.1 Materials for *in vivo* studies

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-0 Ethilon</td>
<td>Surgical suture</td>
<td>Ethicon, Germany</td>
</tr>
<tr>
<td>4-0 PremiCron</td>
<td>Ligation suture</td>
<td>B. Baun AG, Germany</td>
</tr>
<tr>
<td>Loctite 4011 (biological tested)</td>
<td>Adhesive probe glue</td>
<td>Henkel, Germany</td>
</tr>
<tr>
<td>Accelerator</td>
<td>Glue polymerisation</td>
<td>Bob Smith Industries, USA</td>
</tr>
<tr>
<td>ATC 1000</td>
<td>Heating plate</td>
<td>World Precision Instruments, USA</td>
</tr>
<tr>
<td>Isofluran inhalator (animals)</td>
<td>Vaporisor</td>
<td>Eickemeyer, Germany</td>
</tr>
<tr>
<td>Fluovac</td>
<td>Isofluran aspirator</td>
<td>Harvard Apparatus, USA</td>
</tr>
</tbody>
</table>

4.1.2 Materials for nucleotide analytic

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td>RNAse Away</td>
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<tr>
<td>96-well Multiply PCR-plates</td>
<td>RT-rtPCR</td>
<td>BIOplastics, Netherlands</td>
</tr>
<tr>
<td>optical adhesive film</td>
<td>RT-rtPCR</td>
<td>BIOplastics, Netherlands</td>
</tr>
<tr>
<td>Easy Ladder I</td>
<td>RT-rtPCR</td>
<td>Bioline, Germany</td>
</tr>
</tbody>
</table>

4.1.2.1 Primers

**Table 1: List of primers for gene expression analysis**

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxanthin-Guanine-Phosphoribosyltransferase (HPRT)</td>
<td>s 5’-GGTCCATTCTCCTATGACTGTAGATTTT-3’</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>as 5’-CAATCAAGACGGTCTTTTCCAGTT-3’</td>
<td></td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>s 5’-GGCAATGTGGCTGGACCAACACAC-3’</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>as 5’-TTAGAGTTGCTCCACAGTCGGAGATG-3’</td>
<td></td>
</tr>
<tr>
<td>Interleukin 6 (IL 6)</td>
<td>s 5’-ACAGTGCACTCGATGCTGC-3’</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>as 5’-CCGGAGAGAAGACTTCACAG-3’</td>
<td></td>
</tr>
<tr>
<td>Cluster of Differentiation 3 (CD 3)</td>
<td>s 5’-AGAAGTGCACTGGAGGTGGAC-3’</td>
<td>231</td>
</tr>
<tr>
<td></td>
<td>as 5’-TTTCCGGATGGGCTCAGATGTC-3’</td>
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</tr>
</tbody>
</table>
## Materials and methods

| **Vascular Endothelial Growth Factor A (VEGF-A)** | s 5’-CACATAGGAGAGATGAGCTTC-3’ | 227 |
| | as 5’-CCGCCTTGCTTGCTACAT-3’ | |
| **Nerve Growth Factor (NGF)** | s 5’-GGCATGCTGGACCCAAGCTC-3’ | 460 |
| | as 5’-GCCCTTGCTCAGCTGAGTC-3’ | |
| **Estrogen Receptor Alpha (ERα)** | s 5’-CCAAAGCCTCGGGAATGG-3’ | 73 |
| | as 5’-AGCTGCGGGAGCGAAA-3’ | |
| **Estrogen Receptor Beta (ERβ)** | s 5’-TGCTGGAGGAGGGTCAATG-3’ | 81 |
| | as 5’-CGAGGTCGAGGAGGGA-3’ | |
| **Progesteron Receptor (PR)** | s 5’-AGCTCCCAGACGAAAAGACA-3’ | 191 |
| | as 5’-GAGGGCTGGTGTTTTGCTT-3’ | |
| **Chemokine (C-C motif) ligand 2 (CCL2)** | s 5’-CACATAGGAGAGATGAGCTTC-3’ | 191 |
| | as 5’-CCGCCTTGCTTGCTACAT-3’ | |
| **Chemokine (C-C motif) ligand 3 (CCL3)** | s 5’-CTGTTACCTGCTCAGCA-3’ | 187 |
| | as 5’-AAAAGGCTGGCTGTCGCTA-3’ | |
| **Chemokine (C-C motif) ligand 5 (CCL5)** | s 5’-TGCCCACGTGAAGGATTTTA-3’ | 80 |
| | as 5’-TGGCGGTCTTTCGATGCA-3’ | |
| **Cluster of Differentiation 68 (CD68)** | s 5’-TGCCCACGTGAAGGATTTTA-3’ | 232 |
| | as 5’-TGGCGGTCTTTCGATGCA-3’ | |
| **NAD(P)H: quinone oxidoreductase (NQO1)** | s 5’-GCCCGATATTGTAGCTGAA-3’ | 201 |
| | as 5’-GTGGGTGATGCAAGCGAGT-3’ | |
| **Heme oxygenase (decycling) 1 (Hamox1)** | s 5’-CACGCATATACCCGCTAC-3’ | 226 |
| | as 5’-AAGGCGGTCTTAGCCTC-3’ | |

### 4.1.3 Materials for protein analytic

| Whatman paper | Western blot | Whatman, Brentford, UK |
| Hypercassette™ | Western blot | Amersham Biosciences, Switzerland |
| Tubes for homogenisation | ELISA (tissue preparation) | Precellys, peqlab, Germany |
4.1.4 Chemicals and solutions

Ammoniumpersulfat       Merck KGaA, Germany
DNAse/RNAse free destilled H₂O       Gibco, Germany
Ethanol, pro Analysis       Merck KGaA, Germany
Glycin       Roth, Germany
Methanol       Roth, Germany
Milk powder       Roth, Germany
Phosphate buffered saline (PBS)       BiochromeAG, Germany
Sodium chloride, liquid (physiological 0.9 % NaCl)       B. Braun AG, Germany
Sodium chloride, solid (NaCl)       Roth, Germany
Sodiumdodecylsulfat (SDS)       Roth, Germany
Tris(hydroxymethyl)-aminomethan (Tris)       Roth, Germany
Tris-HCl       Roth, Germany
Tris-OH       Roth, Germany
Triton X       Roth, Germany
Tween 20       Roth, Germany
Xylol       Roth, Germany

4.1.5 Buffers and solutions

4.1.5.1 Buffers and solutions for nucleotid analytic

RNA isolation

for 100 ml of 50 % (v/v) EtOH (washing solution)       EtOH, p.A. 50 ml
DNAse/RNAse free destilled H₂O 50 ml

for 100 ml of 70 % (v/v) EtOH (washing solution)       EtOH, p.A. 70 ml
DNAse/RNAse free destilled H₂O 30 ml

4.1.5.2 Buffers and solutions for protein analytic

Western Blot

4 x Stacking buffer (1.5 M Tris-Base, 0.4% SDS, pH 8.8)       Tris-Base 36.94 g
Aqua bidest       ad 200 ml
SDS       0.8 g
4 x Running buffer
(0.5 M Tris-Base, 0.4% SDS, pH 6.8)

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<tr>
<td>Tris-Base</td>
<td>18.91 g</td>
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<tr>
<td>SDS</td>
<td>ad 200 ml</td>
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<tr>
<td>Aqua bidest</td>
<td>0.8 g</td>
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10 % APS

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<td>Ammoniumpersulfat</td>
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Blotting Buffer

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<td>Glycin</td>
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<td>Methanol</td>
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10 x TBST

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<td>Aqua bidest</td>
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5 % Blocking Buffer

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<td>1 x TBST</td>
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<td>Milk powder</td>
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Immunhistochemistry

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<td>1 x PBS</td>
<td>9.55 g</td>
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<table>
<thead>
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</tr>
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<tbody>
<tr>
<td>1 x PBST</td>
<td>1000 ml</td>
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<tr>
<td>Tween 20</td>
<td>500 µl</td>
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Citrate buffer (pH 6.0)

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<tr>
<td>Citrate acid</td>
<td>2.1 g</td>
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<tr>
<td>Tween 20</td>
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Double-immunofluorescence

<table>
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<tr>
<td>10 x Tris-HCl (pH 7.6)</td>
<td>61 g</td>
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<tr>
<td>Aqua bidest</td>
<td>1000 ml</td>
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4.2 Methods

4.2.1 Induction of focal ischemia using the tMCAO model

Experiments included albino Wistar rat strain, belonging to the species *Rattus norvegicus*, which were purchased from Charles River (Sulzfeld, Germany). Male rats were 12-14 weeks old and weighted 380-410 g, ovariectomized female rats were at the same age, but weighted
270-290 g. Ovariectomy was performed by the animal provider approximately 2–3 weeks before tMCAO. The animals underwent routine cage maintenance and were held in a pathogen-free and climate-controlled environment with access to water and food *ad libitum*. Animal conditions were according to the rules of ‘Care of Animal Subjects’ (North Rhine-Westphalia, Germany). Research and animal care procedures were approved by the Review Board for the Care of Animal Subjects of the district government (North Rhine-Westphalia, Germany). This study was performed with 70 animals (58 males and 12 females). 12 animals died (9 males and 3 females) after tMCAO surgery.

For surgery, narcosis of the animals was initiated by free-flow inhalation of 5% isoflurane (Abbott, Ludwigshafen, Germany) through a face mask for 2-3 min and maintained at 2-2.5%. Surplus of isoflurane was aspirated by a tube-in-tube system connected to the face mask. Core body temperature was stabilized during operation at 37 ± 0.5 °C using a heating tablet and a lamp. By the use of Laser-Doppler flowmetry (Moor Instruments VMS-LDF2, Axminster, UK) cerebral blood flow was monitored on the ipsi- and contralateral side for 1 h.

Laser-Doppler sensors were fixed 1-2 mm posterior and 4-5 mm lateral to the bregma on the right and on the left skull hemisphere depending on the highest blood perfusion units (BPU). Skull thickness was ablated on the coordinates to assure that the BPU are digitized from the cortical vascular territory of the middle cerebral artery and not from the external carotid artery (ECA) blood flow before placing the sensors. Transient MCAO was induced with minor changes as described previously (Herrmann, O. 2005; Kramer, M., 2010). The left common, the internal and the external carotid artery (CCA, ICA, and ECA) were exposed through a small midline skin incision in the neck region. To ensure that the BPU digitized the cortical vascular territory of the MCA, the common carotid artery was clipped temporary resulting in a decrease of BPU. The ECA was clipped transiently during the whole operation and the proximal CCA was ligated permanently. The vagus nerve was carefully preserved. Insertion of a commercially available catheter (Asahi PTCA Guide Wire Soft, Abbott Vascular) was performed into the ICA (Fig. 5). To avoid protrusion of the *A pterygopalatina*, the head was bended aside and the catheter was introduced from the lumen of the distal CCA at the bifurcation to the cranial part of the ICA until an immediate drop of cerebral blood flow (CBF) was observed. Only those animals were included which showed a reduction of regional CBF by >50% compared to baseline before tMCAO.
4.2.2 Hormone treatment and analysis

For hormone treatment, rats subjected to tMCAO received application of vehicle, E, P or E/P in a randomized manner. Hormones were dissolved initially in ethanol as a solvent and were further diluted in sesame oil to achieve the final concentration. Following doses were routinely used: E (25 µg/kg body weight) and P (10 mg/kg body weight). Vehicle treated animals received only sesame oil + ethanol in concentrations related to the body weight. For studying dose-dependency, the following concentrations were applied: E (2.5 µg/kg and 0.25 µg/kg body weight) and P (1 mg/kg and 0.1 mg/kg body weight). All steroid hormones were purchased from Sigma–Aldrich, Munich. Applications were performed subcutaneously as a neck depot (500 µl) direct after the end of surgery and 12 h later. The application time points and the concentration of hormones were determined empirically in preliminary dose-finding studies to yield stable plasma hormone levels corresponding to typical female estrous hormone levels already after 4 h of application and throughout the 23 h recovery period (data not shown, studies performed by Dr Kipp, Institute of Neuroanatomy, RWTH Aachen). In the final stage of 23 h survival period, blood samples were collected by cardiac puncture. Control animals (i.e. sham-operated; the catheter was not inserted in the ICA) underwent the same experimental protocol and receive the same vehicle volume without hormones. Individual steroid hormone plasma levels were analyzed by Dialog Service GmbH (Frankfurt am Main, Germany). Total plasma hormone titer of E and P were evaluated by using a standardized electrochemical luminescence technique and commercially available kits (Roche Diagnostic GmbH, Mannheim, Germany with detection limits of 5.0 pg/ml for E and 30 pg/ml for P).
4.2.3 Tissue preparation

Twenty three hours after MCAO, all animals were killed by deep narcotization with 5% isoflurane and a subsequent ntraperitoneal injection with an overdose of pentobarbital (330 mg/kg). Following transcardial perfusion with physiological sodium chloride, brains were dissected out of the skull rapidly and were placed into a rat brain matrix (Alto Brain Matrix stainless steel, 1 mm rat coronal 300–600 g; Harvard Apparatus, Holliston, MA, USA). Brains were subsequently cut into 2 mm coronal slices and were incubated in 2% (w/v) 2,3,5-Triphenyl-tetrazolium chloride solution (TTC, Fluka, Germany) for 20 min at 37°C. TTC is a marker for metabolic function and represents a reliable indicator of ischemic areas for up to 3 days after ischemia (Benedek, A., 2006; Kramer, M.., 2010; Lin, T.-N., 1993) Metabolic active tissue is typically stained red after TTC exposure, whereas necrotic tissue remains pale. Coronal slices were arranged in a frontal to occipital order, and digital photographs of all stained slices were taken using a Canon Digital IXUS 9015 camera (Canon, Tokyo, Japan). Infarct volume was estimated in a blinded manner using the freeware programm ImageJ 1.41 (NIH, Bethesda, MD, USA). Lesion volume was calculated by summing up the infarct area of each slice and multiplied by 2 mm (thickness of the sections). Edema-induced volume was corrected using following equation (Swanson, R.A., 1990):

Infarct Volume x (contralateral Volume/ipsilateral Volume).

Infarct volume was calculated separately for the cerebral cortex (CX) and basal ganglia (BG).

Figure 6: Schematic time line of experimental set up after 1 h tMCAO with subsequent hormone substitution and tissue processing at the end. BS, blood sampling; FT, functional testing; rCBF, regional cerebral blood flow; A1, first hormone application; A2, second hormone application; Final., finalization; OP, operation; occ, occlusion.
4.2.4 Gene expression analysis

4.2.4.1 RNA isolation

Isolation of RNA was performed after TTC-staining under RNAse-free conditions. Cerebral cortical tissue was stamped out with a biopsy puncher (pfm medical ag, Cologne, Germany) using a stereomicroscope (KL 200 LED, Leica, Germany) referring to the penumbra region. Corresponding contralateral tissue was sampled as a reference (Fig. 6). Tissue processing was accomplished using RNA/protein extraction kit (NucleoSpin RNA/Protein kit, Machery-Nagel, Germany). Briefly, tissue was homogenized with 1.4 mm diameter ceramic beads (Precellys 24, Peqlab, Germany) at 5000 rpm for 15 s in lysis buffer delivered by the manufacturer. RNA was extracted with kit-provided columns. Purity was controlled using 260:280 OD ratios (NanoDrop 1000, Peqlab, Germany).

![Figure 7](image)

Figure 7: Coronal 2 mm male rat brain slices after 23 h tMCAO stained with TTC viability dye. (A) Ipsilateral lesion side (core) remains pale after staining, whereas metabolic active tissue is indicated in red. Dashed line mark the penumbral transition zone, which comprised cerebral cortex (CX) and basalganglia (BG). (B) For gene expression analysis, penumbra is punched out in the CX for the ipsilateral and contralateral side.

4.2.4.2 Reverse transcription and RT-rtPCR

Reverse transcription reactions were performed using the MMLV RT-kit and random hexanucleotide primers (all from Invitrogen, Germany) and standardized protocols (Appendix). Real-time rtPCRs (RT-rtPCRs) were carried out in a mixture consisting of 2 µl cDNA, 2 µl RNAse-free water (Invitrogen, Germany), 5 µl 2xSensi Mix x Plus SYBR & Fluorescein (Quantace, San Francisco, USA), and 0.5 µl primers (10 pmol/µl). Reactions were conducted in standard tubes using the MyIQ single colour real-time detection system (Bio-Rad, Germany) under following conditions: 10 min enzyme activation at 95 °C, 40 cycles of 15 s denaturation at 95 °C, 30 s annealing at individual temperatures, 30 s
amplification at 72 °C, and 5 s fluorescence measurement at 72 °C. External standard curves were generated by several fold dilutions of the target genes. The concentration of the target genes was calculated by comparing Ct values in each sample with Ct values of the internal standard curve as described previously (Acs, P. 2009; Kramer, M., 2010). Values were normalized using mean expression values of two housekeeping genes (hypoxanthin-guanine-phosphoribosyltransferase) HPRT and cyclophilin A. All PCR reactions were performed in duplicate. Melting curve and electrophoretical analysis of the RT-rtPCR products (10 µl) plus ethidium bromide staining were routinely performed to specificity RT-rtPCR. For illustration, expression values from the ipsilateral side of vehicle-treated MCAO animals were set to 100% and the corresponding data from hormone groups were always shown as percentage of this normalized value. The contralateral side of the hormone groups were included in the statistical analysis, but not shown in the graphs. A list of all primers (Invitrogen, Germany) designed in the free-access internet program “Primer 3” is given in 3.1.2.1.

4.2.5 Analysis of protein expression

4.2.5.1 Western blotting

For Western blotting, proteins were purified with the same extraction kit used for mRNA isolation (NucleoSpin RNA/Protein, Machery-Nagel, Düren, Germany). Briefly, after homogenization of tissue samples solubilised in lysis buffer and RNA extracted by kit-provided RNA columns, flow-through was collected and proteins precipitated followed by the manufacture recommendation with one change in the protocol working flow for protein extraction. After precipitation proteins were washed with 95% ethanol instead of 50% to remove TTC staining residuals (Kramer, M, 2010). Proteins were denaturized with reducing agents and Protein solving buffer and subsequently incubated for 30 min with Quantification Reagent (all constituents of the kit). After incubation, light extinction was measured photometrically at 570 nm. Light extinction is caused by turbidity appearing after the addition of Quantification Reagent. The protein concentration is determined in reference to a Bovine Serum Albumin calibration curve.

A total amount of 40µg proteins were applied and separated by SDS-PAGE using 14 % running gel, blotted onto nitrocellulose membranes (Hybond, Amersham) for 30 min at 10 V (Trans-Blot SD, Semi-Dry Transfer Cell, Bio-Rad, Germany), and incubated with primary antibodies diluted in 5% milk TBST over night. Unspecific binding was prevented by blocking with 5% milk in TBST for 30 min. Blots were incubated with primary antibodies
diluted in 5% milk TBST overnight. Primary antibodies were: anti-β-actin, Sigma, USA, 1:1000, 42 kDa; anti-CD3, Abcam, Cambridge, UK, 1:100; 16 kDa, anti-IL-6, Abcam, Cambridge, UK 1:100, 22 kDa. After washing and incubating with horseradish peroxidase (HRP) conjugated secondary antibodies (anti-rabbit-HRP, BioRad, Germany, or anti-mouse-HRP, Abcam, both 1:5000), labelled proteins were visualized with the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Amersham, UK). Band intensities were determined with Alpha-EaseFC™ software V 4.0.0 (Alpha Innotech, CA, USA) on autoradiography films (Eastman Kodak, Rochester, NY, USA). Quantitative analysis of Western blots was densitometrically accomplished with using ImageJ 1.41 (NIH, Bethesda, MD, USA).

4.2.5.2 Enzyme-linked immunosorbent assay (ELISA)

For ELISA measurements, penumbrial tissue samples were homogenized by heavily shaking with 1.4mm diameter ceramic beads (Precellys, peqlab, Erlangen, Germany) at 5000 rpm for 15 s using a homogeniser (Precellys® 24, peqlab, Erlangen, Germany) in 1 ml PBS (pH 7.2) + several protease inhibitors (complete Mini, Roche Diagnostics GmbH, Mannheim, Germany). Whole lysate were taken for protein quantification (Pierce BCA Protein Assay Kit, Thermo SCIENTIFIC, US) and ELISA analysis. Turbidity of TTC-staining did not disturb the quantification procedure. Protein levels were normalized to entire protein content determined by BCA assay (Pierce, USA). ELISA (PeproTech GmbH, Hamburg, Germany) measurements were performed by quantifying the absorbance at 450 nm with corrections for 540 nm using a microplate reader (infinite 500M, Tecan, Germany). For quantification, standard concentrations of the corresponding proteins were routinely included.

4.2.6 Immunohistochemistry

Rats were narcotized as mentioned above. Before exitus letalis, thorax was opened and rats were first transcardially perfused with physiological sodium chloride and afterwards with fixative solution, which contained 2% (w/v) paraformaldehyde (Roth, Germany) with 15% (v/v) saturated picric acid at pH 7.4 (AppliedChem GmbH, Germany). Whole caput was post-fixed in the same fixative solution overnight. On the following day brains were dissected out of the skull and were embedded in paraffin (Merck KGaA, Germany) using standardized protocols (Appendix). 5 µm coronal slices were cut (Jung Supercut 2065, Leica, Germany) and deparaffinised 10 min in Xylol. Rehydration of the slices was achieved by decreasing
alcohol steps from 100% for 3 min, 1 min in 95% and 70%. Finally, slices were kept in deionized water for 3 min, subsequently antigen retrieval was performed by boiling with citrate buffer for 20 min. After several washing steps for 5 min in PBST, slices were blocked 1 h in serum (depending on source of primary antibody, blocking solution was chosen in a species-specific manner, Vector Laboratories, USA). Primary antibody (anti-NeuN (1:500, Abcam, Cambridge, UK), anti-ERα (1:250, Abcam, Cambridge, UK), and anti-ionized calcium-binding adaptor molecule 1 (Iba-1, 1:250, Wako, Osaka, Japan) were diluted in blocking serum and exposed to the slices overnight. After several washing steps with PBST, enzymatic activity of endogenous peroxidases was inhibited by 3% hydrogen peroxide (Roth, Germany) diluted in methanol for 30 min, following washing with PBST three times. Biotin-conjugated secondary antibody (depending on primary antibody, biotinylated anti-mouse- or anti-rabbit was used, all 1:100, Vector Laboratories, USA) was diluted in blocking serum and incubated for 1 h with 5 min PBS washing step afterwards. Following incubation with a solution of biotin-avidin-peroxidase complexes for 1 h, antibody binding was visualized by substrate exposure (AEC Substrate kit, Invitrogen, Camarillo, USA).

4.2.7 Double-immunofluorescence

For double-immunofluorescence, 5 µm coronal paraffin-embedded rat brain slices were incubated with 0.1 % Triton X in PBS for 10 min room temperature and antigen retrieval using microwave, sections were blocked in Tris-HCl containing 1.5 % bovine serum albumin (BSA; Sigma Chemical Company, Germany) for 10 min. Slices were incubated at 4 °C overnight with a pair of primary antibodies diluted in TRIS containing 1.5 % BSA. The following primary antibodies were used for this study: rabbit polyclonal anti-Nrf2 antibody (abcam, ab31163, Cambridge, UK), 1:50, mouse monoclonal anti-SMI32 antibody (abcam, ab73273, Cambridge, UK), 1:1000, mouse monoclonal anti-GFAP antibody (abcam, ab10062 Cambridge, UK), 1:250, and rabbit polyclonal anti-Iba1 antibody, 1:100, Wako, Japan). Finally, the slices were incubated with donkey anti-rabbit AlexaFluor 488 (Molecular Probes, USA), goat anti mouse Cy3 (Sigma, Germany) or Donkey anti-goat AlexaFluor 555 (all 1:250; Molecular Probes, USA) for 1 h at room temperature. Nuclear staining was performed with bisbenzimide (Sigma, Germany). Cells were digitally photographed using a Zeiss Axio Z1 Imager microscope (Zeiss, Göttingen, Germany).
4.2.8  Morphometric quantification

4.2.8.1  Immunhistochemistry

Quantification of cell numbers was performed by manual counting of Iba-1-positive cells in a blinded manner. Cells were judged positive when the cell nucleus was clearly visible. Three different phenotypes of Iba-1-positive cells according to the number of branches were quantified following the given criteria: Appearing round or egg-shaped, microglia was determined amoeboid-like. Microglia with 1-2 branches were grouped separately. Finally Iba-1-positive cells with more than 2 branches were also included and counted. For the quantification and ramification study 3 animals were subjected without hormone, whereas 2 animals underwent hormone application. For each animal independent from treatment 6 consecutive slices between the *comissura anterior pars anterior* and the *comissura anterior* were analysed. Penumbra area was determined by NeuN-staining and was carried over to the next corresponding slice for IBA-1 staining (Fig.7). Cell numbers were calculated and expressed per mm$^2$. Cell counting was performed with a Nikon ECLIPSE 55i microscope and a 20 x objective.

![Image](image.png)

**Figure 8:** NeuN-stained coronal 5 µm brain slice of a vehicle-treated male rat after 23 h tMCAO.

(1) Staining allows delineation of the penumbra region. Morphological changes define the difference of vital and dying/dead neurons. (2) Dystrophic cells (↑) and parenchymal vacuoles (*) in the core region are presented.

4.2.8.2  Double-immunofluorescence

The stained slides from three tMCAO rats, three sham operated control rats and three control rats, without surgery, were independently scored in a double-blind fashion. The Nrf2 immunostaining was evaluated by means of a four point scale: 0, no expression; 1, low expression; 2, moderate expression; 3, high expression. The semiquantitative basis for the
scoring was based on the apparent intensity of the labeling of specific cells and not the percentage of the section surface, which was positively labeled. Two observers independently reviewed and compared the stained slides. Using this approach inter-observer variation in histomorphometric scoring was minimal and the majority of scores were identical. In cases where variation was found, the difference was never more than one unit.

4.2.9 Behavioral testing

We assessed post-ischemic neurological deficits using motor and sensory behavioral tests with minor modifications as described (Garcia, J.H., 1995). Testing was performed prior sacrificing by two blinded readers. The following six tests were performed. Spontaneous activity was analyzed for 3 min within an unfamiliar environment by placing the animals in the middle of a 35 cm x 55 cm sized cage (scores: 3 = rat moving around, exploring the environment, and approaching at least three walls of the cage; 2 = slightly affected rat moving around in the cage but not approaching the walls and hesitating to move further, nonetheless eventually rising to at least one upper rim of the cage; 1 = severely affected rat not rising up at all and barely moving in the cage; 0 = rat not moving). For testing forepaw outstretching, rats were fixed at the tail, and the symmetry of the outstretching of both forelimbs was evaluated (scores: 3 = both forelimbs outstretched symmetrically; 2 = right side moves and outstretches less than left side; 1 = right side moves slightly; and 0 = right forelimb not moving). To assess ability to climb, rats were placed on the wall of a wire cage. Normally, rats use all four limbs to climb the wall (scores: 3 = rat climbing easily and gripping the wire tightly; 2 = right side impaired when climbing or not gripping as tightly as the left side; and 1 = rat failing to climb or tending to circle instead of climbing). To test body proprioception, rats were touched with a blunt stick on each side of the body, and the reaction to the stimulus was evaluated (scores: 3 = rat reacts by turning head and being equally startled by the stimulus on both sides; 2 = rat reacts slowly to stimulus on right side; and 1 = rat not responding to stimulus placed on the right side). Spontaneous walking activity was staged (scores: 3 = rat walking straight ahead; 2 = right circling; 1 = rat tending to walk toward the right side; and 0 = rat not moving). Sensory function was tested by brushing the vibrissae (scores: 3 = rat turns head to the stimulus side; 2 = rat reacts slowly to stimulus on right side; and 1 = rat did not respond to stimulus on right side). Individual scores of all tests were summed. An overall minimum score of 3 and maximum score of 18 points was achievable.
4.2.10 Statistics

All data are given as arithmetic means ± SD/SEM (specified in the figure legends). If not otherwise stated, multiple comparisons (infarct size, brain edema, body weight, neurological deficit, gene expression, microglia number and morphology, protein values) were evaluated by one way ANOVA (analysis of variance) and further evaluated by Tukey post-hoc test or an independent sample t-test subsequently calculated by non-parametric Mann–Whitney U-test with SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA and Graph Prism). The criterion for statistical significance was set at P < 0.05.

Evaluation of double-immunofluorescence Nrf2-positive cells was morphometrical staged in a two blinded manner. No statistics were performed.
5 Results

5.1 Effect of single and combined hormone treatment after MCAO

Laser-Doppler flowmetry was monitored for each animal to ensure reduction of rCBF in the vascular territory of the \textit{A. cerebri media}. Occlusion resulted in abrupt decrease of rCBF more than 50 % of rCBF baseline levels (Fig. 9). Baseline levels were determined 5 min prior MCA blockage by mean of BPU for each 30 seconds. Sham-operated animals showed no change of rCBF (not shown). 58 male rats were included in the study and were grouped as followed: 15 animals after tMCAO and 3 sham-operated animals received vehicle injection, 10 animals were subjected to E, 8 animals received P and 22 animals were treated with E and P.

![Figure 9: Representative Laser-Doppler measurement (tMCAO n = 13, E n = 7, P n = 6, E/P = 17). BPU were monitored for 60 min after occlusion of the MCA. Decrease of BPU was in every animal more than 50 %. Animals, which did not surpass the >50 % threshold of cerebral baseline blood flow are not included within this graph. E =17ß-estradiol; P = progesterone; BPU = blood perfusion units.](image)

5.1.1 Assessment of neurological deficits by functional tests

The extent of neurological deficits was determined using behavioural evaluation scale, which scored sensoric and motoric qualities regarding to Garcia, J.H., 1995, with minor modifications. Functional testing revealed that all hormones and also the combinative application of both improved scoring rate (described in 3.2.9) after 23 h tMCAO (Fig. 10A). tMCAO-induced decrease of maximum scoring from 18 points (sham-operated without tMCAO or hormones) to ~7.5 points (tMCAO) was improved by the application of steroids with scoring rates to ~10.5 points (E), ~12.5 points (P), and ~11.9 points (E/P) in male rats.
The individual scoring performance for the six different tests is shown for males in Fig. 10B. Spontaneous activity, walking, and forepaw outstretching displayed best recovery rates, whereas sensory tasks showed only moderate or no recovery.

Figure 10: Functional testing of male rats 23 h after tMCAO and hormone substitution (E = 25 µg/kg body weight/P = 10 mg/kg body weight). A total of six tests were performed resulting in a maximum of 18 score units in sham-operated animals (no tMCAO, no hormone). Panel (A) summarizes the data of all six scorings. Panel (B) shows the individual scores of the six tests in male animals. E, 17β-estradiol; n, number of tested individuals per group; P, progesterone; *P ≤ 0.05 tMCAO vs. sham; **P ≤ 0.01 tMCAO vs. tMCAO plus E, P, or E/P. Data represent means ± SEM and are given in arbitrary units.

5.1.2 Measurement of infarct volume by TTC viability staining

Brain infarct volume without and with hormone substitution are depicted in Figure 11. Lesion volume in the vehicle-treated group (tMCAO without hormone application) compromises the ipsilateral striatal and the frontal to occipital cerebral cortical region. The tissue damage
Results

precisely corresponds to the vascular territory of the MCA as indicated by the colourless tissue.

Figure 11: Hormone supplementation reduces ischemic infarct area in the cerebral cortex. Serial 2 mm thick TTC-stained brain slices are arranged from frontal to occipital lobe. Staining reveals reduction of the cerebral cortex infarct volume in all hormone groups, whereas the basalganglia region (*) seems not to be protected. Ischemic core region of the cortex (arrowhead) is indicated by unstained brain. Arrows show penumbral tissue. For infarct volume measurement brain slices are laying on squared paper. Applied hormone concentration: E = 25 µg/kg body weight; P = 10 mg/kg body weight.

Administration of steroid hormones significantly decreased the rostral to caudal cerebral cortical damage. Total lesion volume was 238 mm³ for tMCAO in male rats. Lesion volume of E-treated group was 158 mm³, P- as well as E/P-treated group showed similar outcome after 23 h tMCAO indicating lesion size for P by ~ 77 mm³ and for E/P by ~ 83.3 mm³. E diminished cerebral cortical infarct by ~37 %, P and the co-application of both hormones effectively reduced infarct volume by ~ 68 % and 65 % subjected to tMCAO (Fig. 12A). Basal ganglia showed no reduction of infarct volume in all groups (Fig. 12B).
5.1.3 Effect of gonadal steroids in the delineated penumbrial tissue

Prior investigation of putative target genes of E and P, penumbrial tissue was analysed for tMCAO-dependent inflammation. The cytokine IL6 is known to be one of the highest stroke-induced inflammatory markers (Yang, G.Y., 1999, Waje-Andreassen, U., 2005) (Fig.13).

Figure 13: Analysis of the cytokine IL6 in the penumbra. (A) tMCAO-induced expression of IL6 mRNA is elevated in the ipsilateral side. Administration of E and E/P attenuates this increase. (B) Western blots following densitometrical quantification for β-actin and IL6. Note
that no detection (n.d.) is assessed on the contralateral side. E = 17β-estradiol, P = progesterone, n = number of animals, c = contralateral, i = ipsilateral, IL6 = interleukin 6, kDa = kd; **P < 0.01 tMCAO c, E c, P c, E/P c vs. tMCAO i, E i, P i, E/P i; #P ≤ 0.05 tMCAO i vs. E/P i. Data represent means ± SEM.

The treatment with P caused no reduction, whereas E and E/P were able to diminish mRNA levels to the half of expression comparing to tMCAO-group, but only E/P shows significant effects (Fig.13A). Densitometrical quantification of Western blotting confirmed the dampening of IL6 expression under combined hormonal treatment (Fig. 13B). IL6 protein was detected only in the ipsilateral side after tMCAO and tMCAO + E/P treatment, but not in the contralateral side.

Stroke-induced upregulation of CD3, a marker for T-lymphocytes, was abolished by all hormones. The co-application of E/P showed again a significantly decrease of mRNA expression referring to tMCAO (Fig. 14A). Semi-quantitative Western blot analysis supported this observation. Similar to the IL6 Western blot, the protein expression of CD3 was only detectable on the ipsilateral but not on the contralateral side after tMCAO as well as after E/P application (Fig. 14B).

![Figure 14: Analysis of the T-cell specific marker protein CD3 in the penumbra.](image)

(A) High induction of CD3 mRNA on the ipsilateral side after tMCAO is diminished by hormonal exposure. (B) Densitometrical quantification of Western blots for β-actin and CD3. Note that no detection (n.d.) is assessed in the contralateral side as well as after tMCAO + E/P treatment. E = 17β-estradiol, P = progesterone, n = number of animals, c = contralateral, i = ipsilateral, CD = Cluster of differentiation, *P ≤ 0.05 tMCAO c vs. tMCAO i; #P ≤ 0.05 tMCAO i vs. E/P i. Data represent means ± SEM.
GFAP, an indicator for activation of astrocytes, is inhibited by all gonadal hormones. Combined hormone treatment resulted in a significant reduction of reactive astrocytes (Fig. 15B). Infiltration, attraction or/and proliferation of microglia/macrophages, shown by CD11b marker, is attenuated by E/P. However, single application of hormones resulted in enhanced CD11b mRNA expression in the delineated penumbra (Fig. 15A).

More detailed histomorphometrical analysis using well-defined tissue landmarks of the penumbra, were used to analyze the status of microglia. In tMCAO-animals treated with E/P, numbers of Iba1-positive cells are significantly lower (120 vs. 80 cells/mm²; Fig. 16Ai). Cell bodies appeared swollen, processes were enlarged and retracted, whereas E/P abolished signs of acute microglia activation, such as swollen somata and thickened processes as shown in the representative images (Fig. 16 Aiii). Quantification of morphological appearance gave more evidence for an amoboid shape (Fig. 16 Aii). A more specific marker for microglia, CD68, revealed almost no expression on the contralateral side of tMCAO animals and a 100 % induction on the ipsilateral lesion side. Treatment with E/P attenuated this increase resulting in highly significant lower ipsilateral mRNA levels compared to tMCAO alone (Fig. 16B).
Results

Figure 16: Effect of hormone treatment on the number and morphology of Iba1-positive microglia cells in the penumbra of male rats. Panel (A i, ii) shows the quantitative evaluation of cell numbers and branching pattern in (nicht on!!!!) the ipsilateral side. (A iii) Representative sections demonstrating the morphological appearance of Iba1-positive microglial cells in the cerebral cortex penumbra on the contralateral and ipsilateral side after tMCAO and E/P substitution. Panel (C) shows the expression and regulation of the activated microglia marker CD68 (ED1) mRNA in the penumbra. **P \leq 0.01 tMCAO c, E/P c vs. tMCAO i, E/P i; *P \leq 0.05 tMCAO i vs. E/P i. Data represent means ± SEM with n = 3 for morphological analysis/cell quantification and n = 5–6 for rtPCR gene expression study. E = 17ß-estradiol; P = progesterone; n = number of tested individuals per group; c = contralateral; i = ipsilateral.

Chemoattractant molecules such as chemokines play an important role for attraction and accumulation of microglia. The studied chemokine (C-C motif) ligand CCL2 (MCP-1), CCL3
Results

(MIP-1α) and CCL5 (Rantes) were already known to be induced by stroke. All chemokines were up-regulated by tMCAO. E/P inhibited the induction of CCL2 and CCL5 but further potentiated CCL3 expression (Fig. 17A–C). Reduced levels of CCL2 and CCL5 in the penumbra of hormone-treated animals were further verified on the protein level by ELISA (Fig. 17D-E).

**Figure 17:** Expression analysis of the chemokines CCL2, CCL3 and CCL5 in male rats in the penumbra of the cerebral cortex after tMCAO and steroid substitution. (A, D) CCL2 expression; (B) CCL3 expression; (C, E) CCL5 expression; c = contralateral; i = ipsilateral; CCL, chemokine ligand; E = 17ß-estradiol; n = number of tested individuals per group; *P ≤ 0.05 E c vs. E i; **P ≤ 0.01 tMCAO c, E/P c vs. tMCAO i, E/P i; #P ≤ 0.05 tMCAO i vs. E/P i.; ##P ≤ 0.01 tMCAO i vs. E/P i. Data represent means ± SEM.

In addition, tMCAO enhanced the expression of ERα and ER-β but not PR within the cortical penumbra (Fig. 18A-C). This effect was neither abolished nor reinforced by hormone treatment.

Expression of VEGF-A was up-regulated by tMCAO and further stimulated by E/P, whereas tMCAO-induced NGF mRNA expression was not affected by hormones. Protein analysis via
ELISA resulted in potentiated expression of VEGF-A in the ipsilateral side under combined hormone application.

**Figure 18:** Gene expression studies of different steroid hormone receptors in the penumbra and hormone treatment. (A) ERα expression; (B) ERβ expression; (C) PR expression. c, contralateral; i = ipsilateral, E = 17β-estradiol; P, progesterone; ER = estrogen receptor; PR = progesterone receptor; n = number of tested individuals per group; **P ≤ 0.01 tMCAO c vs. tMCAO i; *P ≤ 0.05 E/P i vs. E/P c; #P ≥ 0.01 tMCAO i vs. E/P i. Data represent means ± SEM.

**Figure 19:** Gene expression analysis of the growth factor VEGF-A and NGF. (A, C) VEGF-A expression; (B) NGF expression. c, contralateral; i = ipsilateral, E = 17β-estradiol; P, progesterone; n = number of tested individuals per group; **P ≤ 0.01 tMCAO c vs. tMCAO i; *P ≤ 0.05 tMCAO c, E/P i vs. tMCAO c, E/P c; #P ≥ 0.01 tMCAO i vs. E/P i. Data represent means ± SEM.
5.1.4 Dose-dependent effect of hormone treatment on infarct volume in male rats

Steroid plasma levels were analyzed in male rats immediately before sacrificing. Vehicle-treated animals with tMCAO but without hormone supplementation showed low E and P values (Fig. 21). Substitution with high doses of E or P or E/P (25 µg/kg E body weight and 10 mg/kg body weight P) yielded similar plasma hormone levels. The application of lower E/P doses (2.5 µg/kg, 0.25 µg/kg E body weight; 1.0 mg/kg, 0.10 mg/kg P body weight) resulted in much lower E/P plasma concentrations not differing significantly from untreated controls. Lower E/P doses revealed no or only moderate protection from MCAO in males (see Fig. 20).

Figure 20: Hormone serum values of blood samples without and with hormone treatment in male rats. Injection of steroids occurs immediately after surgery and 12 h postoperative. E = 17ß-estradiol; P = progesterone; n = number of tested animals.

Figure 21: Protection of the infarct volume with lower steroid doses. Measurement of the infarct volume measurement of animals treated with lower hormone concentrations (striped bars) are opposed to the hormone doses of 25 µg E/kg body weight and 10 mg P/kg body weight. E = 17ß-estradiol; P = progesterone; n = number of tested animals. **P < 0.01 MCAO
5.1.5 Effect of combined hormone treatment on infarct volume in ovariectomized female rats

Laser-Doppler flowmetry was monitored in ovariectomized female rats. Each animal was handled as described in section 4.1. Figure 22 shows a representative Laser-Doppler monitoring. 12 female rats were included in the study and were grouped as followed: 5 animals were subjected for tMCAO without hormones and 4 animals received tMCAO + E/P.

Figure 22: Representative Laser-Doppler measurement of ovariectomized female rats (tMCAO n = 5, E/P = 4). Occlusion of MCA results in immediate drop of BPU to more than 50 % of baseline. E =17ß-estradiol; P = progesterone; ovx = ovariectomized; BPU = blood perfusion units.

Vehicle-treated female rats yielded significantly reduced (174 mm$^3$) infarct volumes compared to vehicle-treated males (258 mm$^3$). Co-administration with high doses E/P (25 µg/kg body E weight and 10 mg/kg body weight P) resulted in similar neuroprotective effects as demonstrated earlier in male rats with a decrease of infarct volume by ~55 % (Fig. 23 A-C). Similarly, improvement in behavioural testing in the modified Garcia scoring was observed in female rats treated with E/P after tMCAO compared to tMCAO only. The total scoring from 18 points (sham-operated without tMCAO and hormones) was significantly decreased by tMCAO to approximately 7.5 points and were preserved with similar recovery rates by the steroid application with ~11 points of scoring (Fig. 23D). Endogenous hormone serum levels of ovariectomized female rats indicated higher E and P values in the vehicle-group (tMCAO without hormones) compared to male rats but exhibited also higher hormone serum levels after E/P treatment (Fig. 24). Neither single hormone treatment nor dose-dependent study was performed in ovariectomized female rats.
Results

Figure 23: Cortical infarct volume measurement and functional behavioral scoring in female ovariectomized rats. (A) Representative 2 mm thick and TTC-stained slices were arranged serially. Spread of infarcted cerebral cortex of hormone-treated animals is restricted to the lateral cerebrum cortex (B). Basal ganglia are not protected (*). The hormone-dependent reduction of lesion volume of ovariectomized female is compared with the infarct volume of male rats with tMCAO and tMCAO + E/P (C). The functional outcome between female/male and treatment is shown in D. Signs indicating core of infarct (arrow-head) and penumbra (arrow). E = 17ß-estradiol; P = progesterone; n = number of tested animals; ovx = ovariectomized; (C) *P ≤ 0.05 MCAO vs. + E/P; **P ≤ 0.01 MCAO vs. + E/P; #P ≤ MCAO male vs. MCAO female; (D) *P ≤ 0.05 Sham vs. MCAO; **P ≤ 0.01 MCAO vs. +E/P.

Figure 24: Hormone serum values of male and ovariectomized female rats with and without hormone substitution. Plasma analysis are performed with blood samples collected after 23 h
tMCAO. Application of hormones occurs immediately after surgery and 12 h postoperative with the specified hormone doses. E = 17ß-estradiol, P = progesterone; n = number of tested animals

5.1.6 Evaluation of mortality rate after tMCAO

All animals, which survived after surgery and fulfilled the paradigm of a successful occlusion of the MCA (s. 3.2.1), were include in the evaluation of mortality rate after 23 h of tMCAO. No sham-operated animals are listed in table 2 due to no occlusion of the MCA. Numbers of sham-operated animals were 3 male and 3 ovariectomized female rats.

Table 2: Overview of numbers of tested individuals per group and mortality rate after tMCAO.

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
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<th>Total n</th>
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<td>+P</td>
<td>+E/P</td>
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<tr>
<td>Mortality (n)</td>
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<td>7</td>
<td>19</td>
<td>51</td>
</tr>
<tr>
<td>Female</td>
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<td></td>
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</tr>
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5.2 Regulation of Nrf2 during tMCAO

Among a battery of genes encoding detoxifying and anti-oxidative enzymes, NQO1 and Hamox1 are major players in anti-oxidative stress mechanism and targets of the transcriptional factor nuclear factor erythroid 2-related factor 2 (NRF2). Gene expression analysis revealed de novo tMCAO-induced up-regulation of NQO1. Application of P neither increases nor decreases the mRNA level of NQO1 compared to tMCAO without hormones. Single administration of E initiated upregulation of NQO1, but lacks statistical significance. Only the combined hormone treatment of E/P fortified the expression significantly, when subjected to tMCAO-group (Fig. 26A). The gene expression of Hamox1 was massively induced and was highly significant comparing the ipsi- and the contralateral side in each treated-group. There was no regulation of the mRNA expression by hormones (Fig. 26B).
Results

Figure 25: Gene expression study of NRF2-induced anti-oxidative enzymes NQO1 and Hamox1. (A) tMCAO-elevated expression of NQO1 is significantly potentiated by E/P treatment. (B) No regulation of Hamox1 is triggered by hormone application. E = 17ß-estradiol; P = progesterone; c = contralateral; i = ipsilateral; *P < 0.05 tMCAO c, P c vs. tMCAO i, P i; **P < 0.01 E/P c vs. E/P i; ***P < 0.001 tMCAO c, E c, P c, E/P c vs. tMCAO i, E i, P i, E/P i; #P < 0.05 tMCAO i vs. E/P i.

Furthermore, evaluation of cell type-specific expression of NRF2 in the penumbra and in the contralateral cerebral cortex side, as well as on the ipsilateral healthy part of the cerebral cortex and corresponding contralateral side was performed with markers for astrocytes, microglia and neurons. To identify Nrf2 positive cell types we performed double-immunofluorescence studies for Nrf2 in combination with a neuronal cell marker (SMI32), an astroglial cell marker, GFAP, or a microglial cell marker, Iba1 which also labels peripheral macrophages.

Brain slices from untreated control and sham operated control rats showed only sparse immunoreactivity for Nrf2 (data not shown). Astrocytes (Fig. 25C), microglia (Fig. 25H) as well as neurons (Fig. 25M) were stained distinctly positive for Nrf2 in the penumbra region. In addition, we observed immunopositive Nrf2 cells in the ipsilateral healthy cerebral cortex which were microglia (Fig. 25 E), astrocytes (Fig. 25 J), and neurons (Fig. 25M). In the contralateral cortex, Nrf2 was significantly up-regulated only in neurons but not in astrocytes (Fig. 25B, D) or microglia (Fig. 25G, I) compared to sham operated controls (Fig.25B, D).

Morphometric staging of these cell parameters is indicated in Figure 24A, F and K.
Figure 26: Immunofluorescence co-staining and staging of Nrf2-positive astrocytes.
Panel (A) indicates scoring of NRF2-positive astroglia quantified in the penumbra of cerebral cortex in the ipsilateral and corresponding contralateral side, respectively (B, C). NRF2-positive astroglia are shown in the healthy part of the ipsilateral cerebral cortex and the corresponding contralateral side (D, E). For labelling anti-NRF2 (green) and anti-GFAP (red) antibodies against astrocytes are used for detection. Nuclei are stained with DAPI (blue). Overview of quantified regions in the cerebral cortex are given in the upper left microimage of a coronal rat slice and box-marked. Representative images show higher magnification of co-localisation of NRF2 and astrocytes are given as boxes in the left bottom, C-E. Scale bar = 20 µm.

Figure 27: Immunofluorescence co-staining and staging of Nrf2-positive microglia.
Panel (A) indicates scoring of NRF2-positive microglia/macrophages stained in the penumbra of cerebral cortex on the ipsilateral and on the corresponding contralateral side (B, C). NRF2-positive microglia/macrophages are shown in the healthy part of the ipsilateral cerebral cortex and the corresponding contralateral side (D, E). For labelling, anti-NRF2 (green) and anti-IBA1 (red) antibodies against microglia/macrophages are used for detection. Nuclei are stained with DAPI (blue). Overview of quantified regions in the cerebral cortex are given in the upper left microimage of a coronal rat slice and box-marked. Representative images show higher magnification of co-localisation of NRF2 and microglia/macrophages and given as boxes in the left bottom, C-E. Scale bar = 20 µm.

**Figure 28: Immunofluorescence co-staining and staging of Nrf2-positive neurons.**

Panel (A) indicates scoring of NRF2-positive neurons quantified in the penumbra of cerebral cortex on the ipsilateral and on the corresponding contralateral side (B, C). NRF2-positive neurons are stained in the healthy part of the ipsilateral cerebral cortex and the corresponding contralateral side (D, E). For labelling anti-NRF2 (green) and anti-SMI 32 (red) antibodies against neurons are used for detection. Nuclei are stained with DAPI (blue). Overview of quantified regions in the cerebral cortex are given in the upper left microimage of a coronal rat slice and box-marked. Scale bar = 20 µm.
6 Discussion

6.1 Transient MCAO in rat as a model for human stroke pathology

The transient occlusion of the \textit{A. cerebri media} in rats is a reliable animal model close to the human stroke pathology. One major feature of this model is reperfusion of the affected brain areas after the reopening of the occluded MCA followed by neuroinflammatory processes, which closely mimics the clinical lysis therapy.

In our system, the tested animal breathes independently and is narcotized free-flowed with isoflurane using a face mask. Controlling of regional cerebral blood flow is laser-monitored and shows a reduction of regional blood flow by more than $>50\%$ of baseline levels recorded several minutes before blocking the MCA. As demonstrated by TTC-staining or immunohistochemical approaches, tMCAO for 1 h results in loss of function and destruction of tissue in cortical as well as sub-cortical regions. Noteworthy, tissue damage within subcortical areas is a consistent phenomenon and resistant to therapeutical interventions, at least in the applied model. Similar observations have recently been made in latest tMCAO studies in rats (Ryang, Y.M., 2011).

6.1.1 From “bench to bedside”: choice of the right animal model and application strategy

Despite the large number of potentialy promising medicaments tested in experimental animal models, a number of clinical trials have produced negative results with the same agents (del Zoppo, G.J., 1995, del Zoppo, G.J., 1998, Grotta, J., 1995, Adams, R.J., 1995). Many points have to be regarded as crucial parameters to transfer preclinical observations from “bench to bedside”. The treatments should be applied within the temporal window of efficiency for the drug and is often administered in wrong therepeutical timing frames (The American Nimodipine Study Group, 1992). Most experimental studies are conducted on young, healthy animals under maintained laboratory conditions. However, the typical stroke patient is elderly with age-related risk factors for instance artheroscleroses, hypertension and heart diseases. Those factors should be included in experimental setting to study stroke pathology (van der Worp, H.B., 2010). Also, morphological and functional differences between the brain of humans and animals are pathophysiological comparable to cerebral ischemia. Cerebral energy metabolism and, thus, blood flow in mammals is inversely related to their body
weight. For example, in the rat, glucose and oxygen metabolism as well as blood flow, are three times as high as in humans (Armstrong, E., 1983, Nieuwenhuys, R., 1998). Neuronal and glial densities are also quantitatively different in various mammals (Pelvig, D.P., 2008). Furthermore, the human brain is gyrated and larger. Therefore, greater emphasis should be placed on studying experimental stroke and neuroprotection in species that are phylogenetically closer to humans such as non-human primates. However, those studies often carry ethical problems. Furthermore, several tested drugs, in most cases anti-excitotoxic compounds, caused severe side effects in clinical trials such as psychosis, worsen functional outcome or even increased mortality (Wahlgren, N.G., 2004). For instance, MK-801 (Dizocilpine), a non-competitive antagonist of NMDA receptor, has been abandoned during clinical trials due to significant side-effects and is ironically discussed as agent to induce schizophrenia in animal models (Wahlgren, N.G. 2004, Eyjolfsson, E.M., 2006). Therefore, it is important to design drugs with better safety profiles and more-favourable pharmacokinetics with fewer side-effects.

6.2 Effect of short-time hormone treatment on infarct size after tMCAO

At present, only few studies have been scope on the role of co-treatment with both gonadal hormones compared to individual administration, and most studies adressed the neuroprotective potency of E and not P in experimentally induced cerebral stroke.

In the present study, the analysis of neuroprotective properties of gonadal hormone treatment right after tMCAO and 12 h after surgery in physiological high doses demonstrates decisive results after a short-term survival period of 23 h. These results define the concurrence application of both gonadal hormones as the optimal choice to prevent ischemic damage in the cerebral cortex but not the subcortical brain structures such as the basal ganglia. E and P in single administration before, during or after stroke has been proven to be effective to protect cerebral cortex to a certain degree from ischemic damage (Chiappetta, O., 2007, Elzer, J.G., 2010, Gibson, C.L., 2004, Lebesgue, D., 2009). However, the results of our studies, convincingly demonstrates that both hormones are neuroprotective and the combined application appears to be favourable over any single treatment strategy.
6.2.1 Reduction of the cortical infarct volume by gonadal hormones in both genders

Irrespective of the effectiveness to protect cerebral cortex from ischemia-induced cell death by combined E/P administration, single hormone substitution of E and P alone decreases ischemic infarct volume. Remarkably, single P treatment reduces cerebral cortical lesion more effectively than E alone and is even similar protective as E/P in combination.

Impairments of different neurological parameters depend on the size of infarct volume and on the affected brain area. Assessment of the lesion volume as well as anatomical location by TTC-staining and immunohistochemistry clearly reveals no protection in the basal ganglia but protection in the dorsal parts of the cerebral cortex. The infarct core area is mainly restricted to the lateral cortical regions. Steroid treatment resulted in a reduction of infarct volume which can mainly be attributed to the more medially located motor area. In contrast, the more laterally located sensory areas are less protected by hormone treatment. In line with this anatomical distribution of infarct areas the neurological behavioral scoring displays improved motor skills (Fig. 10B, Fig. 11), whereas sensory functions are less preserved (Paxinos, G., 2004). These findings emphasize recently published reports, where P treatment alone could ameliorate motor function in mice up to several days after stroke which was not paralleled by any clear reduction of the infarct volume by P (Gibson, C.L., 2004). Moreover, single E administration attenuates cognitive performance after stroke (Söderström, I., 2009) and functional outcome after a long-term period (Li, X., 2004). Recently, long-term studies (14 days after tMCAO) reveal an equal degree of cortical infarct prevention similar to those observed during short-term recovery (23 hours after tMCAO) and using the same hormone treatment protocol indicating that cell protection is initially started during the first 23h and continues for at least 14 days. Behavioral recovery is also in this long-term study (Ulbrich, C. 2012).

In a male versus ovariectomized female comparison, protection of cerebral cortex is similarly preserved in both genders. In a former publication, protection of ischemic brain injury in young adult female rats is shown to be abolished in reproductive senescence female rats. Substitution with both ovarian hormones could alleviate stroke injury suggesting responsible role of protection by the steroids in stroke-induced cerebral damage (Alkayed, N.J., 2000). Similar observations were described using a long-lasting pretreatment hormone application strategy (at least 7 days) before the onset of MCAO (Toung, T.J.K., 2004).
6.2.2 Dose-dependent protection of cortical infarct size by gonadal hormones

The applied hormone concentrations are chosen to yield steroid plasma levels corresponding to those known to be present in pregnant female rats. The used hormone concentrations were obtained in our own pre-studies (performed by Dr. Kipp). The application strategy by repetitive neck depot injections of the steroids necessarily needs high doses of hormones to yield these steroid plasma levels. Lower concentrations of hormones fail to show neuroprotection of the cerebral cortex after tMCAO supporting beneficial and sustained protective effects by high doses of hormones. Moreover, other \textit{in vivo} studies (Acs, P., 2009; Ivanova, T., 2003, Trotter, A., 2006) and also a pilot study in extremely premature infants (Trotter, A., 1999) reveal that substitution of high dosis of steroids are crucial to obtain stable and beneficial protective effects.

6.3 Protective regulation by 17ß-estradiol and progesterone after tMCAO

To understand the underlying cellular and molecular mechanisms wich are involved in steroid-mediated neuroprotection within the penumbra, expression analysis for several inflammatory mediators, growth factors and markers against oxidative stress was performed and revealed possible new candidate proteins which might orchestrate the observed neuroprotective effects.

6.3.1 Reduction of ischemia-induced neuroinflammation by sex hormones

Stroke-induced expression of CCL2, CCL5 and IL6 is suppressed by E/P treatment. The chemokine CC ligand-2 (CCL2) (synonym monocyte chemoattractant protein-1, MCP-1) has a particular role in chemo-attraction of blood-borne cells to the injury site (Schilling, M., 2009, Conductier, G., 2010, Semple, B.D., 2010). Improved functional recovery, reduction of lesion volume, macrophage accumulation and astrogliosis were evident in CCL2-deficient (-/-) mice after traumatic brain injury (Semple, B.D., 2010). Only little is known about the role of the chemokine (C-C motif) ligand 5 (CCL5) (synonym for “Regulated upon Activation, Normal T-cell Expressed, and Secreted”, RANTES) in stroke. Recent research shows a particular role for CCL5 in athereosclerosis and indicates angiogenetic properties. In the contrary, chronic infections aggravate ischemic neuronal injury, which enhanced microvascular dysfunction by CCL5-mediated systemic inflammatory responses (Denes, A., 2010b, Suffee, N., 2011). Noteworthy, E inhibits the lipopolysaccaride-induced rise of CCL5
levels within the brain during systemic inflammation. (Brown, C.M., 2010). Although the expression of the chemokines CCL2 and CCL5 is attenuated by E/P, CCL3 is potentiated concomitantly. The exact role of CCL3 during ischemia is still unknown. CCL3 plasma levels are elevated in patients after acute ischemic stroke. This indicates that CCL3 might be a prognostic marker for post-stroke intervention (Zaremba, J., 2006). Concerning chemoattraction, stem cells derived from the umbilical cord blood and implanted into the brain parenchyma directly migrate towards ischemic regions promoting migration by CCL3 (Jiang, L., 2008).

Cytokines, which are also found in the peri-lesioned area of cerebral tissue of post-traumatic patients caused by brain contusion, co-operate with chemokines in the infarct region (Stefini, R., 2008, Denes, A., 2010a, Brown, C.M. 2010). While the role of IL6 in atherosclerosis and as a genetic risk factor is well known, direct regulation of IL6 during acute stroke and regulation of IL6 by gonadal hormones are sparse. (Yamada, Y. 2008, Vikman, P., 2007). Interestingly, invasion, proliferation or/and differentiation of local Iba-1-positive microglia/macrophages and CD3 expression, which represent a marker for lymphocytes (Perterfalvi, A. 2009), are inhibited by combined hormone substitution in the penumbra region. CD11b and CD68 mRNA levels, both established markers for activated microglia/macrophages (Graeber, M.B., 2009), are significantly reduced in E/P-treated compared to vehicle-treated animals. These findings strongly support the hypothesis that dampening of a chemokine response by E/P prevents neuroinflammatory processes within the penumbra region. In other in vivo models of neurodegeneration such as in experimental allergic encephalomyelitis (EAE) or during cuprizone intoxication, both models for MS, microglia are implicated in this (autoimmune) pathology and respond on E and/or P treatment (Jansson, L., 1994, Sandyk, R., 1996, Acs, P., 2009). Beside decreased numbers of Iba-1-positive microglia/macrophages in E/P treated animals, more detailed morphological analysis indicates a shift of Iba-1-positive microglia/macrophages appearance from a swollen activated to a more round-shape, ameboid microglia phenotyp, which is almost indistinguishable from activated invading macrophages (Guillemin, G.J., 2004, Tambuyzer, B.T., 2009). It is well accepted that steroid hormones or respective derivatives regulate inflammatory response of microglia in an ER-dependent manner (Mor, G., 1999, Saijo, K., 2011). It is also accepted that activation of microglia results in a macrophage-similar round-shape ameboid phenotyp (Christensen, R.H., 2006). In addition, activated microglia can secrete cytokines and chemokines, proteases as well as ROS and therefore fortify an inflammatory response (Hanisch, U.K., 2004). However, a growing body of evidence assign microglia
neuroprotective properties such as uptake of excessive glutamate by expression of glutamate receptors/transporters preventing neurons from excitotoxicity (Nakajima, K. 2008), phagocyte cell debris, or secreting growth- as well as neurotrophic factors (Lalancette-Hebert, M., 2007, Napoli, I., 2010) Finally, these data suggest the possibility that sex hormones might promote selectively the activation of microglia but coevally inhibit the mobilization of macrophages. It remains to be clarified in future studies whether the observed effect of E/P administration on microglia morphology is functionally involved directly or is a secondary neuroprotective effect in the observed steroid-mediated reduction in infarct volumes.

6.3.1.1 Suppression of ischemia-induced astrocyte activation

Suppression of cell activation, proliferation or/and infiltration is not restricted to microglia/macrophages and peripheral lymphocytes by E/P, but also activation of astrocytes is attenuated by the hormones. Since astrocytes are also capable to secrete chemokines and prostanoids, major biological mediators for direction of microglia/macrophages and peripheral leukocytes or neutrophiles to regions of cerebral damage (Babcock, A.A., 2003, Cross, A.K., 1999, Gao, Y. J., 2010, Johann, S., 2008, Ziebell, J.M., 2010), hormone-mediated dampening of attraction/infiltration and proliferation of brain resident or blood-borne immunocompetent cells seems to create prerequisite conditions for a neuroprotective microenvironment (Denes, A., 2010a, Kriz, J., 2006, Wang, Q., 2007).

6.3.2 Neuroprotective mechanism by 17β-estradiol and progesterone

The neuroprotective action of both hormones during ischemic cerebral insults has versatile targets and triggers numerous of mechanisms and this aspect has been summarized in excellent review article by our group and others (Arnold, S., 2009, Kajta, M., 2003, Liu, M., 2010a, Liu, R.L., 2010b). Independent of the hormone-mediated signaling pathways, neuroprotection during a set of brain insults is operant by both gonadal hormones. A major role is the controlling of brain-intrinsic inflammation, which is mediated via invasion of peripheral lymphatic cells after blood–brain-barrier breakdown. After tMCAO in mice, P influences directly certain mediators of the inflammatory network such as attenuating TNFα, IL1b and TGFβ induction after MCAO likely due to nitric oxidase 3 regulation (Gibson, C.L., 2005), antagonizing NMDA receptor Ca2+ influx and activating Src–ERK signalling depending on the timing of hormone application before MCAO (Cai, W., 2008), increasing anti-apoptotic and decreasing pro-apoptotic molecules (Yao, X.L., 2005), preventing lipid
oxidation (Roof, R.L., 1997), and suppressing ischemia-stimulated proliferation but improving survival of newborn neurons (Zhang, Z., 2010b).

Recent studies demonstrate that E maintains the endothelial function and mitochondrial integrity after hypoxic conditions which can be interpreted as a stabilization of the blood-brain-barrier (Guo, J., 2010, Liu, M., 2010a, Liu, R.L., 2010b). The latter comprises reduction of the Ca2+-dependent excitotoxic mechanisms, which is a tremendous post-stroke cellular reaction (Lebesgue, D., 2009; Liu, M., 2009; Suzuki, S., 2009), supression of NADPH oxidase activation (Zhang, Q.-G., 2009), and counteracting oxidative stress mechanisms by preventing from surplus of free radicals through thioredoxin-regulated protection in the brain (Chen, T.Y., 2010).

6.3.3 Presence of steroid hormone receptors indicating classical nuclear hormone receptor pathways

Gene expression analysis reveals the presence of ERalpha and beta within the penumbra, suggesting classical or non-classical steroid-mediating pathways. Hormones are not capable to further increase the expression of receptors. Nevertheless, there is convincing evidence that E protects from ischemic injury by acting via classical ERs (Elzer, J.G., 2010, Dubal, D.B., 2006, Merchenthaler, I., 2003). After focal ischemia, the expression of ERs and thereby responsiveness of damaged tissue for E is locally induced showing early ERalpha and late ERbeta modulation (Dubal, D.B., 2006). Such an ischemia autonomous tissue response may be seen in conjunction with known aromatase induction after MCAO in local astrocytes which occurs mainly one day after stroke and persists for several days (Carswell, H.V., 2005). The importance of astroglia for steroid-mediated neuroprotection in the stroke area is well-accepted (Arevalo, M.A., 2009, Dhandapani, K.M., 2007). Interestingly, PR is neither increased by tMCAO-induction nor by hormones, which has not been described in the literature so far. Also, non-classical G-protein coupled steroid-mediating pathways interact with different intracellular cell survival and death signal cascades (Arnold, S., 2009; Dhandapani, K.M., 2007, Kipp, M., 2009, Lebesgue, D., 2009, Misiak, M., 2010).

6.3.4 Neuroprotection by Nrf2 signaling

Here, the role of the nuclear factor erythroid 2-related factor 2 (Nrf2) should be underlined as an important player in the oxidative stress-related stroke pathogenesis. Using double-immunofluorescence staining with antibodies directed against Nrf2 and cell type-specific
markers for neurons, astroglia, and microglia the cell-specific distribution of Nrf2 in the peri-infarct area was demonstrated. During the post-stroke episode, reoxygenation of the penumbra results in high degree of oxidative stress, which can cause brain tissue damage at a sublethal level (Carbonell, T., 2007). Hence, Nrf2 activation might be beneficial and subsequently contribute to cell protection and survival.

Under normal conditions inactive Nrf2 is typically proteosomal degraded within minutes but stabilized during activation under damaging conditions (Tanaka, N., 2010). We were able to demonstrate that astrocytes, microglia and neurons are immunofluorescence-positiv for Nrf2 in the penumbra. Quantification revealed high expression rates for Nrf2 in the ipsilateral side (Fig. 26A, K, F) for all cell types studied. In particular astrocytes reveal high levels of Nrf2 expression after 23 h of tMCAO. Recent studies show preventing effects by sulforaphane or 1,5-diCQA (1,5-dicafeoylquinic acid) from oxidative stress in astrocytes after oxygen/glucose deprivation in vitro and in vivo. These effects are coupled with the Nrf2-ARE signaling and are mainly contributed to the upregulated expression of gluthation or NQO1 (Danilov, C.A., 2009, Cao, X., 2010), target genes of Nrf2. Furthermore, oxidative stress is followed by a destruction of nigrostriatal dopaminergic neurons and reactive gliosis in the damaged basal ganglia caused by methamphetamine (METH) toxicity. Nrf2-deficient (-/-) mice exhibit increased inflammatory responses and glial activation in the striatum after METH administration (Granado, N., 2011). The tMCAO-induced upregulation of NQO1 is significantly potentiated by E/P. The expression of Hamox1 is massively induced in the ipsilateral side of tMCAO but shows no further up-regulation by sex hormones. It is most likely that expression of Nrf2-mediates anti-oxidative phase II enzymes such as NQO1 for scavenging ROS. Steroid hormones might aggravate translation of these typ of enzymes to an early time point of post-ischemic period and accelerate following cellular recovery by inhibiting ROS-damaging molecular processes.

Surprisingly, we observed significant neuronal NRF2 expression in the peri-penumbral as well as contralateral cortex region. Therefore, unknown intrinsic Nrf2 activators might exist. Our analysis show abundant expression of Nrf2 in neurons resided ipsi- and contralateral in the healthy regions of cerebral cortex ispi- and contralaterally. In a recent study, cortical primary neurons have shown to expresses high levels of Nrf2 after ethanol (ETOH) exposure, and this effect is mainly involved in cell survival in the ETOH-related apoptotic challenge (Narasimhan, M., 2011). More speculative, growth factors or cyto- and chemokines which are typically active during ischemia may be involved in Nrf2 regulation via MAP-kinase signaling without occurrence of ROS. In addition to neurochemical mediators, cross-
hemispheral neural connections might also be a possible way to activate Nrf2 in the contralateral side. This possibility is suggested by MRI evidence in humans undergoing stroke rehabilitation, as well as by experimental studies in rats (Kim, Y.R., 2005). This effect might play a role to prone primarily not affected brain tissue to forthcoming oxidative stress caused by nearby cell degeneration.

6.3.5 Growth factor expression in the delineated penumbra

Growth factors (GFs) ameliorate stroke outcome by attenuating ischemic-induced cerebral damage through their anti-apoptotic and anti-inflammatory effects, and by inducing angiogenesis and/or neurogenesis (Lanfranconi, S., 2011, Costa, C., 2004, Dempsey, R.J., 2007, Solagol, I., 2006). In the present study, an induction of NGF and VEGF after 23 h tMCAO in the penumbra was observed. The expression of VEGF is potentiated by E/P after tMCAO which has been already demonstrated in a previous study using lung tissue (Trotter, A., 2009). In the brain, VEGF is expressed ubiquitously, mostly by epithelial cells from the plexus choroideus but also by astrocytes and neurons (Monacci, W.T., 1993, Marti, H.H., 1999). VEGF administration mitigates the infarct volume and improved stroke outcome without an increase in brain oedema (Harrigan, M.R., 2003, Kaya, D. 2005).

6.4 Sex hormones in different models of neurological disorders

The neuroprotective properties by sex hormones has been proven to be effective in preventing cerebral tissue from ischemic damage (Chiappetta, O., 2007, Elzer, J.G., 2010, Gibson, C.L., 2004, Lebesgue, D., 2009, Ulbrich, C., 2012). Moreover, E and P reduce accumulation of β-amyloid plaques in an animal model of Alzheimer’s disease (AD) (Caroll, J.C., 2007, Behl, C., 2002). In addition, cognitive decline is attenuated after the administration of estradiol benzoate, a derivate of E, and progesterone before hippocampal damaging by intra-hippocampal colchicine infusions suggesting neuroprotective actions on learning, attenuating neuronal loss and memory impairment (Vongher, J.M., 1999). Corresponding to recently published manuscripts, the synchronous application of both hormones prevent from myelin loss and restored functional outcome in in vivo model of multiple sclerosis (2009, Acs, P., 2009, Kipp, M., 2009). In a MPTP (1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine) rodent model for Parkinson’s disease (PD), E preserved loss of dopamine neurons in the substantia nigra, pars compacta and neurons within the striatal compartment (Dluzen, D.E., 2001,
Horstink, M.W., 2003). In addition, following TBI in a stab-wound model, P suppresses the inflammatory response. Furthermore, P affects the sodium transport from blood to brain via Na-K-ATPase and, thus, reduces brain edema after lesion (Grossman, K.J. 2004). Neuroprotective properties of steroid hormones under different neurological paradigms comprises not only animal models for stroke, AD, PD and TBI but are also seen in other neurodegenerative challenges such as epilepsy, schizophrenia and spinal cord injury (Velísková, J., 2000, Gogos, A., 2012, Labombarda, F. 2003).

6.5 Gonadal hormones as a possible therapeutical intervention directly after stroke

Several studies of long-term hormone replacement therapy (treatment over several years) in women revealed an increase of risk for stroke (Lisabeth, L., 2012, Wassertheil-Smoller, S., 2003). Nevertheless, in the present study combined hormones are injected in high doses right after the release of MCAO in a short-range application strategy. Although the term “hormone substitution” is used here, the application of hormones occurs always in a short-time setting, exactly two times, and not continuously for a long-range. The administration of combined hormones early after tMCAO shows a high potency of neuroprotection which reflects the possible therapeutical use in cooperation with lysis therapy or even without. Colateral-perfusion of the penumbra region might be sufficient to obtain relevant plasma levels in the compromised compartment. Again, treatment could proceed right after or concomitantly to the lysis to gain maximum effect in a short-range way with high doses.

6.6 Conclusion

The present study demonstrates several unique and novel aspects concerning the neuroprotective potency of gonadal steroid hormones after tMCAO. First, the synchronous two-times application of steroid hormones immediately after tMCAO and 12 h later appears to be effective for alleviating cell damage and restoring behavioral functions in male and ovariectomized female young rats. Concurrently, a single application of P only shows a similar remarkably protective effect, although it needs to be mentioned that E is more protective concerning behavior dysfunction, whereas P better protects cell damage. Cerebral cortical tissue protection and preservation of functional recovery is accompanied with a significant raise of hormone plasma values to levels observed in pregnant female rats.
Those levels could only be achieved using high doses of E and P which have been evaluated in preliminary studies.

Second, the exact delineation of the ischemic core and adjacent salvageable tissue by viability TTC- and immunohistochemical stainings allows pinpointing the “tissue at risk” for distinct isolation of tissues for further molecular and cellular analysis. As a result, analysis of gene different expression patterns without and with the combined steroid hormone application uncovers putative targets for both steroids and identifies the regulation of local and peripheral inflammatory processes such as attenuating the chemokines CCL2, CCL5 and the cytokine IL6. Coevally, hormone stimulation diminishes markers for astrocytes (GFAP), microglia/macrophages (CD11b, Iba-1, CD68) and lymphocytes (CD3).

Thus, these findings can be interpreted as a steroid-dependent phenomenon to inhibit or dampen several pro-inflammatory aspects and simultaneously induce growth factors such as VEGF-A in an early timepoint after onset of ischemia. This condition might be a prerequisite for early recovery and rehabilitation and is operant after 14 days of tMCAO with combined hormone treatment indicated by an unchanged infarct volume size and improved motor behavior outcome. It is suggested that this short-range steroid application soon after the onset of ischemia at high concentrations stabilizes neuronal activity/performance until reperfusion is sufficient to supply neurons again. At the same time, oxidative stress-related cell damage by reperfusion is decreased by an enhanced expression of phase II anti-oxidative enzymes such as NQO1 under hormone treatment. The expression of the transcriptional factor Nrf2 in astrocytes, microglia/macrophages, and especially in neurons after tMCAO might be the predominant signaling pathway which leads to the activation of these enzymes.

In summary, the results of this thesis suggest that a combined, high-dose hormone treatment for a short period of a few days might be used as an additional therapy, besides thrombolysis. Further experimental studies are required to analyze the long-term efficiency of both steroids under ischemic conditions before translational studies in human patients should be initiated.
Summary

Stroke is the third leading cause of mortality in the Western civilization and the first listed for severe disability and intensive aftercare. It is classified by the World Health Organisation as a pathological acute brain disease of cerebrovascular origin that can lead to long-lasting neurological impairments or even death.

Two major forms of stroke exist, namely embolism of brain arteries and rupture of a brain blood vessel. For embolism, hitherto, lysis of blood clots by tissue plasminogen activator is the only accepted therapeutic option for acute stroke. Many other compounds have been tested in animals and pilot human trials but finally failed to prove effectiveness in human stroke therapy. The gonadal hormones 17ß-estradiol (E) and progesterone (P) are known to be neuroprotective factors in the brain preventing neuronal death or damage under different injury paradigms. The present study demonstrates that both steroids in combination derogate brain damage, improve behavioral function, and regulate decisive cellular/molecular routes of neuroprotective mechanism such as neuroinflammatory responses or induction of growth factor expression after 1 h transient middle cerebral artery occlusion (tMCAO). 51 male and 12 ovariectomized female rats were included in the study and subjected either to single or combined hormone treatment. The survival period was 24 h. Effective doses of steroid hormones were 25 µg/kg body weight E and 10 mg/kg body weight P. The steroid hormones were applied two-times, immediately after tMCAO and 12 h later. The combined application of both gonadal hormones diminished the cerebral cortical infarct volume to a similar extent in both sexes by ~70%. In this context, P was more effective as E, when applied seperately. Evaluation of neurological scoring revealed an equal degree of improved functional motor performance after hormone treatment in both genders. Expression studies in the delineated penumbra indicated that the combined gonadal hormone treatment dampended tMCAO-induced pro-inflammatory mediators such as interleukin 6, and the chemokines CCL2 and CCL5. Equally, the numbers of infiltrated microglial cells, macrophages, and lymphocytes were reduced after hormone application. Beside steroid-dependent anti-inflammatory effects, the expression of vascular endothelial growth factor A was potentiated by steroid hormones. In addition, nuclear factor erythroid 2-related factor 2 (Nrf2), a anti-oxidative transcriptional marker, was activated in the penumbra after stroke as indicated by immunofluorescence staining. Nrf2 was discovered in microglia, astrocytes, and neurons within the penumbra. The evaluation of the putative target genes of Nrf2, NAD(P)H: quinone oxidoreductase (NQO1) and heme oxygenase (decycling) 1 (Hamox1), revealed a tMCAO-induced upregulation of
these anti-oxidative enzymes on mRNA. Gonadal hormones significantly intensified NQO1 but not Hamox1 expression.

These data clearly indicate the short-term neuroprotective potency of a combined treatment with E and P under ischemic conditions in both genders in a rat model. We assume that the dampening of the early local neuroinflammation and the reduction of oxidative stress are both crucial for this neuroprotective action. Further experimental studies are required to analyze the long-term efficacy of both steroids under ischemic conditions before translational studies in human patients can be started.
Zusammenfassung


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer disease</td>
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<tr>
<td>ADP</td>
<td>adenosindiphosphat</td>
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
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<tr>
<td>ANT</td>
<td>adenine nucleotide translocase</td>
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<td>APS</td>
<td>ammoniumpersulfat</td>
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<tr>
<td>ARE</td>
<td>antioxidant responsive element</td>
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<tr>
<td>ATP</td>
<td>adenosintriphosphat</td>
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<tr>
<td>BBB</td>
<td>blood-brain-barrier</td>
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<td>BPU</td>
<td>blood perfusion units</td>
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<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>CAA</td>
<td>cerebral amyloid angiopathy</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphat</td>
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<tr>
<td>Ca²⁺</td>
<td>calcium-cation</td>
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<tr>
<td>CBF</td>
<td>cerebral blood flow</td>
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<tr>
<td>CCL</td>
<td>chemokine (C-C motif) ligand</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<td>Cl⁻</td>
<td>chloride-anion</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>CSD</td>
<td>cortical spreading depression</td>
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<td>CT</td>
<td>computer tomography</td>
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<tr>
<td>CVT</td>
<td>cerebral venous thrombosis</td>
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<td>DNA</td>
<td>desoxyribonuclein acid</td>
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<td>E</td>
<td>17ß-estradiol</td>
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<td>EAAT</td>
<td>excitatory amino acid transporter</td>
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<td>ECA</td>
<td>external carotid artery</td>
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<td>ECL</td>
<td>enhanced chemo-luminescence</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbtent assay</td>
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<tr>
<td>EPSP</td>
<td>excitatoric post-synaptical potential</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>ERa</td>
<td>estrogen receptor alpha</td>
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<td>estrogen receptor beta</td>
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<td>EREs</td>
<td>estrogen response elements</td>
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<td>ER-X</td>
<td>membrane associated estrogen receptors</td>
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<td>ESPro</td>
<td>erlangen stroke project</td>
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<td>etc.</td>
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<td>ETOH</td>
<td>ethanol</td>
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<td>GABA</td>
<td>gamma-Aminobutyric acid</td>
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<td>GF</td>
<td>growth factor</td>
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<td>GFAP</td>
<td>glial fibrillic acidic protein</td>
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<td>GST</td>
<td>glutathione S-transferase</td>
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<td>h</td>
<td>hour</td>
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<td>H⁺</td>
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<td>Hamox 1</td>
<td>Heme oxygenase (decycling) 1</td>
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<td>hydrogen chloride</td>
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<td>HCO₃⁻</td>
<td>hydrogen-carbonate-Anion</td>
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<td>Hif1α</td>
<td>hypoxic inducible-factor 1 alpha</td>
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<td>HO-1</td>
<td>heme oxygenase 1</td>
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<tr>
<td>HPRT</td>
<td>hypoxanthin-Guanine-Phosphoribosyltransferase</td>
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<td>Abbreviation</td>
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<tr>
<td>Iba1</td>
<td>ionized calcium-binding adaptor molecule 1</td>
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<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
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<td>ICP</td>
<td>intracranial pressure</td>
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<td>IL</td>
<td>interleukin</td>
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<td>K+</td>
<td>potassium-cation</td>
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<td>kg</td>
<td>kilogram</td>
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<tr>
<td>NQO1</td>
<td>nAD(P)H quinone oxidoreductase-1</td>
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<tr>
<td>OH</td>
<td>hydroxy group</td>
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<td>P</td>
<td>progesterone</td>
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<td>pH</td>
<td>potential hydrogen</td>
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<td>PI3K</td>
<td>phosphoinositide-2-kinase</td>
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<td>PKA</td>
<td>protein kinase A</td>
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<td>protein kinase C</td>
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<td>PLC</td>
<td>phospholipase C</td>
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<td>Parkinson disease</td>
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<td>PR</td>
<td>progesteron receptor</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative- Real-Time-PCR</td>
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<tr>
<td>rCBF</td>
<td>regional cerebral blood flow</td>
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<tr>
<td>RIND</td>
<td>reversible ischemic neurological deficit</td>
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<td>RNA</td>
<td>ribonuclein acid</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>mGluRs</td>
<td>metabotropic glutamate receptors</td>
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<td>M</td>
<td>molar</td>
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<td>MAP-kinase</td>
<td>mitogen-activated protein kinase</td>
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<td>MCA</td>
<td>middle cerebral artery</td>
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<tr>
<td>MCAO</td>
<td>middle cerebral artery occlusion</td>
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<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
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<td>METH</td>
<td>methamphetamine</td>
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<td>mg</td>
<td>milligram</td>
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<td>ml</td>
<td>millilitres</td>
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<td>min</td>
<td>minutes</td>
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<tr>
<td>MIP-1α</td>
<td>macrophage inflammatory protein 1α</td>
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<tr>
<td>MISS</td>
<td>membrane-initiated steroid signalling</td>
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<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine</td>
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<td>MPT</td>
<td>mitochondrial permeability transition</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<td>MS</td>
<td>multiple sclerosis</td>
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<td>mV</td>
<td>millivolt</td>
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<td>Na+</td>
<td>sodium-cation</td>
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<td>NaCl</td>
<td>sodium chloride</td>
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<td>NF-κB</td>
<td>nuclear factor- κB</td>
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<td>NGF</td>
<td>nerve growth factor</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<td>NMDA</td>
<td>n-methyl d-aspartate</td>
</tr>
<tr>
<td>NQ1</td>
<td>NAD(P)H: quinone oxidoreductase</td>
</tr>
<tr>
<td>Nrf2</td>
<td>nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>P</td>
<td>progesterone</td>
</tr>
<tr>
<td>p.A.</td>
<td>pro analysis</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered salt solution</td>
</tr>
<tr>
<td>rt-PA</td>
<td>recombinant tissue plasminogen activator</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutan</td>
</tr>
<tr>
<td>SAH</td>
<td>subarachnoid hemorrhage</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffer saline</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffer saline + Tween 20</td>
</tr>
<tr>
<td>TIA</td>
<td>transient ischemic attack</td>
</tr>
<tr>
<td>tMCAO</td>
<td>transient middle cerebral artery occlusion</td>
</tr>
<tr>
<td>t-PA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)-aminomethan</td>
</tr>
<tr>
<td>TTC</td>
<td>2,3,5-triphenyl-tetrazolium chloride</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>VDAC</td>
<td>voltage-dependent anion channel</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WHO</td>
<td>world health organisation</td>
</tr>
<tr>
<td>1,5-diCQA</td>
<td>1,5-Dicaffeoylquinic acid</td>
</tr>
</tbody>
</table>
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Caplan, L.R. (1983) Are terms such as completed stroke or RIND of continued usefulness? *Stroke* 14: 431.


Coomber, B., Gibson, C.L. (2010) Sustained levels of progesterone prior to the onset of cerebral ischemia are not beneficial to female mice. *Brain research* Nov 18;1361:124-32.


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References


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Curriculum Vitae

Due to reasons of data protection, the electronical version of my dissertation content not my Curriculum Vitae.
**Publication list**


# equal contribution as first author
Reviews


Appendix

Embedding of tissue in paraffin

1. For embedding procedure, tissue should be placed in appropriate boxes after fixation following hydration step over night with tap water.

2. For dehydrating of the tissue, following steps are suggested:
   (Cave: methylated absolute alcohol should be used; numbers (1, 2, and 3) after Aceton and Parafin indicate new/fresh Aceton/Parafin)

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<table>
<thead>
<tr>
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<tbody>
<tr>
<td>70% EtOH</td>
<td>3-4 days or longer, daily change of alcohol</td>
</tr>
<tr>
<td>96% EtOH</td>
<td>0:45 min</td>
</tr>
<tr>
<td>96% EtOH</td>
<td>0:45 min</td>
</tr>
<tr>
<td>96% EtOH</td>
<td>1:00 min</td>
</tr>
<tr>
<td>abs. EtOH</td>
<td>0:45 min</td>
</tr>
<tr>
<td>abs. EtOH</td>
<td>0:45 min</td>
</tr>
<tr>
<td>abs. EtOH</td>
<td>1:00 h</td>
</tr>
<tr>
<td>Aceton 1</td>
<td>1:00 h</td>
</tr>
<tr>
<td>Aceton 2</td>
<td>1:00 h</td>
</tr>
<tr>
<td>Aceton 3</td>
<td>1:00 h</td>
</tr>
<tr>
<td>Parafin 1</td>
<td>1-2 h</td>
</tr>
<tr>
<td>Parafin 2</td>
<td>1 day</td>
</tr>
<tr>
<td>Parafin 3</td>
<td>Over night or longer</td>
</tr>
</tbody>
</table>

3. Tissue should be replaced in special metal parfin-embedding forms (bottom is wiped with Glycerin (Roth, Germany) to separate easily the parafin-tissue block from the metal form later) and should be totally covered with paraffin by an embedding machine (Histo Embedder, Leica, Germany). Afterwards the forms must cool down for hours or max. 1 day at room temperature or at 4 degrees in the freezer.