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DISSERTATION

Expression of Angiotensin Receptors in the rat brain after focal cerebral ischemia

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To Ina, George, Jutta and Kaka Minka,

to whom I am greatly indebted

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Abstract

The renin-angiotensin-system is known for its function as a regulator of blood pressure and fluid-electrolyte homeostasis. Beside the systemic RAS, a lot of other tissues have been shown to produce Ang II. The development of selective receptor antagonists led to the discovery of different angiotensin receptors. Among these, the AT_1 receptor has been studied extensively while much less was known about the AT_2 receptor. The majority of the studies were done in vitro. Our aim was to show the distribution of the two receptors in the rat brain in vivo and to identify the cells of their expression.

Using the method of transient MCAO we induced a 90 min focal cerebral ischemia in rats from the “stroke group”, while the “sham animals” underwent only a superficial surgery. Immunohistological, molecular and protein biochemical methods showed significant differences in receptor expression.

We were able to demonstrate that AT_2 receptors were expressed exclusively in neurons and that there was an upregulation of their expression in the ischemic brain hemisphere. Furthermore, some AT_2 receptor-positive cells had long neurites and co-expressed MAP_2. We found no co-expression of the AT_2 receptor and markers of inflammation.

There was no significant change in the expression of AT_1 receptors between stroke and sham animals. Many of the AT_1 receptors were located in astrocytes and only sparsely in cortical neurons. Astrocytes in the periinfarct area appeared stouter, had powerful projections and were located around neuron-like cells. These „activated” astrocytes were significantly increased in number in both hemispheres when compared to the sham group, especially in the right hemisphere. A lot of these activated AT_1 receptors were positive for cCasp-3. We could not find any co-localisation of AT_1 receptor with cCasp-3.

Thus we hope that we were able to throw new light on the importance of the AT_2 receptor expression in the brain. Our experiments hint at the neuroprotective and neuroregenerative effect of the AT_2 receptor in the periinfarct area as well as at a possible influence of the AT_1 receptor on reactive astrogliosis and apoptosis. These results are in good agreement with in vitro studies. Further new insights into the signaling mechanisms that take place in the neuronal and glial cells via the AT_1 and AT_2 receptors will surely give the medical world the possibility to treat patients effectively and quickly, and even provide some preventive therapy.

Abstrakt

Das Renin-Angiotensin-System (RAS) ist mit seinen Wirkungen als Regulator des Blutdruckes und der Flüssigkeit-Elektrolyt-Balance bekannt. Die Mehrheit seiner Wirkungen wird über Angiotensin II vermittelt. Zusätzlich zu dem „zirkulierenden“ RAS existiert auch die so genannte „Gewebe RAS“. Mit der Entwicklung selektiver Rezeptor-Antagonisten wurden die AT_1- und AT_2-Rezeptoren charakterisiert. Dabei ist der AT_1-Rezeptor gut erforscht, während über den AT_2-Rezeptor zum jetzigen Zeitpunkt weniger Daten vorliegen. Größtenteils handelt es sich dabei um in vitro Modelle. Unser Ziel war es die Expression und das Verteilungsmuster beider Rezeptoren in vivo nachzuweisen, und die exprimierenden Zelltypen zu identifizieren.

Mittels einer 90 min. MCAO wurde eine transiente fokale zerebrale Ischämie in der Schlaganfallgruppe induzierten, während die Kontrollgruppe nur eine Haut-OP unterzogen wurde. Immunohistologische, molekulare und proteinbiochemische Methoden zeigten signifikante Unterschiede in der Rezeptorexpression nach Ischämie.

Der AT_2-Rezeptor zeigte postischämisch eine deutlich erhöhte Expression und wurde ausschließlich in Neuronen exprimiert. Einige der striatalen Neurone um die Infarktzone zeigten lange Neuriten und waren MAP_2-positiv. Es wurde keine Koexpression von AT_2-Rezeptoren und Entzündungs- oder Apoptosemarkern gefunden.

Im Unterschied dazu konnte keine relevante Differenz in der Expression des AT_1-Rezeptors festgestellt werden. Viele der AT_1-Rezeptoren wurden in Astrozyten und nur wenige in Neuronen exprimiert. Viele der Astrozyten waren verplumpt, zeigten prominente Ausläufer und umgaben neuronenähnliche Zellen. Diese aktivierten Astrozyten waren deutlich vermehrt in beiden Hemisphären der Schlaganfallgruppe im Vergleich zu der Schein-operierten Gruppe, rechts stärker als links, und waren positive für cCasp-3. Es konnte keine Kolokalisation der AT_1-Rezeptoren und der cCasp-3 darsgetellt werden.

Unsere Ergebnisse weisen auf die neuroprotektive und neuroregenerative Wirkung des AT_2-Rezeptors in der Periinfarktzone hin, sowie möglicherweise auf einen Einfluß des AT_1-Rezeptors auf die Apoptose und die reaktive Astrogliose in diesem Areal. Diese Ergebnisse stimmen gut mit den in vitro Untersuchungen überein. Weitere neue Erkenntnisse über die Signalmechanismen in den Neuronen und Gliazellen vermittelt über die AT_1- und AT_2-Rezeptoren würden einen großen Beitrag für die bessere und schnellere Behandlung der Patienten leisten und möglicherweise sogar eine präventive Therapie ermöglichen.

The most insatiable thirst
is the thirst for knowledge!

Foreword

Among the diseases that are of great importance in public health, we certainly find stroke in the foremost ranks. Stroke is a leading cause of death and considerable disability, and in our modern society full of stress, bad nourishment and little exercise, it takes its toll among young and old. In Germany, as well as in the USA, stroke is the third most common cause of death, (Taylor *et al.*, 1996), (Bundesamt, 2007), whereas worldwide it is the second leading cause of death (Murray *et al.*, 1997). In Germany, the number of new stroke patients per year varies between 150 000 and 200 000, depending on whether the asymptomatic cases which had been discovered by chance during other investigations are taken into account. In 1995, over 100 000 persons died due to ischemic stroke, and the incidence of stroke amounted to around 250 persons per 100,000 individuals. Adding the transient ischemic attacks (TIA), the number increases to 300 cases per year (Bundesministerium, 1998). Epidemiological studies from the year 2004 report an incidence of 182 per 100,000, and mortality of 66,000 in the first year. 64 000 patients remain with permanent disability. (www.dsg-info.de, www.public-health.de, Kolominsky-Rabas, P. Erlangen Schlaganfall Register: Schlaganfall in Deutschland. 11.10.2004. doc)

The rapid rise in the elderly population in both developed and developing countries prompted predictions of an increase in stroke incidence. In relation to age, problems with the blood supply of the brain occur in at least 5 percent of all persons over 65 years of age have, in 10 percent with those above 75 years and in 20 percent with those above 85 years. Since the 1960s, stroke mortality rates in Western Europe and North America have been decreasing continuously. This may be seen as a result of the prevention and effective treatment of high blood pressure and hyperlipidemia, and substantial reductions in the number of smokers. However, some researchers note a levelling-off of the decline, (Gillum *et al.*, 1997; Reitsma *et al.*, 1998; Stegmayr *et al.*, 1996) or even an increase, for example in Eastern Europe (Sarti *et al.*, 2003). Stroke incidence seemed to be decreasing as well. But findings of most subsequent studies have shown either no change (Bonita *et al.*, 1993; Feigin *et al.*, 2003; Harmsen *et al.*, 1992; Stegmayr *et al.*, 1994; Wolf *et al.*, 1992) or even an increase in age- and sex-adjusted incidence (Brown *et al.*, 1996; Johansson *et al.*, 2000; Jorgensen *et al.*, 1992; Lemesle *et al.*, 1999; Medin *et al.*, 2004; Rothwell *et al.*, 2004; Terent, 1988). For example, despite progress in the prevention and treatment of risk factors for stroke with the use of antihypertensive therapy, the increasing prevalence of ischemic heart disease, diabetes mellitus, and obesity in the United States leads to increased risk of stroke, particularly among blacks (Gillum, 1999). Thus, in order

to obtain a greater stroke incidence reduction, the search for widespread preventive strategies continues.

There have been many attempts to clearly describe and classify the different types of stroke as well as to define their origin, risk factors and prevention.

Definition/Aetiology:

The formal medical term for stroke is apoplexy. It is a sudden and severe attack, which represents as a sudden loss of muscular control, with diminution or loss of sensation and consciousness, resulting from rupture or blocking of a blood vessel in the brain (<*Gk. apoplēxiā, from apoplēssein, to cripple by a stroke: apo- (intensive) + plēssein, to strike*) *(American Heritage Dictionary, 2006).*

There are two major groups of cerebral damage: intracerebral haemorrhage and ischemic brain infarction. In the first case there is usually a rupturing of a vessel wall followed by bleeding into the brain parenchyma. In the second one there is no sufficient blood supply to the brain tissue with the result of insufficient oxygen and glucose supply, cell damage and consequently cell death.

The unmodifiable risk factors for stroke include increasing age, gender, positive family history/genetic susceptibility, and ethnicity. In contrast, modifiable risk factors, which can be controlled or treated, include high blood pressure, cigarette smoking, heart disease, diabetes mellitus, carotid or other artery diseases and transient ischemic attacks (TIAs) (National Institute of Neurological Disorders and Stroke, 2004). Identifying and modifying risk factors for stroke may reduce mortality and morbidity.

High blood pressure is by far the dominant cause of vascular disease of the brain, the heart, the legs and other organs (Fisher, 2002). High blood pressure is a risk factor for stroke and coronary artery disease mainly because it promotes vascular deposits of atherosclerosis in the form of cholesterol plaques. Furthermore, as C.M. Fisher and R.D. Adams were able to show in a study that it is only in the presence of high blood pressure that atherosclerosis of the intracranial arteries can take place. The incidence of stroke is related to hemodynamic, vascular and cardiac mechanism, and events such as hypertension, low brain perfusion, atherothrombosis and atrial fibrillation. Treatment of hypertension and associated pathological states presents a significant advantage in the struggle against cerebrovascular disease.

The renin-angiotensin system (RAS) is central to the control of blood pressure and the maintenance of volume and salt homeostasis. Thus, inappropriate RAS activity may lead to

continuing hypertension with ensuing renal pathologies, myocardial infarction, sudden death and cerebrovascular events such as stroke. During the past decade, a number of studies have indicated that the brain RAS may interfere with processes occurring during and after brain ischemia (Culman *et al.*, 2002).

This thesis is an attempt to study in detail the function and influence of RAS in the brain and, more specifically, to provide more precise information about the regulation of AT_1 and AT_2 Ang II receptor subtypes and their potential relevance for parenchymal changes after focal cerebral ischemia. For this purpose, transient unilateral medial cerebral artery occlusion in rats was performed, simulating as closely as possible an ischemic brain incident in humans (Dai *et al.*, 1999). Since the stroke outcome depends on the extent of brain tissue changes— glial and neuronal death or regeneration— and later tissue repair and convalescence, we were looking for processes of inflammation, apoptosis and regeneration. We hope we have been able to contribute to a better understanding of the pathophysiological processes in the brain that take place after ischemic stroke and to discovering and improving effective prevention and treatment of stroke.

List of abbreviations

Ab	antibody
AC	adenylate cyclase
ACE	angiotensin-converting enzyme
Ang I	angiotensin I
Ang II	angiotensin II
AT_1	angiotensin II receptor type 1
AT_2	angiotensin II receptor type 2
BBB	Blood-brain barrier
cAMP	adenosine 3', 5'-cyclic monophosphate
CBF	cerebral blood flow
cCasp-3	cleaved caspase-3
CCA	common carotid artery
CNS	central nervous system
DAG	diacylglycerol
DEPC	diethyl pyrocarbonate
dNTP	deoxyribonucleotide triphosphate
ECA	external carotid artery
EtBr	ethidium bromide
FCI	focal cerebral ischemia
GFAP	glial fibrillary acidic protein
ICA	internal carotid artery
IP-3	inositol 1,4,5-triphosphate
ITFs	inducible transcription factors
LDF	laser-Doppler flowmetry
MAP	mean arterial blood pressure
MAP_2	microtubule-associated protein
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PNS	Peripheral Nervous System
RAS	renin-angiotensin system
rCBF	regional cerebral blood flow
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SHRSP	stoke-prone spontaneously hypertensive rat
TBE	Tris/Borate/EDTA
TNF-α	tumour necrosis factor α
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling

1. Introduction

1.1 The renin-angiotensin system

The renin-angiotensin system (RAS) plays a major role in the regulation of blood pressure and in maintaining fluid and electrolyte balance (Phillips, 1987).

The first insight into the regulation of blood pressure came from the discovery of a pressor principle by Tigerstedt and Bergman in 1897. They called the factor "renin" because it was extracted from the kidney. In 1940, two separate groups, Braun-Menéndez in Argentina and Page and Helmer in the United States, isolated simultaneously and independently the same vasoconstrictor substance. They named it "hypertensin" and "angiotonin", respectively, but later agreed on the hybrid term "angiotensin". It was soon recognised that not renin but angiotensin II was the major effective peptide of RAS (Skeggs *et al.*, 1956). Its sequence is Asp-Arg-Val-Tyr-Ile-His-Pro-Phe in the human, horse, and pig (de Gasparo *et al.*, 2000).

Ang II is a highly active octapeptide, which is built via several mechanisms. The traditional one is when circulating renin synthesised and released from the juxtaglomerular cells of the kidney cleaves the macroglobulin precursor angiotensinogen to produce the inactive decapeptide angiotensin I. With the help of angiotensin-converting enzyme (ACE), a dipeptidyl-carboxypeptidase, the latter is further degraded to the active octapeptide Ang II (at the same time, ACE regulates the conversion of bradykinin, substance P, enkephalins and other peptides into inactive fragments). Other angiotensin-derived metabolites such as angiotensin 2–8 (Ang III), angiotensin 1–7, or angiotensin 3–8 (Ang IV) have all been shown to have biological activities (Ferrario *et al.*, 1991; Ferrario *et al.*, 1998; Peach, 1977; Schiavone *et al.*, 1990; Timmermans *et al.*, 1993; Wright *et al.*, 1995).

Furthermore, Ang II may also be formed in ACE- and renin-independent pathways via chymase, cathepsin G, chymostatin-sensitive AngII-generating enzyme (CAGE), tissue plasminogen activator (tPA) and tonin, a fact that clarifies the limitations of ACE-inhibitors' effects (see Fig 2).

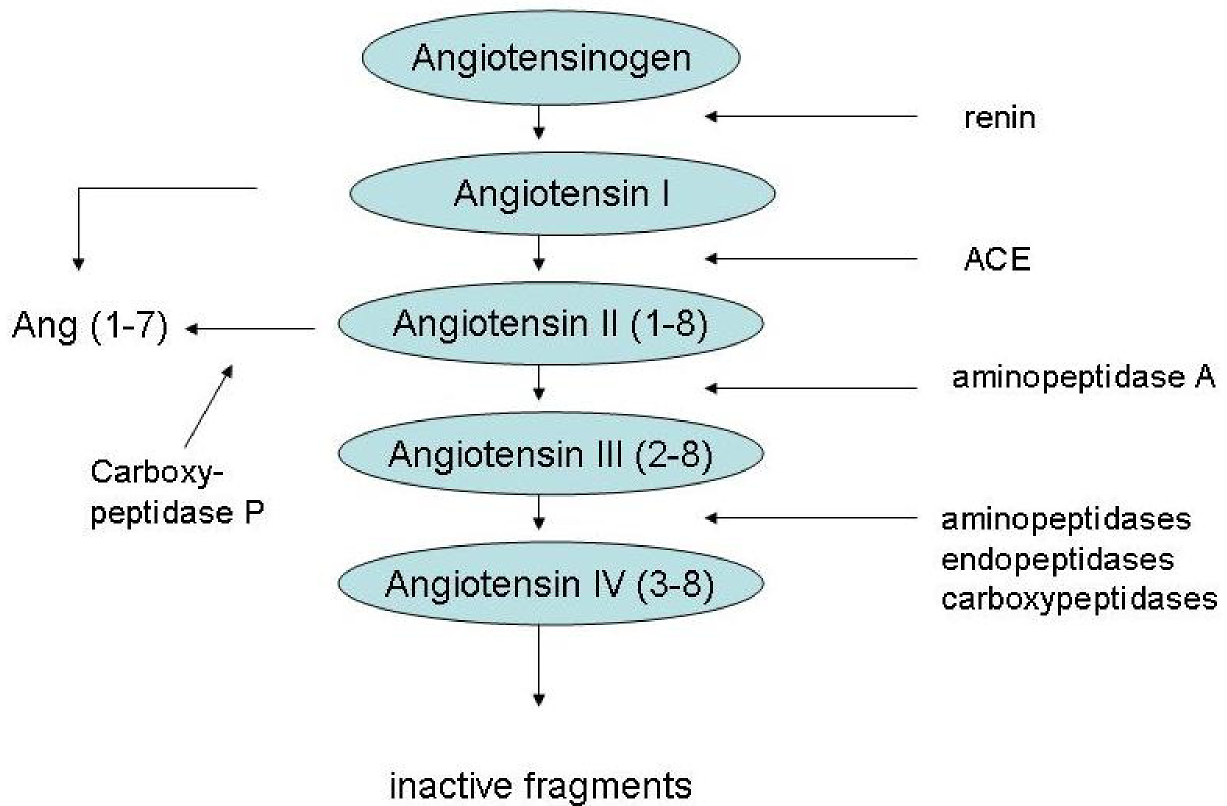


Figure 1: The Renin-angiotensin system: enzymatic synthesis and degradation.

Apart from the systemic RAS, there is evidence that indicates production of Ang II by other tissues such as the heart, the vasculature and the brain. This fact explains its autocrine, paracrine and intracrine hormonal properties which may influence cell growth and proliferation and the formation of extracellular matrix (Campbell, 1987; Johnston, 1992; Unger *et al.*, 1988; Unger *et al.*, 1991), thus contributing to myocard hypertrophy and interstitial connective tissue proliferation in heart insufficiency patients, for example.

Since RAS plays an important role not only in regulating cardiovascular homeostasis but also in promoting the development of various cardiovascular diseases, such as hypertension, congestive heart failure, coronary ischemia, renal failure (Dzau, 1994) and stroke, it is necessary to study the angiotensin receptors and their pathways to be able to interfere with its over stimulation and adverse effects.

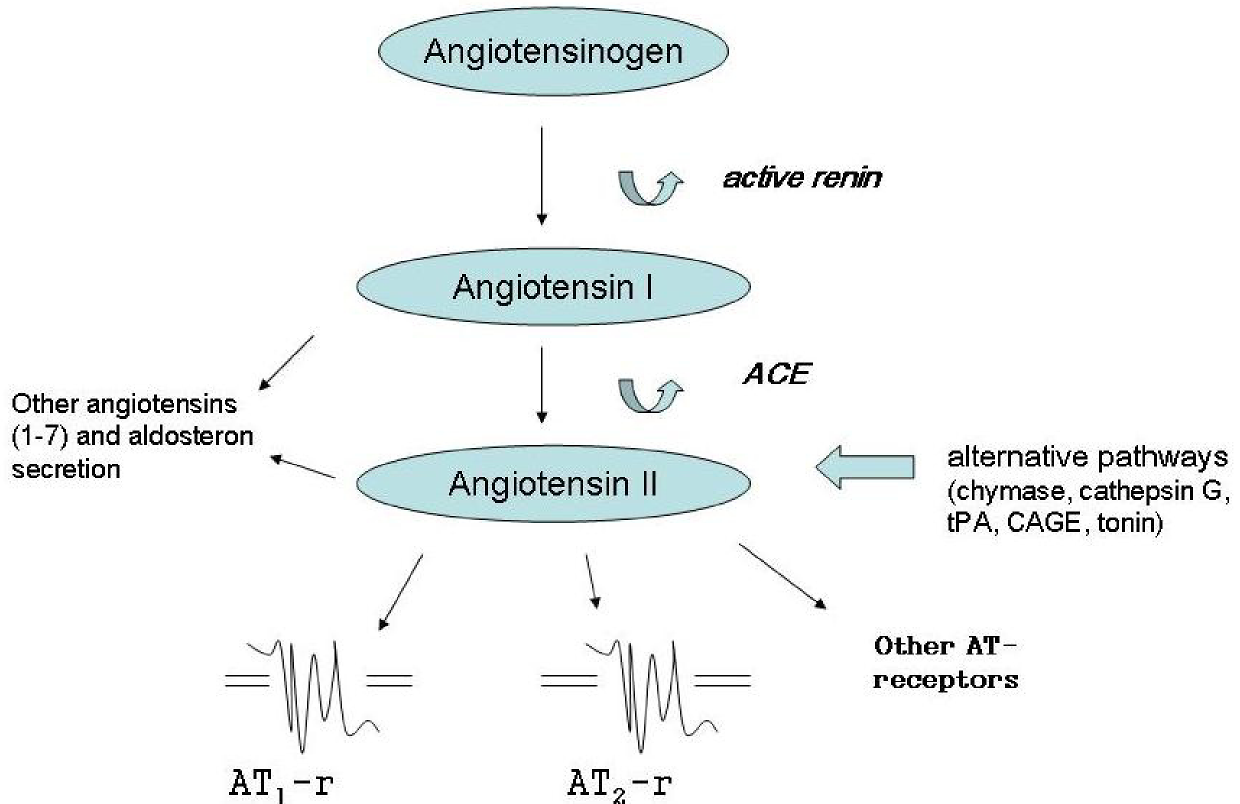


Figure 2: Formation of Angiotensin I and II.

1.2 Angiotensin II receptor subtypes and their functions

In the body, Ang II exerts its effects on blood pressure and fluid homeostasis by binding to receptors. Lin and Goodfriend made the discovery of the latter in 1970 with a binding reaction of radioiodinated Ang II (monoiodoangiotensin-^{125}I). They were able to show various target tissues of Ang II and typical hormone-receptor specific binding, but lacked antagonistic drugs or antibodies to receptors to prove the relation between hormone binding and response (Lin *et al.*, 1970). In the years following, four Ang II receptors have been proposed, but not all fulfil the required classification criteria established by Humphrey et al., 1994, to identify and characterise distinct receptors. Only the AT_1 and AT_2 receptors types fulfil the three main criteria - operational, transductional and structural. The AT_3 and AT_4 receptor types could be isolated based on operational criteria, but their transduction mechanisms are unknown and they have not yet been cloned (de Gasparo *et al.*, 2000).

Only the discovery of non-peptidergic selective receptor blockers, DuP753 and PD123319, made possible the differentiation between the two main receptor subtypes: AT_1 and AT_2 (Blankley *et al.*, 1991; Duncia *et al.*, 1990). DuP753 selectively inhibits the AT_1 receptor

whereas PD123319 shows properties of effectively blocking the AT_2 receptor (Sumners *et al.*, 1991).

Both AT_1 and AT_2 receptors contain seven hydrophobic transmembrane segments forming α helices in the lipid bilayer of the cell membrane but differ in their chromosomal locations, amino acid sequences, coupling mechanisms and effects, and tissue distribution (Allen *et al.*, 1999b; Dinh *et al.*, 2001).

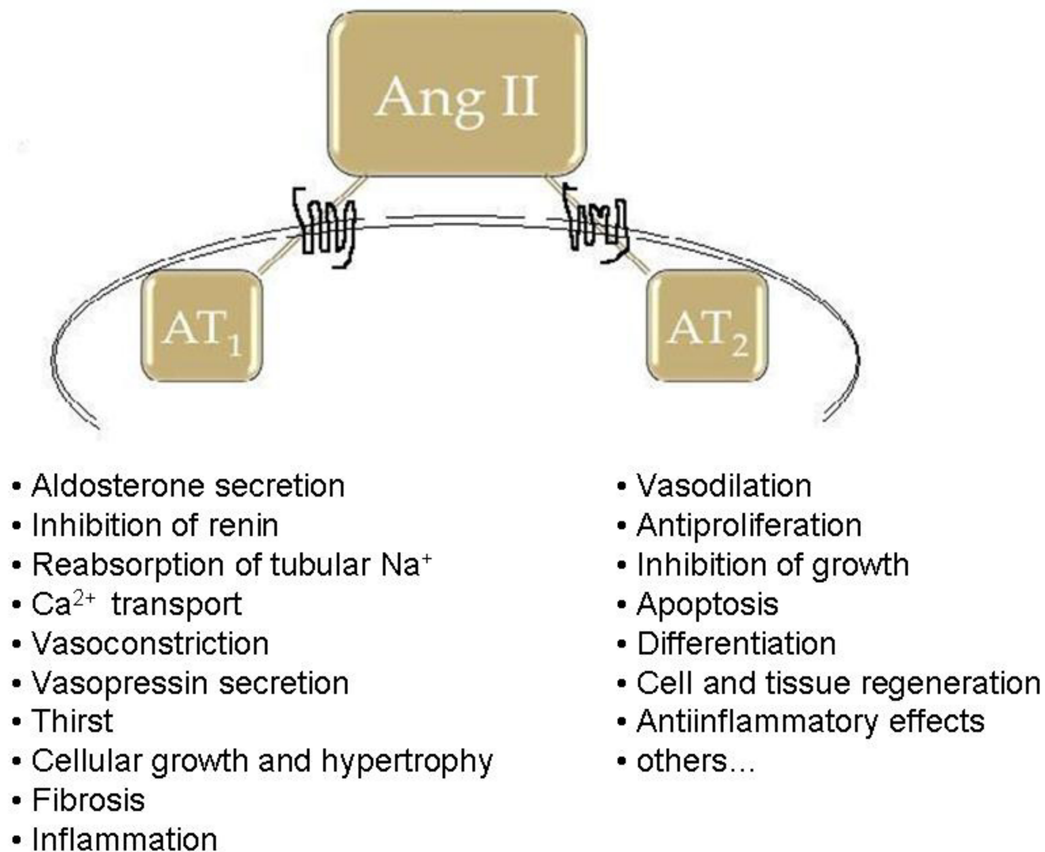


Figure 3: Effects of AT_1 and AT_2 receptor subtypes, respectively.

1.2.1 AT_1 receptor subtype

Properties

The angiotensin AT_1 receptor mediates practically all of the known physiological actions of Ang II, such as the regulation of arterial blood pressure, ADH release and electrolyte and water balance, thirst, hormone secretion, renal function and cellular growth (See Fig.3) (de Gasparo *et al.*, 2000). It is widely distributed in diverse adult tissues: in vascular smooth muscle cells, heart, kidneys and adrenal glands, as well as in the brain— a fact that correlates with its fundamental role in body physiology (Allen *et al.*, 2000).

The AT_1 receptor was cloned and localised on chromosome 3 in humans, and on chromosomes 2 and 17 in rats. The human AT_1 receptor contains 359 amino acids (Inagami *et al.*, 1994).

AT_1 cDNA clones and proteins isolated from mouse, rat and human tissues show significant homologies, as well as the two isoforms AT_{1a} and AT_{1b}. The latter are found only in rodents and share 95% similarity of amino acid sequences (Sasamura *et al.*, 1992). AT_{1a} and AT_{1b} receptors exhibit similar ligand binding and signal transduction properties but differ in their tissue distribution and transcriptional regulation, including the CNS (de Gasparo *et al.*, 2000). Whereas AT_{1a} mRNA is expressed predominantly in the hypothalamus and the median eminence, AT_{1b} mRNA is found in the cerebellum, subfornical organ and lamina terminalis (Kakar *et al.*, 1992).

The two AT_1 isoforms in the rodent genome may be the consequence of a gene duplication event that occurred during evolution after the branching of rodents from the mammalian phylogenetic tree (Aiyar *et al.*, 1994).

Structure and signal transduction:

The AT_1 receptor belongs to the G protein-coupled seven transmembrane receptor subtypes (GPCRs) (Griendling *et al.*, 1993). Four cysteine residues located in the extracellular domain represent sites of disulfide bridge formation and are essential for Ang II binding. The transmembrane domain, the N terminus and the first and third extracellular loops play an important role in Ang II binding, whereas the intracellular third loop domains of Ang II receptors are key regions for G protein coupling. The binding site for AT_1 receptor antagonists is different from the one for Ang II and interacts with the transmembrane domains of the receptor.

A proposed model of GPCR activation predicts that rigid body movement of the third, sixth, and seventh transmembrane domains induces conformational changes in the cytoplasmic loops that permit G protein interaction with the agonist-activated receptor (Gether *et al.*, 1998). Recent studies reported that the intracellular third loop domains of Ang II receptors is not only the key region for G protein coupling and the subsequent enzyme cascades but also the critical determinant for the mutually antagonistic AT_1 and AT_2 receptors' signalling pathways (Daviet *et al.*, 2001).

The AT_1 receptor is coupled to the heterotrimeric G_q or G_i proteins. Like most of the G-protein coupled receptors, when stimulated by Ang II, the AT_1 receptor undergoes internalization in endosomes and after dissociation from the ligand is transported back to the membrane (Hein *et al.*, 1997).

There are five classic signal transduction pathways for the AT_1 receptor: activation of phospholipase A_2 (PLA_2), phospholipase C and D (PLC/D) and L-type Ca^{2+} channels, and inhibition of adenylate cyclase (AC) (Dinh *et al.*, 2001).

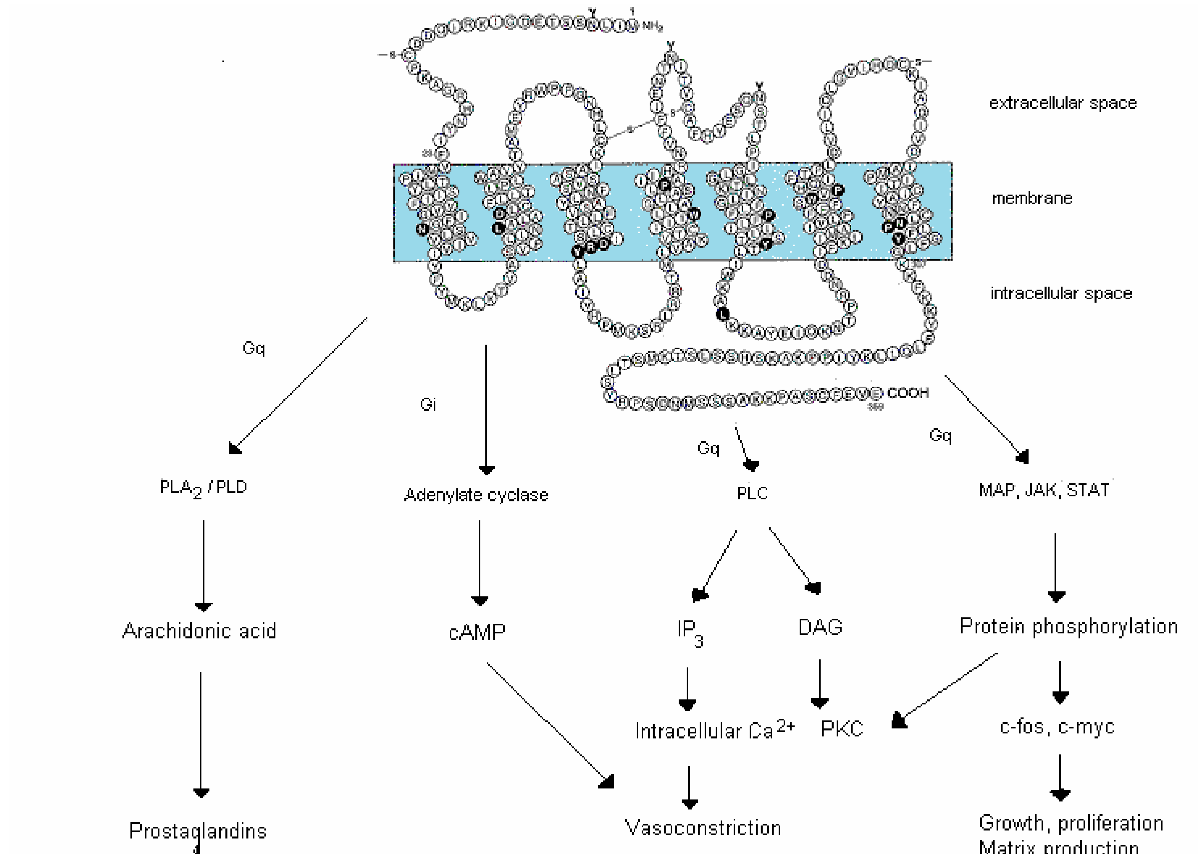


Figure 4: Signal transduction pathways and AT_1-receptor mediated effects (modified after D.T. Dinh et al.).

Activation of PLD and PLA_2 through the G_q protein releases arachidonic acid from membrane phospholipids and initiates synthesis of eicosanoids.

The inhibition of AC via G_i protein reduces the cAMP amount in several tissues (kidney, liver, adrenals) thus also leading to vasoconstriction (Dinh *et al.*, 2001).

Although the AT_1 receptor has been reported to interact with several G proteins, its major physiological functions are expressed through G_q-mediated activation of phospholipase C-β (PLC-β) followed by formation of diacylglycerol (DAG) and inositol trisphosphate— Ins (1,4,5)P_3. The first induces protein kinase C (PKC) activation and influx of extracellular Ca^{2+},

while tyrosine phosphorylation of PLC-γ stimulates release of stored Ca^{2+}, both causing vasoconstriction and expression of inducible transcription factors (ITF) (Blume *et al.*, 1999).

It has been reported that activation of AT_1 receptor induces tyrosine phosphorylation and phospholipase C-γ, thus activating downstream proteins: the phosphorylation-dependent mitogen activated protein (MAP) kinase, the Janus kinases (Jak) and the signal transducers and activators of transcription (STAT) proteins (Marrero *et al.*, 1995). These pathways involve increased expression of ITFs, such as *c-fos, c-myc* and *c-jun,* and are believed to be involved in AT_1 receptor-mediated cell proliferation and growth (Marrero *et al.*, 1995; Schieffer *et al.*, 1996). They have been linked in this way to hypertension, cardiac failure and atherosclerosis (Dinh *et al.*, 2001).

Furthermore, Ang II-mediated activation of the c-jun N-terminal kinase- stress-activated protein kinases (JNK/SAPK) is reported to be responsible for apoptotic (Xia *et al.*, 1995) and neuroprotective processes (Reimold *et al.*, 1996).

Distribution and function

The distribution of the AT_1 receptor correlates closely with the function and effects of Ang II. The wide spectrum of Ang II target tissues includes the adrenals, kidney, vascular smooth muscle, brain and the sympathetic nervous system (de Gasparo *et al.*, 2000). Respectively, AT_1 receptors, though ubiquitously distributed in adult tissues, are primarily found in the adrenals, kidney, heart, vasculature and brain, and serve to control blood pressure through vasoconstriction and regulate fluid and electrolyte balance through sodium reabsorption (Dinh *et al.*, 2001).

As mentioned above, the angiotensin receptor was identified as a functional entity by Lin and Goodfriend (1970) in the adrenal gland. In the adrenal cortex, Ang II via AT_1 receptors induces aldosterone secretion, consequent sodium reabsorption and rise in the blood pressure.

In the human kidney, AT_1 receptors are expressed in the renal vasculature, glomeruli, and the vasa recta bundles in the inner stripe of the outer medulla (Goldfarb *et al.*, 1994). Apart from controlling salt homeostasis, they may be involved in the pathogenesis of glomerulosclerosis due to Ang II-induced hypertrophy and proliferative responses (Orth *et al.*, 1995).

The same pathomechanism is found in cardiac myocytes and fibroblasts (de Gasparo *et al.*, 2000). Along with cardiomyocyte hypertrophy, cardiac fibroblast stimulation appears to facilitate the accumulation and proliferation of collagen in the extracellular matrix of the heart (Brilla *et al.*, 1995) and both contribute to the development of ventricular hypertrophy independently of blood pressure.

Being one of the most potent endogenic pressor substances, Ang II owes this to a great extent to its effect on the vasculature. AT_1 receptors have been found in the aorta, pulmonary and mesenteric arteries (Zhuo J *et al.*, 1995), in the endometrial blood vessels, the human placenta and the fetal vascular endothelial cells (Cooper *et al.*, 1999), where they cause a contraction of the vascular smooth muscle and mediate the contractile and hypertrophic effects of Ang II.

In the brain, AT_1 receptors are abundant in the hypothalamus, more precisely, in the parvocellular region of the paraventricular nucleus (PVN), which regulates anterior pituitary hormone secretion, cardiovascular function and fluid/electrolyte balance (Allen *et al.*, 1998; Culman *et al.*, 1995). Additionally, AT_1 receptors exist in the brain in areas devoid of blood brain barrier, such as the circumventricular organs (subfornical organ, median eminence, vascular organ of lamina terminalis, anterior pituitary and area postrema of the hindbrain) (de Gasparo *et al.*, 2000), which allow access to blood-borne hormones from the periphery (Steward, 2000).

1.2.2 AT_2 receptor subtype

Properties

While the AT_1 receptor has been extensively studied, less is known about the signalling pathways and various properties of the AT_2 receptor (Chung *et al.*, 1998). The AT_2 receptor shows significant differences in molecular weight, tissue distribution, regulation of expression and signalling pathways. Although the AT_1 receptor has been considered the main mediator of the Ang II effects, studies in the recent years revealed important AT_2-mediated effects (Csikos *et al.*, 1998).

The development of cell lines expressing only the AT_2 but not the AT_1 receptor, such as PC12W and R3T3, facilitated the cloning and studying of that "new" Ang II receptor type. In rat, it has been cloned and studied by Nakajima, Mukoyama et al. in 1993 and Koike et al. in 1995, (Koike *et al.*, 1995; Mukoyama *et al.*, 1993) followed by the decoding of mouse and human AT_2 genome by Ichiki and Koike in 1994 (Ichiki *et al.*, 1994; Koike *et al.*, 1994).

The coding sequence of the human AT_2 receptor and other fragments could be cloned too, and it was found to be 72% identical with the rat one (Koike *et al.*, 1994; Martin *et al.*, 1994; Tsuzuki *et al.*, 1994). It is encoded by 363-aminoacid protein and is located on chromosome X.

Although Ang II binds to AT_2 receptor with similar affinity as it does to the AT_1 receptor, it initiates different, even contrary, effects. Where the AT_1 receptor promotes inflammation, tissue proliferation and hypertrophy, the AT_2 receptor promotes anti-inflammatory, anti-

proliferative events and cell apoptosis, or tissue regeneration and remodelling (See Fig. 3) (Culman *et al.*, 2002; Volpe *et al.*, 2003).

Thus we may look upon it as an identical twin when regarding its structure and Ang II binding affinity, but one having a lot of contrary actions (Berk, 2003).

Structure and signal transduction:

The AT_2 receptor, too, belongs to the seven transmembrane domain receptors but its sequence is only 34% identical with that of its AT_1 counterpart. It comprises five potential N-glycolysation sites in the extracellular N-terminal domain and 14 cystein residues. AT_1 and AT_2 receptors differ in the structure of the third intracellular loop and the carboxyl terminal tail (C-tail) (de Gasparo *et al.*, 2000). These were shown to be essential for coupling with G_i and SHP-1, respectively (Feng *et al.*, 2002). In addition, on the C-tail there is a binding domain for the transcription factor promyelocytic zinc finger protein (PLZF) which can modulate the signalling mechanism (Senbonmatsu *et al.*, 2003). Some studies proved that the fifth and sixth transmembrane domains interact directly with the agonist Ang II (Kurfis *et al.*, 1999; Lehtonen *et al.*, 1999a; Pulakat *et al.*, 1998; Turner *et al.*, 1999). Prolonged binding of Ang II to the AT_2 receptor, however, does not induce desensitization or downregulation as it is typical with other GPCRs (Hein *et al.*, 1997; Hunyady *et al.*, 1994). There is rather an upregulation of its surface expression which is followed by the activation of various phosphatases: MAP kinase phosphatase 1 (MKP-1) (Fischer *et al.*, 1998; Horiuchi *et al.*, 1997), SH2-domain containing phosphatase 1 (SHP-1) (Bedecs *et al.*, 1997) and serine/threonine phosphatase 2A, and results in the inactivation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Huang *et al.*, 1996; Shenoy *et al.*, 1999).

Furthermore, extensive studies showed that the AT_2 receptor does not require Ang II binding to initiate signalling but it is constitutively active (Miura *et al.*, 2002). Feng et al. presented evidence that AT_2 couples to $G\alpha_s$ independently of $G\beta$ and $G\gamma$ (Feng *et al.*, 2002). The presence of $G\alpha_s$ alone leads to activation of SHP-1, which dissociates from the AT_2 receptor and remains bound to $G\alpha_s$. Another important discovery was made by Abdalla et al.. The group showed that the AT_2 receptor binds directly to the AT_1 receptor, and probably by means of changing its conformation, directly antagonises it (Abdalla *et al.*, 2000).

Because of this and other properties, the AT_2 receptor is viewed as an atypical, unique GPCR.

Yet in comparison to AT_1, relatively little is known about its signal transduction pathways: activation and/or induction of a series of phosphatases (SHP-1, MKP-1 and PP2A), stimulation of ceramide synthesis, followed by MKP-1 activation, Bcl-2 dephosphorylation and induction of caspase-3- eventually leading to apoptosis (Lehtonen *et al.*, 1999b). Studies on neuroregeneration in vitro showed upregulation of polymerized β-tubulin (Laflamme *et al.*, 1996) and MAP_2 (Stroth *et al.*, 1998). In models of sciatic nerve lesion, AT_2 receptor stimulation leads to activation of NF-κB in Schwann cells (Reinecke *et al.*, 2003). The development of selective receptor ligands enables scientists to further characterise the structure and functioning of the AT_2 receptor.

Distribution and function

Distribution of the AT_2 receptor appears to be tissue- and species-specific.

The AT_2 receptor is highly expressed during foetal development in most tissues. It rapidly decreases after birth and in the adult organisms its distribution is limited mostly to the brain, heart, adrenal medulla, kidney and reproductive tissues (Gehlert *et al.*, 1991; Grady *et al.*, 1991; Millan *et al.*, 1991).

Yet, a strong AT_2 receptor expression may reappear in adults under pathological conditions, such as skin or vascular injury, heart failure and cardiac remodelling after infarction, nerve injury, brain lesions and post-ischemic recovery (Akishita *et al.*, 2000; Unger *et al.*, 1996; Yamada *et al.*, 1996).

Although the distribution of the AT_2 receptor in the CNS differs among the species, in the human brain it is predominantly found in the sensory areas: thalamic nuclei, medial geniculate nucleus and inferior colliculus (Allen *et al.*, 1999a), and in the cerebellar nuclei, inferior olive, and locus caeruleus (Obermuller *et al.*, 1991). This could be important for cognitive functions and modulation of behaviour such as exploration or drinking (Hein *et al.*, 1995), and antagonism of AT_2 receptor-mediated central osmoregulation (Culman *et al.*, 1995; Hohle *et al.*, 1995; Hohle *et al.*, 1996). Furthermore, after nerve lesions, the AT_2 receptors seem to take part in the neuroregenerative and neuroprotective processes (Gallinat *et al.*, 1998; Unger, 1999).

The presence of low-density AT_2 receptors was demonstrated in the myocardium and coronary vessels in the rat heart (Sechi *et al.*, 1992). Here the fibroblasts are strongly AT_2-receptor-positive, while only about 10% of the cardiomyocytes express the AT_2 receptor compared to the 50% AT_1-receptor-positive myocytes (Busche *et al.*, 2000). As mentioned above, in cases of pathological processes, such as cardiac hypertrophy, myocardial infarction, cardiomyopathy, and congestive heart failure (Matsubara, 1998; Unger, 1999), there is an

increase in AT_2 receptor expression. Typically, it is the border zone between infarcted and non-infarcted areas, which is rich in perivascular AT_2 receptors. At the same time the level of AT_1 receptor expression becomes extremely low (Tsutsumi *et al.*, 1998). This all the more supports the idea that the AT_2 receptor subtype can modulate and even counteract the effects of the AT_1 receptor, in particular, those on blood pressure (Hein *et al.*, 1995; Ichiki *et al.*, 1995; Masaki *et al.*, 1998; Oliverio *et al.*, 1998; Siragy *et al.*, 1999) and cardiac and vascular cell growth (Akishita *et al.*, 1999; Bartunek *et al.*, 1999; Stoll *et al.*, 1995), as well as on tissue regeneration after injury (Janiak *et al.*, 1992; Lucius *et al.*, 1998; Nakajima *et al.*, 1995; Reinecke *et al.*, 2003; Unger, 1999).

In the canine and primate pancreas, AT_2 receptor expression was shown to be particularly high, especially in acinar cells, and the AT_2 receptor appeared to have high binding activity for Ang II (Chappell *et al.*, 1992; Chappell *et al.*, 1991).

In the adrenal medulla of adult organisms AT_2 receptor expression remains relatively high and in rats the ratio of AT_1 to AT_2 receptors reaches about 20:80 (Chang *et al.*, 1990).

The AT_2 receptor expression in the kidney strongly varies during the different developmental stages. Ozono et al. (Ozono *et al.*, 1997) observed AT_2 receptor expression in the mesenchymal cells of differentiating cortex and medulla in the foetal kidney (14th day), while at a later stage and in newborns it was in the glomeruli of the outer cortex layers, tubules and vessels. Later on, in young and adult rats, the glomeruli showed decreased expression, which however increased again under low dietary sodium intake. In human kidney, AT_2 receptors are found in large preglomerular vessels of the cortex (Grone *et al.*, 1992) and in the tubular interstitium (Chansel *et al.*, 1992; Goldfarb *et al.*, 1994).

Another site of high AT_2 receptor expression is the reproductive organs, in particular the nonpregnant uterus. Interestingly, AT_2 receptor expression in myometrium decreases during pregnancy but rises back to its previous level of expression after parturition (de Gasparo *et al.*, 1994).

1.3 RAS and the brain

The chemical transmission in the brain was first clearly stated by John Newport Langley in 1905. He was the first to introduce the idea of a “receptor substance”, or “synaptic substance” probably not in the nerves, but “in the cells in which they end”. Before this reigned the general conviction that neural transmission is solely electrical (Marshall, 1998).

The RAS, however, was first known to act only in the periphery. In 1961 Bickerton and Buckley revealed an Ang II-induced hypertension via a central mechanism, while the existence of an endogenous brain renin-angiotensin system separately from the systemic system─ the kidneys, liver, and lungs─ was demonstrated some 10 years later. In 1971, Fisher-Ferraro et al. were able to demonstrate the presence of renin and angiotensin II in the brain; soon followed the recognition of an autonomous brain RAS that selectively regulates the cardiovascular system and several other brain functions─ sympathetic nerve activity, drinking behaviour, salt appetite, and pituitary hormone secretion (Ganong, 1984; Ganten *et al.*, 1978; Phillips, 1987). Components of the RAS were then found in both neurons and astrocytes (Sumners *et al.*, 1984; Wilkin *et al.*, 1995). These new facts initiated the search for specific Ang II receptors and their function in different brain areas (Ganong, 1993; Phillips, 1987; Wright *et al.*, 1994).

This "central" RAS was found to be activated in spontaneously hypertensive rats (SHR) accompanied by raised Ang II levels and AT_1 receptor expression in the brain (Saavedra, 1992), implying that an overactive brain RAS might be responsible for the development and maintenance of high blood pressure in SHR (Culman *et al.*, 2002).

In recent years, numerous studies indicated that the brain RAS might be the pivot of the processes occurring during and after brain ischemia (Culman *et al.*, 2002). All components of RAS─ angiotensinogen, ACE, renin, Ang I and Ang II─ have been shown by immunohistochemical and biochemical methods. There is evidence for both intra- and extracellular Ang II formation directly from angiotensinogen, that is, without interaction with renin, as well as for non-ACE pathways via acid proteases (Culman *et al.*, 2002; Saye *et al.*, 1993). The distribution of angiotensin peptides and receptors corresponds to the brain areas associated with the above mentioned functions. Ang II presence in lamina terminalis and the hypothalamic supraoptical and paraventricular nuclei mirrors its effect on cardiovascular control; Ang II positive cells in the nucleus tractus solitarius may be related to the modulation of sympathetic vasomotor tone and those in the amygdala and stria terminalis to the effects on learning and memory, and sensory function, respectively (Lind *et al.*, 1985).

The brain angiotensin receptors have been studied and reviewed extensively by Gehlert et al. (1991), Rowe et al. (1992), Saavedra (1992), Song et al. (1992), Höhle et al. (1995). Most of the well defined physiological actions of Ang II in the brain are mediated by AT_1 receptors (Lenkei *et al.*, 1998) and these are found at sites that influence cardiovascular function, fluid and electrolyte homeostasis, and pituitary hormone secretion (Allen *et al.*, 1999a; Allen *et al.*, 1998) or in areas exposed to blood-borne Ang II. Therefore, AT_1 receptors are most abundant in the hypothalamus and the circumventricular organs (see 1.2.1 above). The AT_1-receptor-rich area of

the paraventricular nucleus has been implicated in the control of anterior pituitary hormone secretion, ingestive behavior, and autonomic regulation of the cardiovascular system. In contrast, AT_2 receptors are found in areas with sensory functions, including the thalamic nuclei, medial geniculate nucleus and inferior colliculus (Song *et al.*, 1992).

Furthermore, while in the adult brain the majority of brain areas express predominantly the AT_1 receptor, the AT_2 receptors are highly expressed in fetal (E18) and neonatal brain tissues, and little in adult brain (Tsutsumi *et al.*, 1993). In the adult rat brain, AT_2 receptor expression, which is not correlated with the AT_1 expression, is mainly located in the cerebral cortex (Lenkei *et al.*, 1997).

1.4 Brain damage; cell injury and death

Normal cell function requires a balance between the physiologic demands imposed on it and the constraints of the cell's structure and metabolic capability, which provides for its homeostatic steady state (Kumar V. *et al.*, 1997). Under excessive physiologic stresses or harmful stimuli, cells may undergo adaptation, thus achieving a new steady state and preserve their viability; they may sustain reversible injury, or, beyond a certain point, when the cell's adaptive capabilities are exceeded, they may suffer irreversible injury and subsequently die.

The principal adaptive responses─ hyperplasia, hypertrophy, metaplasia and atrophy─ put the cell in a new state, and allow its viability.

Reversible cell injury tolerates pathologic stimuli or adverse changes to a certain extent. When the stimulus is removed or the injury mild, the process can be reversed.

Irreversible injury denotes pathologic changes that are permanent and fatal and lead ultimately to cell death.

There are two morphologically distinct patterns of cell death: necrosis and apoptosis.

■ Necrosis occurs after exposure to harmful exogenous stimuli and is characterized by membrane dysfunction, stopping of the membrane ion pumps, severe cell swelling, denaturation and coagulation of proteins, breakdown of organelles and cell rupture. The occurring events do not kill the cell immediately, but initiate a series of intra/extracellular cascades that lead unavoidably to cell death (Fawcett *et al.*, 2001). A crucial event may be the generation of free radicals within the cell, particularly due to mitochondrial damage. Usually, the necrotic process causes an inflammatory response with further accumulation of cytokines and rapid cell demise (Choi, 1992; Kumar V. *et al.*, 1997).

■ Apoptosis is manifested by activation of an internal suicide program and is energy-dependent. It is a carefully orchestrated disassembly of cellular components while the cell membrane is relatively preserved, and it serves the elimination of unwanted cells with minimal effects on surrounding tissues and inflammatory progress. It plays an important part in embryogenesis and development by selectively eliminating unwanted or superfluous cells. It has a physiological action in the involution of endometrium and prostate, or in proliferating cell populations such as in tumours (Kumar V. *et al.*, 1997). It also contributes to death of cytotoxic T cells, neutrophils and virus-infected cells. Chromatin condensation and fragmentation, cellular blebbing and phagocytosis by macrophages without ongoing inflammation are its typical morphological signs (Kumar V. *et al.*, 1997). However, it may be difficult to demonstrate histologically, because it occurs usually in single clusters of cells and does not cause inflammation.

The adverse effects leading to cell injury can be grouped into broad categories, such as:

- oxygen deprivation (hypoxia) resulting from ischemia, inadequate oxygenation or loss of oxygen-carrying capacity of the blood (anaemia);
- physical agents such as mechanical trauma, heat, cold, radiation and others;
- chemical agents and drugs: acetaminophen, lead, alcohol;
- infectious agents: viruses, rickettsiae, bacteria, fungi and parasites;
- genetic derangements as a result of chromosomal aberrations or gene mutations;
- nutritional imbalances, such as protein-calorie deficiency, lack of specific vitamins or over nutrition.

Ischemic and hypoxic injury is the most common type of cell injury in clinical medicine, and the cellular response depends on the duration and severity of ischemia (Kumar V. *et al.*, 1997). At first, the characteristic pathologic changes may be reversed and the injury repaired if oxygen supply is restored. With the progression of the ischemia, however, the energy-generating machinery of the cell becomes irreparably damaged (“point of no return”) and the cell cannot be rescued anymore. Five general biochemical processes are of importance:

- decreased ATP synthesis and ATP depletion leading to slowing or stopping of the sodium/potassium and calcium/hydrogen ion exchange pumps,
- oxygen-derived free radicals due to imbalance between free radical-generating and scavenging systems,
- loss of Ca^{2+} homeostasis and increased intracellular Ca^{2+} leading to activation of phospholipases, endonucleases, proteases, and ATPases,
- defects in membrane permeability and

- irreversible mitochondrial damage with formation of a high conductance channel— the so-called *mitochondrial permeability transition* (MPT)— in the inner mitochondrial membrane. This results in leakage of cytochrome c (cyt c) into the cytosol and activation of the apoptotic death pathways (Kumar V. *et al.*, 1997).

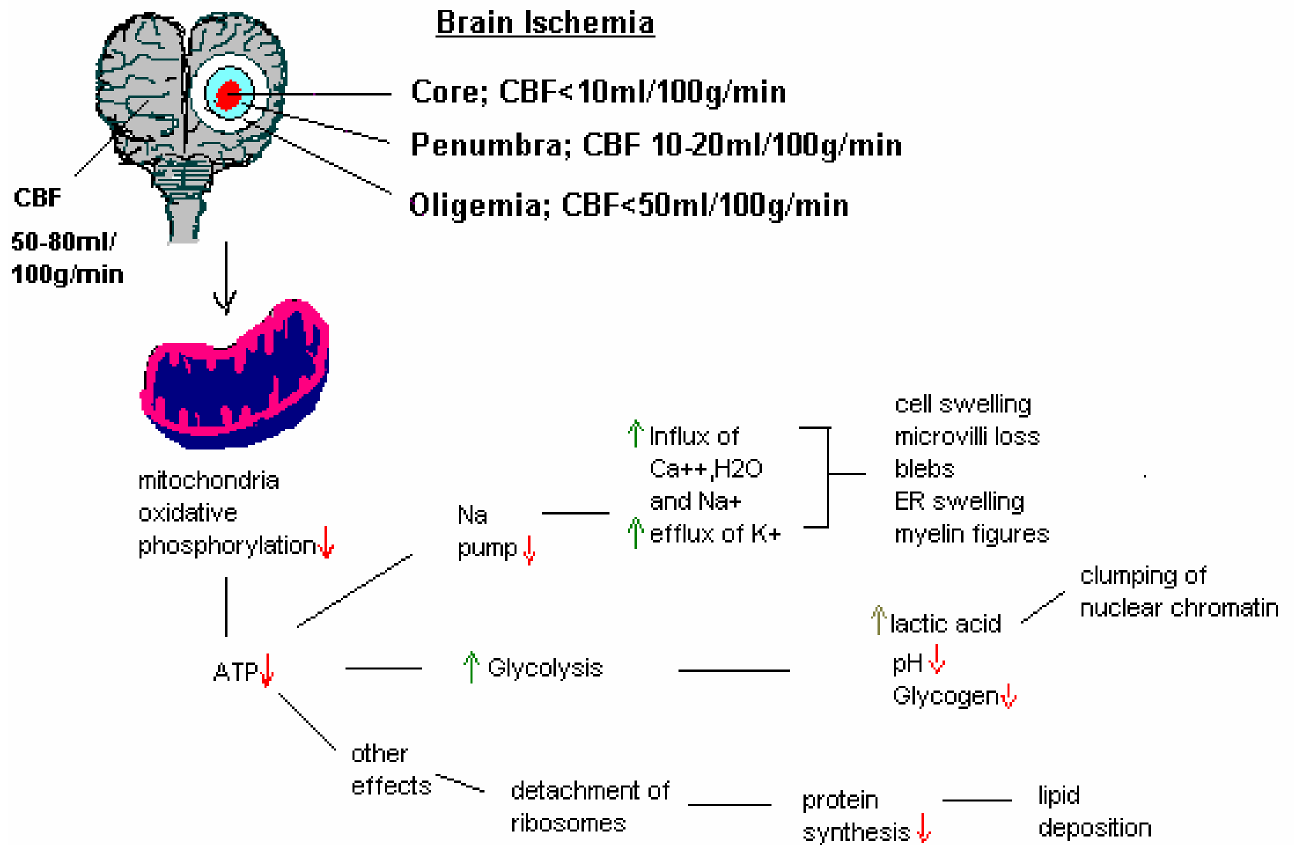


Figure 5: The process of cell injury following decreased intracellular ATP showing still reversible state. Further depletion of ATP will lead to cell death, typically by necrosis.

With the restoration of blood flow, the cells are exposed to further danger: the injury of previously ischemic cells may be exacerbated. This additional damage typically befalls cells that have been irreversibly damaged and is designated ***ischemia-reperfusion*** injury. Cells die either by necrosis or apoptosis, and there is usually a neutrophilic infiltrate. Reperfusion injury is a clinically important process that contributes to tissue damage during myocardial infarction and stroke. Following mechanisms are supposed to lie behind it: ATP depletion, increased cytosolic Ca^{2+}, reactive oxygen species and induction of MPT, release of CytC and accumulation of metabolic intermediates. Furthermore, adjacent parenchymal or endothelial cells produce cytokines, which recruit polymorphic neutrophils and enhance the injury by way of inflammatory response (Kumar V. *et al.*, 1997).

Only recently it was realised how important the part of cell death is in the development, homeostasis and injury responses in the CNS. In the last decade, it could be shown that hypoxic neurons die by way of both necrosis and apoptosis (Ferrer *et al.*, 1994; Okamoto *et al.*, 1993).

Necrosis is the sum of the morphologic changes that follow cell death in the anoxic brain tissue. In the brain, autolysis prevails over protein denaturation and the necrotic area is soft and oedematous (liquefaction necrosis). A rise in glutamate level, released from both neurons and glial cells, increases their membrane permeability for Ca^{2+}, furthering cell damage. A significant rise in intracellular Ca^{2+} in neurons will lead to necrosis, while a moderate one will induce apoptosis.

In 1993, Linnik et al. were the first to suggest the presence of apoptosis in cerebral ischemia, later supported and expanded by Manev et al. and Braun et al. (Braun *et al.*, 1996; Manev *et al.*, 1994).

Typical apoptotic markers are found among the members of the tumour necrosis factor (TNF) family of receptors (TNFR). Among the best-defined signalling pathways for apoptosis are Fas- and Fas ligand-mediated initiation of caspases, and Bax- and Bad-controlled release of cytC from mitochondria with consequent caspase activation. The cytokine TNF and the tumour-suppressor gene, p53, occurring with DNA damage, can likewise lead to apoptosis by way of activating the proteolytic caspase cascade, also called “terminal proteolysis”.

Caspases are cysteine proteases, which exist as zymogens that are activated by cleavage. They can break down nuclear DNA or cytoskeleton proteins (actin filaments, e.g.), or cleave other caspases, thus forming a positive feedback loop. They represent the final common pathway that leads to cell death. Different caspases are involved in different routes to apoptosis but Caspase-3 is one of the key executioners of apoptosis (Cohen, 1997).

There is a certain control stage, in which, depending on the ratio between various positive and negative regulators and factors, the apoptotic threshold is reached or not, and the cell returns to life or dies. This dynamic state is mirrored in the presence of apoptotic neurons several days after ischemia (Kumar V. *et al.*, 1997).

Generally, the central lesion or core in cerebral ischemia is conventionally considered necrotic (Garcia *et al.*, 1995a; Garcia *et al.*, 1995b; Lipton, 1999), whereas the adjacent tissue is said to show predominantly apoptotic mechanisms (Charriaut-Marlangue *et al.*, 1996; Li *et al.*, 1995a; Li *et al.*, 1995b; Linnik *et al.*, 1993). However, accumulating evidence points to a common continuous process of both mechanisms, where injury exposure time, Ca^{2+} or glutamate accumulation, DNA damage and other factors, determine the direction of the pathway.

1.5 Brain repair; neuronal regeneration and neuroprotection

The events that follow CNS damage are either neuron degeneration and death or regeneration and growth. Whereas in the PNS cut mammalian axons regenerate well, it is considered that in the adult CNS these seldom regenerate or spontaneously recover (Fawcett *et al.*, 2001). Obviously not only the axon itself determines which way to take, but its interplay with the environment (permissive or inhibitory) influences the neuronal regeneration. In other words, failure of the neuron to recover might be due to an intrinsic inability or to inhibitory surroundings.

The recognition that Ang II, acting via AT_2 receptors, may modulate tissue regeneration and protection, differentiation and processes leading to programmed cell death is one of the most exciting discoveries of the last decade (Culman *et al.*, 2002; de Gasparo *et al.*, 2000).

1.6 Pharmacological intervention in focal brain ischemia

Owing to the high morbidity and mortality of stroke incidents, there have been many attempts to intervene pharmacologically and reduce or obviate the fatal damages in the brain. The penumbra area has been the main therapeutic target in focal cerebral ischemia (see §1.4, Fig.5) (Ginsberg *et al.*, 1994; Hossmann, 1994). In the present study, we examined the expression of AT_1 and AT_2 Ang II receptor subtypes on different cell populations, as well as other inflammatory or apoptotic and neuroregenerative markers. We thus hoped to acquire more information about future treatment possibilities, such as with AT_1- RB or AT_2- agonists.

1.7 Working hypothesis

The renin-angiotensin system has been involved in the development of end-organ damage, including cerebral ischemia in experimental as well as in clinical conditions. Recently, Walther et al. (Walther *et al.*, 2002) demonstrated a direct correlation between brain Ang II and the severity of ischemic injury in transgenic mice after focal cerebral ischemia. Yet the mechanisms of the protective effects of AT_1 receptor antagonists in cerebral ischemia have not been fully elucidated, nor the role of the AT_2 receptors in the same process.

This gave rise to our several points of interest:

- Influence of MCAO on AT_1 and AT_2 receptor expression

The lack of blood supply, i.e., of oxygen, during focal cerebral ischemia, as well as the impaired auto regulation of CBF, leads to infarction of brain tissue. Clinical studies and animal models provided knowledge about the beneficial effects of the blood-pressure-lowering ACE-

inhibitors and AT_1 receptor antagonists. It was not fully clear whether these effects were due to normalized CBF auto regulation (Culman *et al.*, 2001; Nishimura *et al.*, 2000; Walther *et al.*, 2002) or whether other mechanisms activated by the drugs, independent of blood pressure changes or cerebrovascular regulation, were responsible for reduction in neuronal injury (Nishimura *et al.*, 2000; Paulson *et al.*, 1988; Saavedra *et al.*, 2001; Vraamark *et al.*, 1995). New questions arose about the interaction between Ang II and its AT_1 and AT_2 receptors.

- What quantitative difference exists between AT_1 and AT_2 receptor expression in a normal rat brain, and how does it change after cerebral injury? What is the possible explanation for it?
- How the expression of AT_1 and AT_2 receptors changes after focal cerebral ischemia? Many studies have shown the AT_1 and AT_2 receptor distribution and predominance in different brain areas (see 1.3). However, their exact expression by particular cell types- neuronal, glial or microglia cells- had yet to be determined.
- What other histological changes, promoted by Ang II, can be observed?
- Does brain AT_2 receptor promote inhibition of cell proliferation and apoptosis as well as neuronal regeneration?
- Markers showing AT_2-mediated cell differentiation?

2. Materials

2.1 General chemical substances

Acetone	Baker, Deventer, NL
Acetic acid	Merck, Darmstadt, G
Acrylamide	Roth, Karlsruhe, G
Agarose	Eurogentec, Köln, G
Ammoniumpersulfate	Sigma, Taufkirchen, G
Aqua destillata	Invitrogen Life Technologies, Karlsruhe, G Millipore (Milli Q) water distilling apparatus
2-Deoxy-D-Glucose	Sigma, Taufkirchen, G
96% EtOH	Merck, Darmstadt, G
Glycin	Serva, Heidelberg, G
Guanidine hydrochloride	Sigma, Taufkirchen, G
Isopropanol	Baker, Deventer, NL
Isotone NaCl- 0.9%	B. Braun, Melsungen, G
Potassium chloride	Merck, Darmstadt, G
Magnesium chloride	Merck, Darmstadt, G
Paraformaldehyde	Sigma, Taufkirchen, G
Pyronin G/Y	Sigma, Taufkirchen, G
Skimmed milk powder	Roth, Karlsruhe, G
Sodium lauryl sulphate	Sigma, Taufkirchen, G
Sodiumacetate, -chloride	Merck, Darmstadt, G
TEMED	Sigma, Taufkirchen, G
Tris HCl	Roth, Karlsruhe, G
TritonX 100	Sigma, Taufkirchen, G
Trizol Reagent	Invitrogen life technologies, Darmstadt, G

2.2 Buffers and solutions

Some special solutions are to be found in the corresponding sections.

10 x PBS: 80 g NaCl (Merck), 2 g KCl, 2.5 g KH_2PO_4 and 13.5 g Na_2HPO_4; $2H_2O$ were dissolved in 800 ml distilled water. The pH was adjusted to 7.4 and a final volume of 1000 ml was achieved by adding distilled water.

AEC working solution: 50 mg AEC (Merck, Darmstadt, Germany)
mixed solution consisting of: 150 ml dd H_2O, 35 ml sodium acetate (0.2 M) and 15 ml acetic acid (0.2M). Final step of:
Working solution: The AEC was added to 10 ml dimethylformamid (N-N), 200 ml mixed solution and 100 µl peroxidase substrate (H_2O_2) and everything was well mixed.

10%, 5% or 1% blocking serum: depending on the separate antibodies we used, either a horse, donkey or goat serum was used to block unspecific binding activity. To prepare 6 ml of these in the given concentrations, the serum was diluted as follows:

600 µl serum and 5400 µl PBS, or

300 µl serum and 5700 µl PBS or
60 µl serum and 5940 µl PBS, respectively.

TBE:

we took 108 g Tris Base, 55 g Boric acid, 40 ml EDTA (pH 8.0) to prepare 10x TBE, then dilute 1:9 with deionised water to achieve final concentration: 89 mM Tris base, 89mM Boric acid and 2 mM EDTA (1x solution).

0.25% Triton X-100 in PBS:

2.5 ml Triton X-100 (Sigma) was added to 997.5 ml PBS (1%, pH 7.4)

5% goat serum in PBS:

50 µl normal goat serum (Vector Lab.) was added in 950 µl PBS and mixed.

10% donkey serum diluted in 1xPBS:

100 µl normal donkey serum (Jackson Lab.) was added to 900 µl 1x PBS and well mixed.

0.3% Hydrogen peroxide:

10 µl 30% H_2O_2 (Merck) was added in 990 µl 1x PBS and mixed.

ABC solution:

VECTASTAIN\ *ELITE* ABC Reagent (VECTOR® SG, Vector Laboratories, Inc. Burlingame, CA 94010): a drop of reagent A was added to 5 ml of PBS. Then a drop of reagent B was added, the solution was mixed immediately and allowed to stand for 30 min before use.

2% triphenyl tetrazolium chloride, TTC (Sigma):

200 ml phosphate buffer were prepared by mixing 154.8 ml of Na_2HPO_4 (0.1M) and 45.2 ml of NaH_2PO_4 (0.1M); then 4 g of TTC were added.

2.3 Kits, enzymes, DNA-strands, nucleotides

BCA™ Protein Assay Reagent Kit	Perbio Science, Piece, Bonn, G
ECL Western Blotting Detection Reagents	Amersham-Pharmacia, Freiburg, G
NucleoSpin® RNA II Kit	Macherey-Nagel, Düren, G
ProteoExtract™ Subcellular Proteome ExtractionKit	Calbiochem, Schwalbach, G
Taq DNA Polymerase	Invitek GmbH, G
RQ1 RNase-Free DNase Kit (M6101)	Promega, Mannheim, G
RNase	Invitrogen, Karlsruhe, G
TaqMan® Universal PCR Master Mix	Applied Biosystem

PCR amplification was performed using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems).

2.4 Primers

All primers used in this thesis have been synthesised by TIBMolBiol (Berlin).

AT_1- Receptor forward	CACGATGCTGGTAGCCAAAGT
AT_1- Receptor reverse	GGTAGATGACGGCTGGCAAA
probe	ATCTGGCTAATGGCTGGCTTGGCC
AT_2-Receptor forward	AATCCCTGGCAAGCATCTTATGT
AT_2-Receptor reverse	CGGAAATAAAATGTTGGCAATG
probe	TCCCCTTGTTTGGTGTATGGCTTGTCTGT

2.5 Antibodies and serum

The below listed antibodies and their dilutions have been used in western-blot analysis (WB) and immunofluorescence stainings (IF).

2.5.1 Primary Antibodies

3. Name	Species	Dilution	Blocking serum	Manufacturer
Anti- AT_1 Polyclonal	Rabbit	1:1000 (WB) 1:20 (IF)	10% goat serum in 1xPBS	Santa Cruz Biot.
Anti- AT_2 Polyclonal	Rabbit	1:1000 (WB) 1:20 (IF)	10% goat serum in 1xPBS	Santa Cruz Biot.
Anti- GFAP (H-50), polyclonal	Rabbit	1:50 (IF)	10% donkey serum in PBS	Santa Cruz Biotechnology Inc., Heidelberg, G
CD-68, ED1	Mouse-anti-rat	1:400 (WB) 1:50 (IF)	10% horse serum in PBS	Serotec
Anti-MAP_2	Mouse monoclonal	1:50		Becton Dickinson Co. Biosciences PharMingen
Anti-NeuN biotin conjugated monoclonal	Mouse	1:100		Chemicon International, Temecula, CA 92590
GFAP	Mouse-anti-rat	1:60		BDTransduction Lab
p53	Rabbit-anti-rat	1:50		
cCasp3 monoclonal	Rabbit-anti-rat	1:200	1% blocking donkey serum	Cell Signaling Technology®
Phospho-Akt (Ser473)	Rabbit-anti-rat	1:100		Cell Signaling Technology®

Table 1. Primary ABs used.

2.5.2 Secondary antibodies

In accordance with the primary antibodies, we used various secondary antibodies acquired from Jackson ImmunoResearch, Hamburg, Germany, labelled as shown:

- FITC-anti-goat 1:200 in PBS IH, 1:100 IF; Fluorescein-conjugated AffiniPure Donkey Anti-Goat IgG (H+L)
- FITC-anti-rabbit 1:100; Fluorescein-conjugated AffiniPure Donkey Anti-Rabbit IgG (H+L)
- FITC-anti-mouse 1:100, Fluorescein-conjugated AffiniPure Donkey Anti-Mouse IgG (H+L)
- Cy3- anti-goat 1:200; Cy^{TM}-3 conjugated AffiniPure Donkey anti-mouse IgG (H+L)
- Cy3- anti-rabbit 1:200; Cy^{TM}-3 conjugated AffiniPure Donkey anti-mouse IgG (H+L)
- Cy3-anti-mouse 1:200; Cy^{TM}-3 conjugated AffiniPure Donkey anti-mouse IgG (H+L)
- Rhodamine-anti goat 1:200; Rhodamine Red^{TM}- x-conjugated AffiniPure donkey anti-goat or anti-mouse IgG (H+L)
- Rhodamine-anti-mouse 1:200; Rhodamine Red^{TM}- x-conjugated AffiniPure donkey anti-goat or anti-mouse IgG (H+L).

2.6 Serum

Normal goat serum, Vector Laboratories, Burlingame, CA 94010
Normal horse serum, Vector Laboratories Burlingame, CA 94010
Normal donkey serum, Jackson Immunoresearch Laboratories, Inc..

2.7 Selected apparatus

2.7.1 Microscopic and imaging devices, photometers

Zeiss AXIO Imager.M1 microscope	Carl Zeiss MicroImaging GmbH
Leica Fluorescent Microscope DMIRE 2	Leica Microsystems GmbH, G
Photometer UV-1202	Shimadzu, Japan

2.7.2 Surgery utilities

Stereotactic device Stoelting	Stoelting Co, 620 Wheat Lane Wood Dale, Illinois 60191, USA
Heating pad Präzitherm Type PZ28-1	Störk-tronic, Störk GmbH&Co.KG, Stuttgart, G
Periflux System 5000, type PF5001	PERIMED AB, Box 564, 175 26 Järfälla, Sweden
Jung Frigocut 2800 E cryostat	Leica Instruments GmbH 6907 Nussloch, G
Sutures: Prolene 13 and 17 mm, non absorbable	Ethicon

2.7.3 Electrophoresis, blotting, PCR

Electrophoresis and Blotting systems

Vertical Electrophoresis-System, Midi and Maxi	Harnischmacher, Kassel, G
Horizon™ 58	Bethesda Research Laboratories, Gaithersburg, USA
Horizontal Electrophoresis-System, Mini-Protean®	Bio-Rad, Munich, G
Semi-Dry Blot-System	Bio-Rad, Munich, G

Centrifuges/Rotors

Table centrifuges Mikro 20, Type 2004	Hettich Centrifuges, Tuttlingen, G
Galaxy Mini	Merck KgaA, Darmstadt, G
Centrifuge 5415R	Eppendorf, Hamburg, G
Cooling Centrifuge Mikro 22R	Hettich Centrifuges, Tuttlingen, G
Vortex 3005	G.F.L, Burgwedel®, G

Filmmaterial

Hyperfilm™ ECL	Amersham-Pharmacia, Freiburg, G
Developer	AGFA, G
Rapid Fixer	AGFA, G

Membranes und Filters

Hybond™-C, Nitrocellulose membrane, 0.45 Micron	Amersham Life Science, UK
Sterilfilter 0.2 µm	Schleicher&Schuell, Nürnberg, G
3-MM Whatman Filterpaper	Bio-RAD, Hercules, CA, USA

2.7.4 Ancillaries

Bosworth Trim® temporary Resin Acrylic	Bosworth Company, USA
Xantopren® H green	Heraeus Kulzer, 41538 Dormagen, G
4-0 silicon-coated nylon monofilament	ETHICON GmbH & Co. KG, G
6-0 PROLENE suture	ETHICON GmbH & Co. KG, G
Tissue-Tek®, Cryomold® Standard 4557	Sakura Rinetechnical Co., Ltd., Tokyo, 103-0023 Japan
Jung Tissue Freezing Medium ®	Leica Microsystems Nussloch GmbH, G
Hydro-Mount	Vogel GmbH&Co.KG, 35396 Gießen, G
SuperFrost® Plus Microscope Slides	R.Langenbrinck, 79312 Emmendingen, G
Vectashield® Mounting Medium	Vector Laboratories, Inc., Burlingame, CA 94010, USA
DakoCytomation Fluorescent Mounting Medium	Dako Deutschland GmbH, G
Delimiting water-repellent Pen *(Dako Cytomation Pen)*	Dako Deutschland GmbH, G

2.7.5 Software

Openlab® 3.1 and Mac OS X	Improvision, Tübingen, G
AxioVision 4.1	Carl Zeiss Jena GmbH, G

2.8 Animals and facilities

Male normotensive Wistar rats, weighing between 200 and 250 g, were obtained from Charles River, Sulzfeld, Germany. The animal experiments were done after approval by Landesamt für Arbeitsschutz, Gesundheit und technische Sicherheit (LaGetSi) in accordance with requirements of the Federation of European Laboratory Animal Science Associations (FELASA) in the animal facility of CCR (Center for Cardiovascular Research, Charité Universitätsmedizin Berlin, Institut für Pharmakologie und Toxikologie, Hessische Str. 3-4, 10115 Berlin, Germany). The animals housed in a SPF barrier in stainless polycarbonate cages type IV in groups of 3-4 animals under controlled conditions with respect to temperature (22°C) and humidity (55%), and were kept on a 12h light/ 12h dark cycle with free access to food and water (Sniff standard rat diet).

3. Methods

3.1 Animal experiments

Animal models of focal cerebral ischemia provide insights into the dynamics of ischemic brain injury and are widely used in preclinical trials. The development of reliable animal models of stroke is associated with lesion reproducibility, physiological controls, assessment of neurological outcome and histopathologic changes analogous to those occurring in human cerebral ischemia and allows studying the efficacy of various treatment modalities.

The present study employed the minimal invasive model for transient intraluminal thread MCAO.

3.1.1 Surgical preparation and procedures

Two surgical procedures were performed. Focal cerebral ischemia was induced by unilateral ligation of the internal carotid artery and before that, in order to monitor the cerebral blood flow (CBF) throughout the surgery and 30 min after reperfusion, a Doppler flow probe was implanted on the skull of the animal. During the procedures, each rat was allowed to breathe spontaneously and was kept on a homeothermic heating pad (*Störk* GmbH&Co.KG) to maintain constant body temperature at about 37°C.

3.1.2 Implantation of a Doppler flow probe

After general anaesthesia with chloral hydrate (400 mg/kg body weight: 100g/0.1ml 40% chloral hydrate) injected intraperitoneally, the head of the rat was fixed in a stereotactic device (*Stoelting Co*) so as to prevent any slight movement of body or head. A median longitudinal 1.0-1.5cm cut of the skin, reaching down to the periost, revealed the underlying skull with its sutures. The site of interest was the bregma [*LLat. = top of head <Gr.]:* the craniometric point at the junction of the sagittal and coronal sutures at the top of the cranium. Once located, the opened surface was cleaned and widened laterally using a cotton pad soaked with H_2O_2 so as to prevent bleeding and lay out the skull surface dry and clean and increase adhesion. The micromanipulator of the stereotactic device was adjusted to 1 mm caudal of bregma and 5 mm right lateral to the midline, a point that corresponds to the supply territory of the MCA. The probe was then tightly fixed to the skull using a mixture of dental cement (Bosworth Trim® temporary Resin Acrylic). It hardened immediately and made a smooth bloodless closure of the incision.

3.1.3 Measurement of cerebral blood flow (CBF)

The method of measuring regional blood flow in a rat was introduced by Haywood et al. in 1981 (Haywood *et al.*, 1981). The Doppler principle postulates that a change in the observed frequency of a sound or light wave occurs when the source and the observer are in motion relative to each other, with the frequency increasing when the source and the observer approach each other and decreasing when they move apart. The motion of the source causes a real shift in frequency of the wave, while the motion of the observer produces only an apparent shift in frequency (American Heritage Dictionary, 2006). Thus echoes returning from blood flowing away from the transducer will appear to have a lower frequency and longer wavelength than the original wave. Similarly, blood flowing toward the transducer will appear to have a higher frequency and shorter wavelength than the original wave.

In order to measure the regional cerebral blood flow (rCBF) in the rat brain, a laser-Doppler flowmeter (Periflux system 5000, PERIMED) was connected to the probe, attached to the skull at the above mentioned coordinates.

Cerebral blood flow (CBF) was continuously monitored from 30 min before ischemia, till 30 min after taking the filament out of the carotid artery. The values were recorded 15 min before ischemia begin, immediately after the ligation of ICA and the MCA occlusion, then every 15 min during 90 min of ischemia, and during 30 min of reperfusion. The rCBF values were calculated and expressed as a percentage of the baseline values.

The close monitoring ensured relatively equal alterations in blood supply and dimensions of ischemic area and reduced the likelihood of associated differences in histopathological or neurological results. Rats were included only when the procedure was considered successful when an over 80% drop in CBF (of baseline) was observed after MCAO. At the end of the ischemia period, the obstructing filament was taken out of the carotid artery and a clear reperfusion flow and reestablishment of CBF ensued.

3.1.4 Middle cerebral artery occlusion (MCAO)

For the study of the pathomechanism of stroke various procedures have been used. Animal models of focal cerebral ischemia are generally intended to reproduce the processes and conditions in human focal ischemic stroke produced by occlusion of a single cerebral artery. These more or less reflect the situation in humans. They have been described by several researchers and reviewed by Garcia in 1984 (Garcia, 1984; Ginsberg *et al.*, 1989; Hunter *et al.*, 1998; McAuley, 1995).

A most commonly affected vessel is the middle cerebral artery (MCA), total occlusion of which produces contralateral hemiplegia, hemianaesthesia, and visual field defect (Fawcett *et al.*, 2001). In the ischemic area, three zones can be differentiated (see Fig. 5 in 1.4): central core, penumbra (= 1. an outlying, surrounding region, periphery, fringe 2.A partial shadow, between regions of complete shadow and complete illumination; *Lat. Paene, almost+ umbra, shadow)* (American Heritage Dictionary, 2006) and oligaemia. The core is the site where the flow reduction is most severe (<10ml/100g/min) as compared to normal CBF of 50-80 ml/100g/min, and therefore the ischemia and consequently the neuronal damage there have been most severe. In the penumbra, the CBF comprises 10-20 ml/100g/min, while in the oligaemic zone it is around 50 ml/100g/min. (see graphics). The penumbra is the "border" zone, where the decision-making between survival and death, regeneration and apoptosis takes place. Here the CBF moves on critical levels and if a due reperfusion ensues, the penumbra can be salvaged (Memezawa *et al.*, 1992).

In unison with clinical experience, almost all animal models of focal cerebral ischemia employ the MCA. The most frequently used procedures to achieve occlusion are microsurgical coagulation of the MCA through a temporal craniotomy (Tamura *et al.*, 1981), causing permanent middle cerebral artery occlusion (MCAO), photochemically induced ischemia (photothrombosis approach) introduced by Watson et al. (Watson *et al.*, 1985) and intraluminal thread occlusion without craniotomy via a cervical carotid approach introduced by Koizumi et al. (Koizumi *et al.*, 1986), and later modified by Longa et al. (Longa *et al.*, 1989).

The intraluminal occlusion method may be varied by way of withdrawing the thread and reconstituting reperfusion of MCA territory (transient MCAO). Then, depending on the duration of the occlusion, there is a variable infarct extension. Infarcts ensuing transient MCAO are smaller than in permanent MCAO model and affect mostly the caudato-putamen and to a lesser degree the neocortex (Stoll *et al.*, 1998).

Its several advantages over the other models, such as obviation of craniotomy, easy access to blood vessels, and the option of reperfusion by way of re-establishing blood supply to the ischemic area, have made it an increasingly popular method for studying the pathophysiology and treatment of stroke since its original description (Koizumi et al., 1986).

The general idea behind the procedure of introducing a filament is that after unilateral ligation of a main blood vessel supplying the brain, the ischemic hemisphere receives blood by means of anastomoses with the contralateral side and no grievous ischemia would take place (See Fig. 6). Insertion of a filament into the vessel to the point of where the MCA branches or beyond it, would avoid contralateral blood influx.

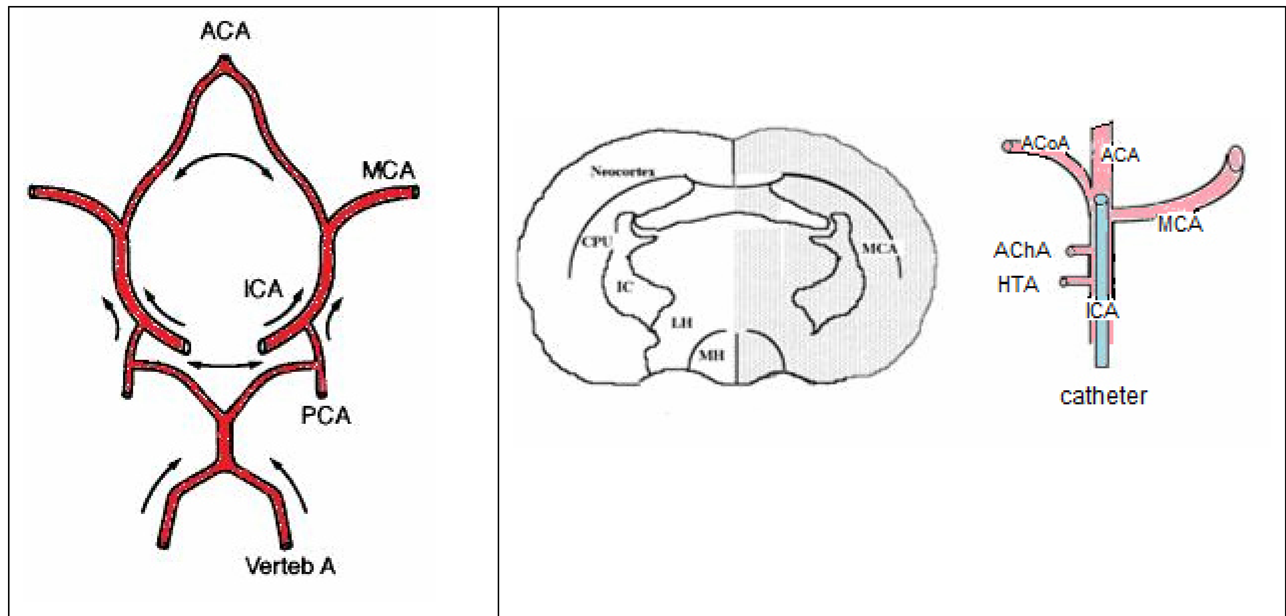


Figure 6: Circulus willisii and model of catheter insertion to stop blood access into the MCA-territory.

Regardless of the species or technique of MCA occlusion, the resulting ischemia shows some typical neuropathological changes and patterns: drop in CBF, area of necrosis and apoptosis, generalized brain oedema, inflammation and gliosis in the surrounding ischemic core (Stoll *et al.*, 1998).

3.1.4.1 Preparation of the carotid arteries

Under general anaesthesia with chloral hydrate (400 mg/kg i.p.), the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed through a midline neck incision and followed up to the cervical carotid bifurcation and a little beyond this point. Then the CCA and its external and internal branches were carefully laid out and separated from the adjacent tissues and structures. Four ligatures were used: three around the CCA and one around the ECA. They were performed with **4-0** silk sutures**.**

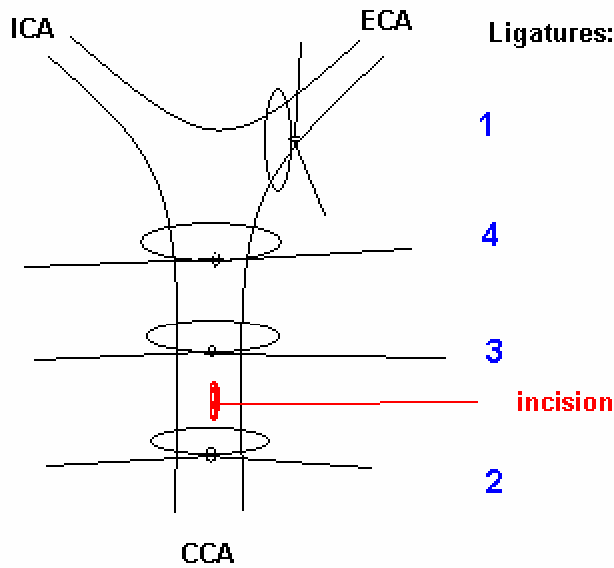


Figure 7: Preparing ligatures.

The first ligature to be closed was the ECA ligature, which was meant to prevent the introduced filament from going into the ECA instead of into the ICA. The second ligature was the one on the proximal CCA, which stopped blood coming from the heart. 1mm cranially from it was made a loose notch, which was to be closed around the introduced catheter and hold it fixed. The last ligature just at the foot of the CCA bifurcation was used only to mobilize the vessel and regulate blood flow. It was held under tension by pulling it slightly upwards; keeping it in this position reduced blood flow coming back from the cranium and prevented an otherwise strong bleeding. A microincision into the CCA between the first and third ligatures was made with scissors to allow insertion of a catheter.

3.1.4.2 Introducing catheter into the middle cerebral artery

A 4-0 silicon-coated nylon monofilament (Ethicon) was gently inserted through the proximal CCA into the ICA and the middle cerebral artery (MCA), thus obstructing collateral blood circuitry. The distal end of the filament had been coated with silicon mixture (Xanthopren®H green) thus forming a blunt ending so as to provide a tight occlusion without any risk of rupturing the vessel. It was inserted to a mark measuring 1.7 cm. Then the third ligature, placed just above the incision, was tightened around the vessel and filament, holding the latter firmly in place, preventing its slipping out.

The ensuing ischemia was confirmed by reduction in CBF of more than 80% of the initial CBF. Wet coverlets were put over the incision for the duration of the induced brain ischemia. After 90 minutes the filament was withdrawn into the stump of the CCA to allow reperfusion. Finally, the vessels were ligated: the ECA stump was closed with 7-0 silk suture and the neck wound was closed with a 6-0 prolene suture. Reperfusion was confirmed by a rise in CBF. The Doppler probe was removed; the rat received analgesia with 0.2 mg/kg body weight buprenorphin (Temgesic®) s.c. and was put into its pre-warmed cage. Body temperature after the surgery procedures was still maintained at 37 ± 0.5 °C by a heating pad.

Sham-operated rats underwent the same surgical procedure except that the occluding monofilament was not inserted and no vessel was ligated.

3.1.5 Evaluation of neurological deficits

After the animals had fully recovered from the effects of anaesthesia, 24 and 48 hours after MCAO, evaluation of neurological deficits was performed by a blinded observer using the 18-point neurological scoring system developed by Garcia et al. (Garcia *et al.*, 1995b). It includes six different parameters:

- spontaneous activity
- symmetry of movement of four limbs
- forepaw outstretching
- climbing
- body proprioception and
- response to vibrissae touch.

The degree of neuronal deficits is expressed as the sum of all six individual scores. Thus, the maximum 18 points of the scale stand for a normally strong and healthy rat, whereas severely handicapped rats have a score of 5 to 8 points. The minimum neurological score is 3 points (see Table below).

Test parameters	Score 0	 1	 2	 3
Spontaneous activity	No movement	Barely moves	Moves but does not approach at least three sides of the cage	Moves and approaches at least three sides of the cage
Symmetry of movement (four limbs)	Left side: No movement	Left side: Slight movement	Left side: Moves slowly	Both sides move symmetrically
Forepaw outstretching	Left side: no movement, no outstretching	Left side: Slight movement to outstretching	Left side: Moves and outreaches less than right side	Symmetrical outreach
Climbing		Fails to climb	Left side is weak	Normal climbing
Body proprioception		No response on left side	Weak response on left side	Symmetrical response
Response to Vibrissae touch		No response on left side	Weak response on left side	Symmetrical response

Table 2. Neurological deficit evaluation scoring system for Wistar rats after MCAO, Garcia et al. Stroke 1995: 26: 627-635.

Spontaneous activity

The rat was observed for 5 minutes in its cage. According to its ability to reach all four walls the assessment showed: score 3 if rat moved around, explored the environment, and approached at least three walls of the cage; score 2 if slightly affected rat moved about in the cage but did not approach all sides and hesitated to move, although it eventually reached at least one upper rim of the cage; score 1 if severely affected rat did not rise at all and barely moved in the cage; 0, rat did not move at all.

Symmetry in the movement of four limbs

The rat was held in the air by the tail to observe the movement of the four limbs. Score 3 was given if all limbs extended symmetrically; score 2 meant that limbs on the left side extended less or more slowly than those on the right one; score 1 meant that limbs on the left side showed only minimal movement and 0, forelimb on the left side did not move at all.

Forepaw outstretching

The rat was held by the tail and made to walk on its forelimbs only. The symmetry in the outstretching of both forepaws was tested by bringing the rat to the edge of the table. The scores indicated: 3, both forelimbs were equally outstretched and rat walked symmetrically; 2, left side was less outstretched than the right one and forepaw walking was impaired; 1, left forelimb moved minimally; and 0, left forelimb did not move.

Climbing

The rat was put on the wire-meshed top of its cage. The strength of attachment of the rat was assessed by trying to pull it off the wire by the tail. The scores indicated: 3, rat climbed easily and had a tight grip on the wire; 2, the left side was not as strong as the right one in climbing and showed a less firm grip; and 1, the rat failed to climb or moved in a circle instead.

Body proprioception

The rat was touched with a blunt stick first on the left and then on the right side to detect any differences in reaction. Scores indicate: 3, rat reacted by turning its head and was equally disturbed by the touch on both sides; 2, rat reacted more slowly to touch on the left side; 1, rat did not respond to the stimulus applied to the left side.

Response to vibrissae touch

The vibrissae of the rat were brushed slightly with a blunt stick. The visual field of the animal was avoided by approaching the vibrissae from the rear. Scores indicated: 3, rat reacted by turning its head or was equally startled by the stimulus on both sides; 2, rat reacted slowly to the stimulus applied to the left whisker; 1, rat did not respond to the stimulus on the left side at all.

3.2 Preparation of tissues

Most neuroanatomical procedures involve the following steps: (1) preparation of solutions, (2) brain preparation, (3) sectioning the brain, (4) labelling and staining of brain sections or tissue processing for protein analysis, and (5) microscopic analysis. Animal protocols followed the German law on animal protection.

3.2.1 Preparation of the brain

After 48 hours and completed neurological evaluation, the rats were given an intraperitoneal injection of overdose of chloral hydrate according to approved animal care protocols.

Immunohistochemical procedures in general require that the brain be perfused via the circulatory system to achieve brain tissue devoid of blood. When the animals failed to respond to a painful stimulus (pinching of the foot), they were perfused transcardially with 0.9% NaCl PBS by using a peristaltic pump. Briefly, the thoracic cavity was opened with two horizontal cuts through the rib cage on either side of the heart, and the cut rib flap was folded headward to expose the heart. A small incision was made into the bottom apex of the left ventricle and a cannula was inserted into the left ventricle to the aorta to allow inflow of saline, while the right atrium was cut open to allow an escape route for blood and saline to leave the body during perfusion. When the effluent became clear, the cranium was carefully opened and the brain taken out with a blunt instrument.

3.2.2 Preparation and sectioning of tissues

The brains were once again washed in PBS and then placed into a 2-mm brain matrix to preserve their natural form. They were coronally dissected into 2 mm sections. Four main sections were taken out for further investigations according to a predetermined scheme. One brain section (bregma +2.0 to 0 mm) was stained in 2% TTC (Sigma) to identify the infarction area. Another one was put into tissue matrix liquid and directly embedded in OCT compound and snap-frozen in liquid nitrogen (bregma 0 to −2.0 mm). The remaining two slices were put in eppendorf tubes and rapidly frozen in liquid nitrogen (bregma −2.0 to −4.0 mm, peri-infarct areas).

Preparation of sections for TTC

The slices designated for infarct area detection were put into the base mold (TissueTec®) without any frozen tissue matrix, then laid on dry ice for some minutes and then stored at -80 C for following TTC-staining.

Preparation of sections for immunohistochemistry

The brain slices designated for IH were laid into base molds (TissueTec®). The molds were labelled accordingly and partially filled with frozen tissue matrix (Jung Tissue Freezing Medium® For Frozen Tissue Specimens). Then the molds were plunged into 2-methylbutane pre-chilled in a Dewar flask of liquid nitrogen until the blocks solidified (approximately 30 sec). Afterwards they were placed on dry ice till transferral to a -80° C freezer and were kept there until sectioning.

Preparation of sections for protein /Western blot / or mRNA analysis

The tissues determined for Western blot and mRNA analysis were put into 2 ml tubes and dipped in liquid nitrogen for 5 minutes. Then they were transferred to -80 C and kept there for further analysis. The labelling and further processing of the specimens are described in the respective sections below.

3.3 Histological analysis

3.3.1 Identification of infarction area

Triphenyltetrazolium chloride (TTC) and cresyl violet (CV) staining are routinely used methods to determine cerebral infarct volume and area. TTC is a reagent for oxidative enzymes and was first introduced by Jestaedt and Sandritter in 1959 (Jestaedt *et al.*, 1959) as a stain to detect ischemic injury of the myocardium. This salt accepts a proton from succinate dehydrogenase in the inner membrane of the mitochondria which reduces it to its red insoluble form formazan (Bederson *et al.*, 1986; Bednar *et al.*, 1994; Belayev *et al.*, 1999; Cole *et al.*, 1990), therefore an area with inactive enzymes, as is the infarction zone, is not stained and appears pale. Some authors have argued that TTC staining may overestimate the infarct area because it can include enzymatically inactive, but vital brain tissue (Bednar *et al.*, 1994; Belayev *et al.*, 1999). The major advantages of TTC staining over other histopathologic staining methods are low cost, technical simplicity, and reproducibility (Dettmers *et al.*, 1994; Hatfield *et al.*, 1991; Liszczak *et al.*, 1984; Okuno *et al.*, 2001; Tureyen *et al.*, 2004; Wexler *et al.*, 2002).

The designated slices of rat brains with parameters bregma +2.0 to 0 millimetres were used for the measurement of infarct size. They were incubated in 2% TTC (Sigma) at 37°C for 20 min and then fixed with 10% paraformaldehyde at 4°C for 10 min. The infarction area could be detected with the naked eye (see also Fig. 15).

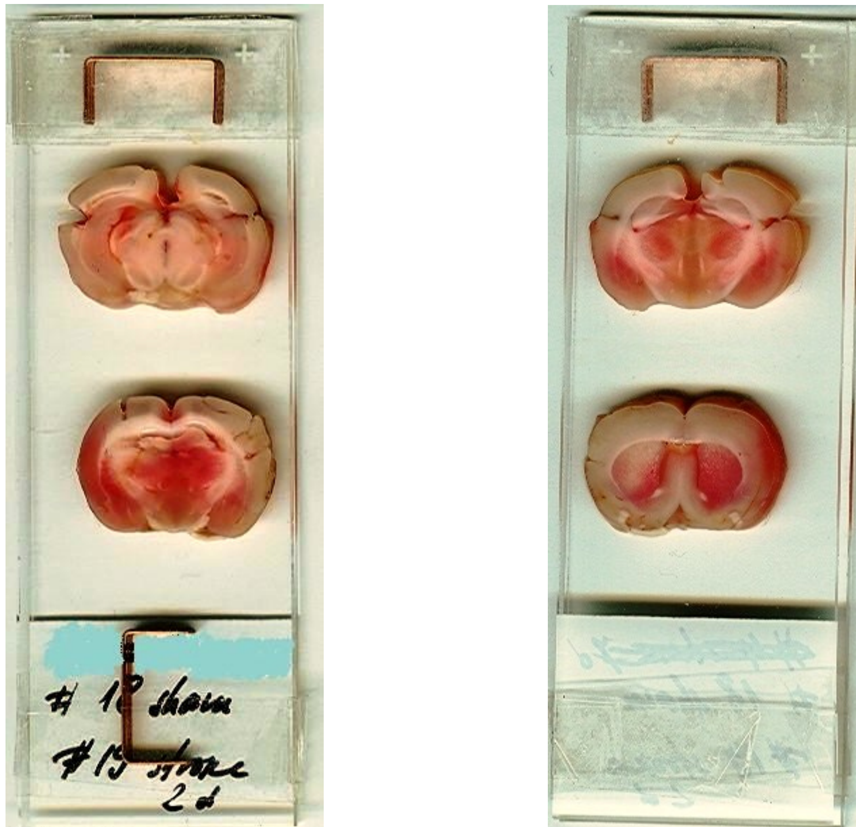


Image 1: Brain slices for TTC staining.

The slices were placed on a glass holder. The cranial and caudal faces of each slice were scanned with a flatbed scanner, the slice images digitalized and the area of infarction identified (Leica image analysis system).

3.3.2 Immunohistochemistry

The method of immunohistochemistry is used to anatomically localise particular molecules or receptors to particular cells. For this purpose, antibodies against the molecule/receptor of interest must be synthesised in and derived from an animal. The molecule/receptor candidate has to have been purified and then injected into an animal, thus causing an immune response and stimulating the generation of antibodies that can bind tightly to specific sites of the candidate. Blood is withdrawn from the animal and the antibodies isolated from the serum. These can be chemically tagged with a colour marker and applied to sections of brain tissue later to be seen with a light or confocal microscope (Bear *et al.*, 1996). The brain slices prepared as described above, are too thick to allow detailed study of receptor distribution and have to be cut into thinner slices. Normally, the choice of sectioning method depends on how the brain has been prepared and what histochemical method is to be used. A cryostat is required for sectioning fresh-frozen brains, as the unfixed brain sections must be maintained in a frozen state until they are affixed to

a microscope slide. A cryostat is a microtome housed in a freezing chamber that allows the sectioning process to be performed at a temperature of −20° to −30°C.

In our experiments, the brain samples were cut into 7µm-thick slices at -20°C (Leica Jung Frigocut 2800E) and then mounted onto microscope slides (SuperFrost® Plus Microscope Slides). These were dried at room temperature and then immersed for 10 min in -20°C acetone. Then they were placed in storage boxes at -20°C, pending further processing.

3.3.2.1 Immunohistochemical detection of ED1, GFAP, MAP_2 and p53

The first staining immunohistochemical method was intended to prove and analyze the process of inflammation (see 3.3.3 for marker information). Each rinsing process was performed thrice.

Immunocytochemistry was performed by the conventional avidin-biotin complex (ABC) method. The cryosections were placed in a humid chamber and each brain tissue was encircled with a liquid repellent slide marker pen (Liquid Blocker Super Pap Pen, Daido Sangyo Co., Ltd. Tokyo, Japan). Then they were incubated 10 min in 0.3 % H_2O_2 in PBS to quench the endogenous peroxidase activity. After rinsing in PBS, there followed a 20-minute incubation with blocking serum, (10% donkey serum diluted in PBS to prevent unspecific binding). The blocking serum was removed and the primary Ab (e.g., ED1) was given and incubated 1 hr in the humidified chamber. Negative control staining in each session was performed by omitting the primary Ab in the 5 % blocking-serum-PBS solution.

After washing the slides three times, these were incubated with biotinylated secondary Ab (e.g., goat-anti-mouse) for another hour. After rinsing thrice with PBS, the reaction was visualized by using the VECTASTAIN® ABC reagent (VECTOR®) for 30 min (see above). After a rinsing step, the AEC working solution (Merck) was added and incubated for 12 min. Then this was washed out twice, the slides were counterstained with Haemalaun (4 min), rinsed again and mounted with hydromounting medium (Vogel GmbH&Co.KG).

Antibody Name	Species	Dilution	Blocking serum	Manufacturer
AT_1 , AT_2	Rabbit-anti-rat	1:40	Horse, goat	Santa Cruz Biot.
CD-68, ED1	Mouse-anti-rat	1:50 (IF)	5% horse serum diluted in PBS	Serotec
GFAP	Mouse-anti-rat	1:50	5% horse serum diluted in PBS	BD Transduction Lab
p53	Rabbit-anti-rat	1:50	5% goat serum diluted in PBS	

Table 3. Primary antibodies for immunohistology.

The secondary antibodies for the immunohistochemical analysis were biotinylated secondary Ab horse-anti-mouse and goat-anti-mouse IgG 1:300 (Vector Lab., Burlingame, CA), depending on the blocking serum (horse or goat serum).

When the slides became dry they were viewed and estimated under light microscope (Zeiss AXIO Imager.M1 microscope).

3.3.2.2 Immunodetection of AT_1 and AT_2

We used the same staining protocol as in 3.3.2.1. The primary antibodies were AT_1 and AT_2 (rabbit-anti-rat), diluted 1:40 at room t°; the secondary Ab was biotinylated goat-anti-rabbit IgG 1:300 (DakoCytomation, Denmark, Produktionsvej 42, DK-2600 Glostrup, Denmark).

However, due to lack of satisfactory staining evidence (See Fig.7), we proceeded by using the method of immunofluorescence.

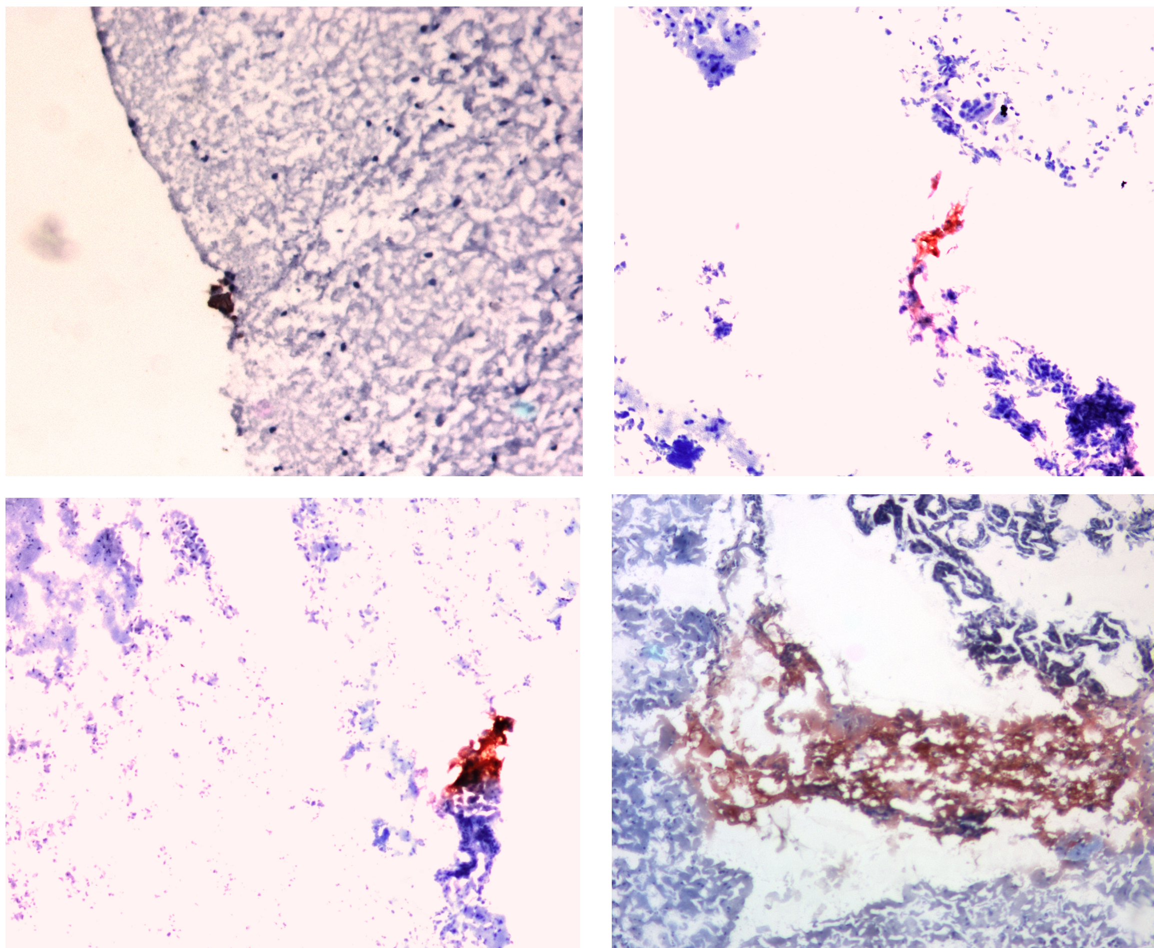


Image 2: Immunohistological staining with AT_1-antibody (upper line) and AT_2-antibody (lower line).

3.3.3 Immunofluorescent labelling

Fluorescence is a luminescence, an optical phenomenon in which a molecule having absorbed a high-energy photon re-emits it as a lower-energy photon, and it is named after the mineral fluorspar (calcium fluoride), which exhibits this phenomenon (Wikipedia). The processes that occur in certain molecules called fluorophores or fluorescent dyes comprise usually 3 stages: excitation, excited-state lifetime and fluorescence emission. We find fluorophores in fluorescent probes designed to localise within a specific region a biological specimen or to respond to a specific stimulus (Invitrogen).

The method of immunofluorescence labelling provides a qualitative analysis and allows relative quantification. The detection of the structure of interest is made possible by a reaction of the first Ab (already bound to the target) with a second one, conjugated to a fluorophore. The most common secondary antibodies used in immunofluorescence stainings are Fluorescein Isothiocyanate (FITC)-conjugated antibodies and Cy3 (Cyanine)-conjugated ones (ACS, ; Kantor).

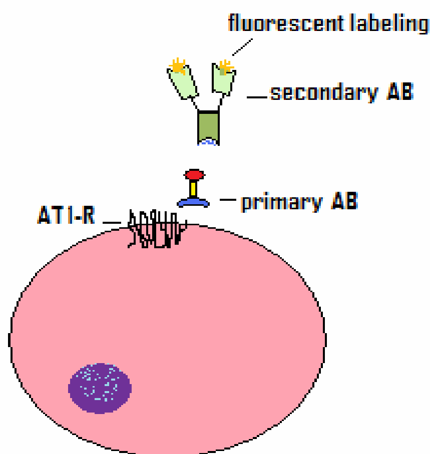


Figure 8: Antibody-labelling.

FITC is among the most widely used fluorescent labelling reagents due to its high quantum efficiency and conjugate stability. It is a small organic molecule and it is conjugated to proteins via primary amines. Usually, three or four FITC molecules are conjugated to each Ab. It is excited by the 488nm line of an argon laser and its emission lies at 530 nm.

The Cy3 dye is an orange fluorescing cyanine that produces an intense signal easily detected with fluorescein filter sets. Cyanine reagents have been shown to be useful as fluorescent labels for biological compounds (Mujumdar *et al.*, 1993). Owing to their properties- intense fluorescence, low hydrophobicity and high photostability- the Cyanine dyes are popular labels for fluorescent microscopy (Wessendorf *et al.*, 1992).

Both fluorescent markers are commercially supplied as pre-dried portion in reaction vials, which are sealed in dry atmosphere to prevent hydrolysis during storage. They were dissolved in 1ml of ultra distilled water before use. In our experiments we used FITC-, Cy3- and Rhodamine-conjugated AffinityPure Donkey anti-rabbit or –mouse IgG in dilutions 1:100, 1:150 and 1:200, respectively.

The cryosections underwent different incubation steps and time depending on Ab-type and structure to be labelled (intracellular versus membrane-bound).

3.3.3.1 Immunofluorescence of AT_1 and AT_2

In general, the standard protocol we used was as follows: the cryosections were placed in a humid chamber and each brain tissue was encircled with a water-repellent pen (*Dako Cytomation Pen*). Then these were incubated for 5 min with 0.2% Triton-X-100 PBS and after blotting on paper, incubated for 20 min in blocking serum (10% donkey serum diluted in PBS). After rinsing thrice for 5 min in PBS, the slides were incubated with the primary Ab (e.g., AT_1 rabbit-anti-rat, diluted 1:20 in PBS containing 5% blocking serum) at room t°. In 45 min, after rinsing the slides 3 x 5 min, the secondary Ab (e.g., FITC donkey-anti-rabbit, diluted 1:100 in 5% blocking serum) was added. After 30 min incubation, the slides were washed 3 x 5 min in PBS, mounted with aqueous medium (Vectashield® Mounting Medium) and coverslips, and examined under a microscope (Leica Light Microscope DMIRE 2).

For the AT_2 labelling we used the same protocol as for the AT_1 one, the only difference being the 1st and 2nd antibodies.

3.3.3.2 Immunofluorescence of NeuN and MAP_2

We postulated that neurons might express AT_2 receptors and therefore used two neuronal markers to confirm our thesis.

NeuN (neuronal nuclei) is a neuron-specific protein present in most neuronal cell types. It is a marker of maturation in the fetal nervous system as well as a reliable marker of proliferative capacity of neurons. Its two isoforms are to be found in the nucleus and in the cytoplasm (Antibody&Beyond).

MAP_2 is a neuron-specific protein, which stabilizes microtubules in the dendrites and plays an important role in the development and maintenance of neuronal morphology, especially in neuronal differentiation (Antibody&Beyond).

3.3.3.3 Immunofluorescence of GFAP, ED1, CD11b and cCasp-3

In order to identify different cell populations in the brain tissues, we used GFAP, ED1, CD11b and cCasp-3 - antibodies to localise astroglial, inflammatory and apoptotic cells, respectively.

GFAP (Glial Fibrillary Acidic Protein) was discovered by Amico Bignami and co-workers as a major fibrous protein of multiple sclerosis plaques (Bignami *et al.*, 1972). It was subsequently found to belong to the intermediate filament protein family Class III along with peripherin, desmin and vimentin. GFAP is specifically expressed in astrocytes, in satellite cells in peripheral ganglia and in non-myelinating Schwann cells in peripheral nerves. Therefore, it is often used to visualize reactive astrogliosis following tissue damage and formation of glial scar.

ED1 is a marker for an ongoing inflammatory process and is expressed by tissue macrophages, Langerhans cells and at low levels by dendritic cells. It could play a role in phagocytic activities of tissue macrophages, both in intracellular lysosomal metabolism and extracellular cell-cell and cell-pathogen interactions. The antigen recognised by ED1 is the rat homologue of human CD68 (abcam). CD11b is implicated in various adhesive interactions of monocytes, macrophages and granulocytes as well as in mediating the uptake of complement coated particles. It is also a receptor for fibrinogen, factor X and ICAM1. Furthermore, it is commonly used as a microglial marker in nervous tissue (abcam).

Caspase-3 plays a key role in apoptotic processes and is responsible for proteolytic changes of some key proteins (ADP-ribose, PARP). Cleaved Caspase-3 (cCasp-3) represents the activated form resulting from cleavage of the little fragment adjacent to (Asp175) (Cell Signaling, ; Fernandes-Alnemri *et al.*, 1994; Nicholson *et al.*, 1995).

3.3.4 Double immunofluorescence labelling

Double-labelling immunofluorescence detects localisation of a protein of interest as well as the distribution of the protein relative to another marker such a neurochemical or organelle marker. For example, co-localisation of a receptor with a typical cell marker determines if the receptor localises to a particular type of cells and a double labelling of receptor with a synaptic vesicle marker determines if the receptor localises to presynaptic terminals. Immunofluorescent imaging using confocal microscopy is superior to conventional microscopy since it allows of high-resolution analysis limited theoretically to 0.1 to 0.2 µm. Depending on the origin of the primary Ab applied to the sections, we combined FITC-anti-mouse Ab and Cy3-anti-rabbit Ab or FITC-anti-rabbit and Cy3-anti-mouse antibodies. The general protocol we used was as follows:

1. Sections were incubated for 20 min with 10% donkey serum diluted in PBS in a humidified chamber.

2. After blotting the blocking serum, sections were incubated with first primary Ab (e.g., rabbit-anti-rat), diluted in PBS containing 5% blocking serum for 45 min.

3. Slides were rinsed thrice with PBS and then incubated with secondary Ab (e.g., FITC-anti-rabbit IgG), diluted in PBS containing 5% blocking serum for 30 min.

4. After rinsing thrice with PBS, sections were again incubated for 20 min with 10% donkey serum in a dark humidified chamber.

5. After blotting the blocking serum, sections were then incubated with second primary Ab (e.g., goat- or mouse-anti-rat), diluted in PBS containing 5% blocking serum for 45 min in a dark humidified chamber.

6. After rinsing thrice with PBS, sections were incubated with diluted RR-X Donkey Anti-goat or –mouse IgG for 30 min in a dark humidified chamber.

7. The slides were then rinsed three times with PBS, mounted with aqueous medium (Vectashield® Mounting Medium) and coverslips, ready to be examined.

Omitting the primary Ab in the 5 % blocking-serum-PBS solution gave our negative control staining.

We made following double stainings:

- AT_1 and GFAP
- AT_1 and cCasp-3
- AT_2 and NeuN and MAP_2
- AT_2 and GFAP
- AT_2 and ED1
- MAP_2 and cCasp-3 and
- cCasp-3 and GFAP.

For the last three mentioned, we added a pre-treatment with Triton-X-100: the sections are incubated for 5 min with 0.2% Triton-X-100 diluted in PBS at room temperature. Then the sections were rinsed thrice and processed as described above. Additionally, depending on their binding activity, some antibodies were incubated overnight in a cold chamber.

Negative control staining consisted of omission of the primary Ab in the 5 % blocking-serum-PBS solution.

3.4 Molecular methods

Molecular biology is the study of biology at the molecular level. It studies the interactions between the various systems of a cell, including the interrelationship of DNA, RNA and protein

biosynthesis and explains how these interactions are regulated. For the purpose it uses methods such as:

- Expression cloning
- PCR
- Gel electrophoresis
- Southern blotting
- Northern blotting
- Western blotting
- Microarrays

and others.

3.4.1 RNA isolation

The isolation of total cell RNA was performed using the Trizol Reagent (U.S.Patent No.5,346,994, *Invitrogen life technologies*). It is a ready-to-use reagent, which allows RNA extraction from cells as well as from tissues and represents an improvement to the single-step method developed by Chomczynski and Sacchi (Chomczynski *et al.*, 1987).

The tissue probes were kept in tubes at -80°C (see 2.4.3.3). They were taken out, put into a mortar onto liquid nitrogen, and pounded. Trizol was added to the probes (1 ml per 50-100 mg brain tissue) in order to maintain the integrity of the RNA while lysing cells and their components. Final homogenization was performed with Polytron (Pellet Pestle® Motor Kontes). Samples were stored for 5 minutes at 15 to 30°C for better dissociation of nucleoprotein complexes. To achieve full separation of the RNA, we added 200µl chloroform, shook the samples vigorously, then incubated them at 15 to 30°C for 3 minutes and centrifuged them (15 min, 12000 x g at 4°C). As a result, the mixture showed 3-phase separation: a colourless RNA phase at the top (=aqueous phase), followed by an interphase containing the DNA, and a red phenol-chloroform, protein-rich phase below. The aqueous phase was transferred to a fresh tube and addition of 500µl isopropyl alcohol precipitated the RNA. After incubation at room temperature for 10-15 min, and centrifugation (10 min, 12000 x g at 4°C), a gel-like pellet formed at the bottom. The supernatant was removed and the RNA pellet washed with 75% ethanol, mixed by vortexing and centrifuged again (≤7500 x g). The supernatant was removed; the pellets were dried at room temperature and dissolved in 20 µl RNase-free water. For the photometrical quantification, we dissolved the (1 µl) RNA in RNase-free water (dilute 1:100). The spectrophotometric analysis was done at 260-280 nm.

The steps in short:

1. Tissue extraction and homogenization
2. Phase separation
3. RNA precipitation
4. RNA wash
5. RNA solubilisation

From the photometrical results we calculated the concentration in mg/ml and the RNA quantity in mg in each probe.

3.4.2 RNA electrophoresis

Gel electrophoresis *(<Gr. phoros= to carry across*) is a method to separate macromolecules (nucleic acids, proteins) on the basis of their size, charge or other properties. The two basic materials used for the gel are agarose and polyacrylamide. One electrode attracts, the other repels the molecules, respectively. The electrical current between the electrodes causes the macromolecules to migrate through the gel pores. The rate of migration depends on the strength of the field, the shape and size of molecules, their hydrophobicity, and other factors.

To investigate the quality of the acquired RNA, we used 1.0 % agarose gel.

Gel preparation: 1 % TBE-buffer diluted agarose (60 ml TBE + 0.6 g agarose for mini-gel) was heated up to boiling until the agarose was melted and dissolved. Then 3 µl EtBr was added, mixed well and let cool down; then the mix was poured into an electrophoretic tray and allowed to harden at room temperature for 1-1.5 hours.

Probes: 1 µg RNA; low volume was filled up with RNase-free water up to 40 µl. Then we supplemented the RNA samples with a gel loading buffer with Orange G (400kb, 4000kb colour markers) with final concentration of 10% (4 µl) and mixed them by vortexing.

Loading and separation: The comb was gently removed from the hardened agarose gel. The gel was placed in the electrophoretic apparatus submerged in TBE buffer.
20 µl of the mixture (ca. 0.5 µg RNA) was loaded into each application slot and incubated for 2 hours at room temperature.

Assessment: DNase, RNase activity: none detected; residues in slots-DNA, fragments
mitochondrial 28 S band ca. 4700 bp
mitochondrial 18 S band ca. 1900 bp

The 28S RNA band had to be twice as intense as the 18S one. This 2:1 ratio was indicative of the intact state of the RNA. Degraded RNA appeared as a smear.

3.4.3 Reverse transcription

In order to study RNA with the PCR method, the RNA sample must first be reversely transcribed to cDNA to provide the necessary DNA template for the thermostable polymerase. The process is called reverse transcription (RT).

For efficient synthesis of cDNA in preparation for PCR amplification, we used Promega products (Promega GmbH). Since the quality and purity of RNA is crucial to RT-PCR, in order to secure such pure solution of high-quality total RNA, the samples had to be first treated with DNase (1U/µg RNA). We used the RQ1 RNase-Free DNase.

DNase Digestion

For the removal of contaminating DNA and proteins we prepared a mixture of:

- 1 µg RNA+ DEPC H2O = 7 µl
- 1 µl DNase (1U/µg RNA) + 1 µl 10xDNase-buffer

The tubes were then incubated for 30 minutes at 37°C, after which each was supplemented with:

-1 µl Stopsolution and kept at 65°C for 10 minutes (each tube with 10 µl)

This procedure was immediately followed by the synthesis of cDNA.

cDNA synthesis

Each 10 µl of digested RNA was supplemented with 1 µl random primer (0,25µg/ml / 1µg RNA) and incubated at 70°C for 5 minutes in a cycler. Then according to the number of probes we prepared a master mix of:

- 5x buffer	5 µl
- dNTP (25mM)	2 µl
- M-MLV (200U/ µl)	0.5 µl
- R'nasin	0.5 µl
- $DEPC_{H2O}$	5 µl
	= 13 µl

This mix was added to the first solution and the whole amount (24-25 µl in all) then incubated at 37°C for 1h in a cycler. The synthesized cDNA was then used for PCR analysis.

To rule out contamination in the PCR, a negative sample RT- , lacking reverse transcriptase, was performed.

3.4.4 Polymerase chain reaction (PCR) and Real-Time PCR

The PCR method was established in 1983 by Kary Mullis and has had a great influence on the enzymatic synthesis of specified DNA sequences ever since. It also enables the detection of nucleic acids, which are only weakly expressed (Saiki *et al.*, 1985).

With the help of the catalysing property of the heat-stable recombinant Taq DNA-polymerase (Thermophilus aquaticus YT1) and two specific oligonucleotides, called primers, the amplification cycles require only few hours.

One such cycle comprises three steps of varying temperatures:

- template denaturation
- primer annealing
- primer extension

The first step is the heating up of the DNA matrix to 94-95°C thus achieving denaturation, meaning cleavage into 2 single strands. Then, at 45-60°C, the annealing of the gene-specific primer with the target single-stranded DNA takes place. Finally, under the influence of Taq polymerase follows the primer extension- the synthesis of the new DNA. The enzyme is most active at 72°C and takes only 1-2 minutes to act. The next amplification cycle begins with raising the temperature to 94°C again. At the end of all cycles, the probes are incubated at for 5 to 10 min so as to enable completion of unfinished DNA strands.

Our experimental design:

PCR was performed using the Invitek protocol, buffers and solutions (Invitek, Berlin, Germany) and two primers (TibMolbiol, Berlin, Germany). To provide identical reaction conditions at amplification, all components, which were to be added to the RNA probe, except for the corresponding cDNA, were mixed into a “master mix”. This **master mix** included:

Component	Volume	Final concentration
Double distilled, sterile water	**18.175 µl**	
10x buffer	**2.5 µl**	**1x**
50 mM $MgCl_2$ solution	**1.0 µl**	**1.5 mM**
50x d NTP-Mix (each 25 mM)	**0.2 µl**	**0.2 mM**
Primer forward (20 µM)	**0.5 µl**	**0.4 µM**

Primer reverse (20 µM)	**0.5 µl**	**0.4 µM**
Taq DNA Polymerase (5U/ µl)	**0.125 µl**	**0.025 U**
Total volume	**23 µl**	

Then, 2.0 µl of template DNA ($3.2x10^{-3}$ µg) was supplemented with 23 µl of the master mix. Amplification (PCR) was performed using the ABI PRISM® (Applied Biosystems).

3.5 Protein biochemical methods

We carried out protein preparation and Western blot analysis in order to show whether post-infarct changes had taken place at receptor and protein expression level.

3.5.1 Protein isolation- trizol method

Proteins were isolated from the phenol-ethanol supernatant obtained after DNA precipitation (see below) and afterwards analyzed by Western blotting.

Solutions

Solution	Amount	Component	
1X electrophoresis buffer	3.03 g	Tris base	
	14.4 g	Glycine	
	10 ml	10% SDS	fill up to 1 L
2X SDS-buffer	2.0 g	sucrose	
	8.0 ml	25% SDS	
	2.4 ml	1M Tris HCl (pH= 6.8)	
	9.6 ml	ddH_2O	
	dip of	pyronin G/Y	
25% SDS pH= 7.2	25 g	sodium lauryl sulfate	
	75 g	dd H_2O	
0.3 M guanidiumhydrochloride/ 95% EtOH (store at -20°C and use ice-cold)	2.9 g	guanidiumhydrochloride	
	90 ml	EtOH	
	10 ml	dd H_2O	
Towbin buffer	3.03 g	Tris base	
	14.4 g	Glycine	
	5.0 ml	10% SDS	
	200 ml	methanol	fill up to 1 L
10 X TBS	24.2 g	Tris base	
	80 g	NaCl	
Coomassie dye solution	1.0 g	Coomassie Blue in 100 ml dd H_2O	
1X TBST	100 ml	10X TBS	
	1.0 ml	Tween (0.1%)	fill up to 1 L
Coomassie working solution	31.25 ml	Coomassie dye solution	
	125 ml	methanol	
	25 ml	acetic acid	

Table 4. Solutions for Western blot.

DNA precipitation

First 30 µl Ethanol (1 ml Trizol) was added to the inter- and organic phase (see 3.4.1) and pipetted up and down. The mixture was incubated for 2-3 min RT and then centrifuged for 5 min at 2000 rpm at 4°C.

Protein precipitation

The supernatant was carefully moved to new tubes; the pellet was further used for DNA isolation. 1.5 ml isopropanol (1 ml Trizol) was added to the new tubes, and they were incubated for 10 min at RT. The protein precipitate was sedimented at 12000 rpm for 10 min at 4°C and the supernatant removed.

Protein wash

The protein pellets were washed thrice with 0.3 M Guanidine hydrochloride in 95% ethanol. After each washing step the samples were incubated for 20 min at RT and then centrifuged at 7500 rpm for 5min at 4°C, the supernatant removed. After the last washing step, the pellets were redissolved in Ethanol and vortexed. After 20 min incubation at RT, they were centrifuged again for 5 min at 7500 rpm at 4°C, the supernatant was removed and the pellets air-dried for 20 min at RT.

Protein solubilisation

The pellet was dissolved in 1x SDS-buffer solution ($V=m*5$) and incubated overnight in the Thermomixer at 37ºC. For this purpose 2X SDS-buffer (see 3.5.1) was diluted 1:1 with dd H_2O.

Protein analysis by BCA method

We used the bicinchoninic acid (BCA) assay in kit form (PIERCE BCA™ Protein Assay Kit, Rockford, IL) and prepared a working solution by mixing reagents A and B in relation 50:1 respectively.

Protein probes were centrifuged for 5 min at 13000 rpm, the supernatant removed to new tubes, the pellets discarded. The new samples were diluted 1:5 (40 µl dH_2O+ 10 µl supernatant, respectively), supplemented each with 1ml working solution and vortexed. Incubation steps of 30 min at 37 ºC, 5min at 4 ºC and 5 min at room temperature followed. Subsequently, the samples were quantified photometrically at 562 nm.

3.5.2 SDS- Polyacrylamide gel electrophoresis (SDS-PAGE)

A very common method used to separate proteins according to their size is the sodium dodecyl sulfate polyacrylamide gel electrophoresis. SDS is an anionic detergent that can dissolve hydrophobic molecules and has a high negative charge attached to it, so that it denatures all proteins and supplies them with a negative charge. Thus they will all migrate unfolded, or linearised, towards the positive pole when placed in an electric field (Experimental Biosciences).

A discontinuous polyacrylamide gel serves as a support medium. It is a polymer of acrylamide monomers which allows differently sized proteins to move at different rates as if through a mesh. Thus polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules.

Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the occurrence of major proteins in a sample, and to determine the distribution of proteins among fractions. It allows estimation of the purity of protein samples and the progress of a fractionation or purification procedure can be followed. Different staining methods can be used to detect rare proteins and their biochemical properties. Furthermore, Western blotting, two-dimensional electrophoresis, and peptide mapping can be used to detect extremely scarce gene products, to find similarities among them, and to detect and separate isoenzymes of proteins (Experimental Biosciences).

Gel preparation

Two different layers of acrylamide between glass plates are required: the lower layer (the separating gel) separates the proteins by size whereas the upper one (the stacking gel) is of very low acrylamide concentration and includes the sample wells into which the proteins are loaded. Thus the proteins are compressed (=stacked) into micrometer thin layers when they reach the separating gel (EnCor Biotechnology, 2006; Experimental Biosciences).

Separating gel

	Volume 12% separating gel	Volume 10% separating gel
H_2O	**3.35 ml**	**4.05 ml**
1.5M Tris HCl, pH= 8.8	**2.5 ml**	**2.5 ml**
Acrylamid	**4 ml**	**3.3 ml**
10% SDS	**100 µl**	**100 µl**
10% ammoniumpersulfate	**50 µl**	**50 µl**
TEMED	**5 µl**	**5 µl**

Stacking gel

	Volume 4 % stacking gel
H_2O	**6.1 ml**
0.5M Tris HCl, pH= 6.8	**2.5 ml**
Acrylamid	**1.3 ml**
10% SDS	**100 µl**
10% ammoniumpersulfate	**50 µl**
TEMED	**10 µl**

Table 5. Gel Preparation.

Sample preparation and electrophoresis

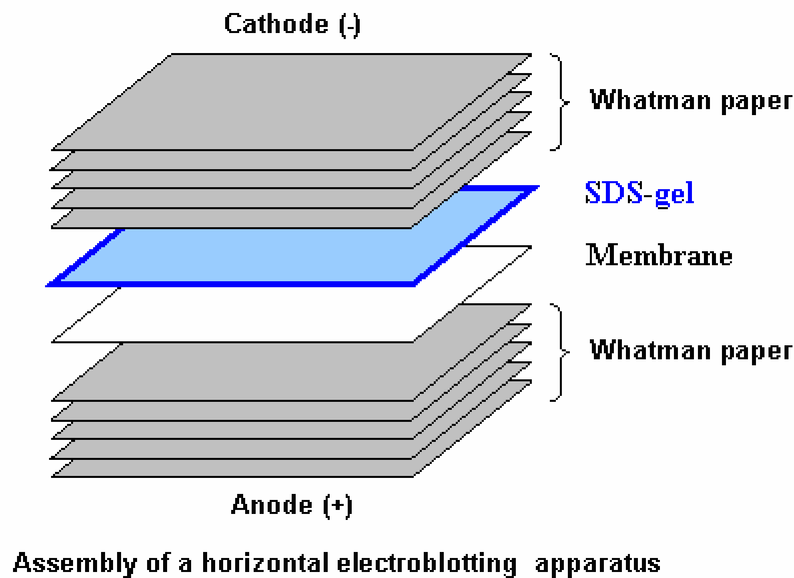
15-20 µg of each protein sample was supplemented with 1x SDS buffer (see 3.5.4), the tubes were incubated for 5 min at 95°C. Meanwhile, the pre-stained protein ladder was also incubated for 1 min at 40°C. The tubes were centrifuged for 1 min at 13000 rpm and left at room temperature until they were ready to be loaded onto the gel. The gel was run at 100V for separation for at least an hour.

3.5.3 Western blot

To transfer the separated proteins before incubation with antibodies, we used the wet-blot technique.

Transfer

The gel was removed from the electrophoresis apparatus and rinsed in transfer buffer (Towbin buffer). A Hybond-C-Extra membrane (Amersham) was wetted in dH_2O and then incubated with 4 layers of Whatman paper for 10 min in the Towbin buffer. The gels were also placed in transfer buffer. The blotting sandwich was assembled as follows:



Assembly of a horizontal electroblotting apparatus

The blotting chamber was filled with Towbin buffer and power applied at a constant current of first 70 V and then 100V for 1-1.5h for the transfer.

Antibody incubation

The blot was removed from the transfer chamber and placed immediately into blocking solution (5% skimmed milk in 1XTBST) in a small tray and allowed to block for an hour at room temperature on a shaker. Equal protein loading and completion of transfer were verified by staining the gel with Coomassie brilliant blue and the membrane with Ponceau S for 10 min and then immersed in dH_2O.

The membranes were then incubated with primary antibodies diluted in 5% skimmed milk and 1X TBST:

rabbit anti-AT_1 (1:100)

rabbit anti-AT_2 (1:100)

and kept overnight at 4 ºC on a shaker. Next, the membrane was washed 3 times with 1X TBST using a shaker: 1 x 10 min, 2 x 5 min, respectively.

The membranes were then incubated with secondary antibodies— goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000, DAKO, diluted in 5% fat-free milk and 1X TBST, see 2.5.2)— for an hour at room temperature on a shaker and then rinsed with 1X TBST as described above.

Detection of protein signal took place in a darkroom. First the membrane was incubated for 1 min in ECL solution (enhanced chemiluminescence detection system, Amersham Pharmacia Biotech, Kit) and then put into a film cartridge and covered with filter paper. The film (Hyperfilm, Amersham) was exposed for 1 to 10 min with respect to intensity and then developed. The relevant band was analysed densitometrically; the relative protein level was expressed as an arbitrary unit.

3.6 Statistical analysis

All data but the CBF-measurements were reported with mean ± sd (standard deviation). Comparisons between the sham and MCAO groups were performed by the two-tailed Student's unpaired t-test for independent samples. CBF-data was analysed using one-way ANOVA.

For all tests, values of $p < 0.05$ were considered to be statistically significant.

4. Results

4.1 Changes in physiological parameters after transient MCAO

The obstruction of the blood flow in the right cerebral hemisphere following MCAO lead to a change in CBF and thus to an ensuing brain injury, which were followed by changes in general physical and neurological status.

4.1.1 Cerebral blood flow measurements

CBF was similar in the rats of all groups at the beginning of the experiment with values of around 90±20 U (units). During the surgery, CBF initially increased slightly, but it decreased immediately after occlusion of the ipsilateral (right) MCA and remained at values between 10 and 20 U. Of significance was considered a drop of more than 80% of baseline CBF (average drop 12.23 %, SD 0.04). After filament withdrawal, the ipsilateral rCBF increased to 53±28% of baseline.

In the sham-operated group, the CBF remained constant and showed only slight fluctuations throughout the surgery procedure.

Three of the animals, which showed a severe drop of CBF (more than 90 %), died a day later.

Animal groups	CBF (baseline)	Baseline During MCAO (min)			Reperfusion period (min) or	
		0	15-30	60	0	30
Sham	91.8 U	86.8 U	83.3 U	87.2 U	93.1 U	-
Stroke	89.3 U	19 U	17.2 U	21.2 U	26.4 U	49.5 U

Table 6. CBF- mean values measured at different points during and after the ischemia or the sham-surgery, respectively.

Ipsilateral cerebral blood flow (CBF) was measured at the beginning, 30 and 60 minutes during MCAO, immediately after removing the catheter and 30 min after reperfusion. The values are mean ± SD. No significant differences between CBFs recorded at individual time points were found (one-way ANOVA).

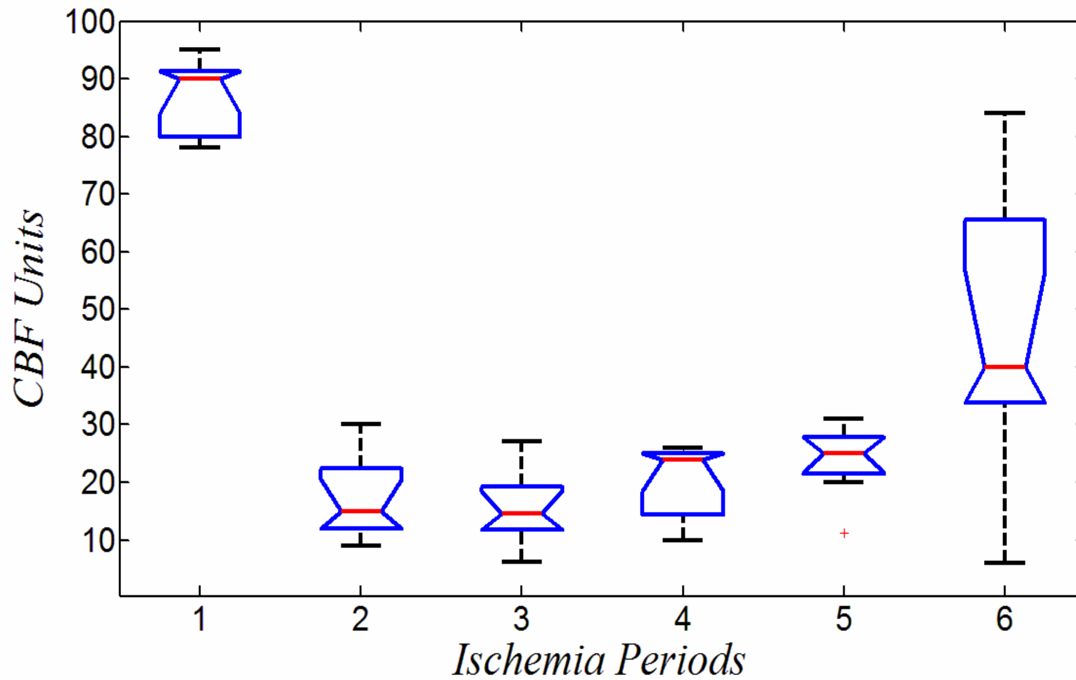


Figure 9: Ischemia periods 1 to 6 according to the table above (surgery begin, ischemia begin, 15-30, 60 min during ischemia and 10 and 20 min after filament withdrawal) using one-way ANOVA (n =9).

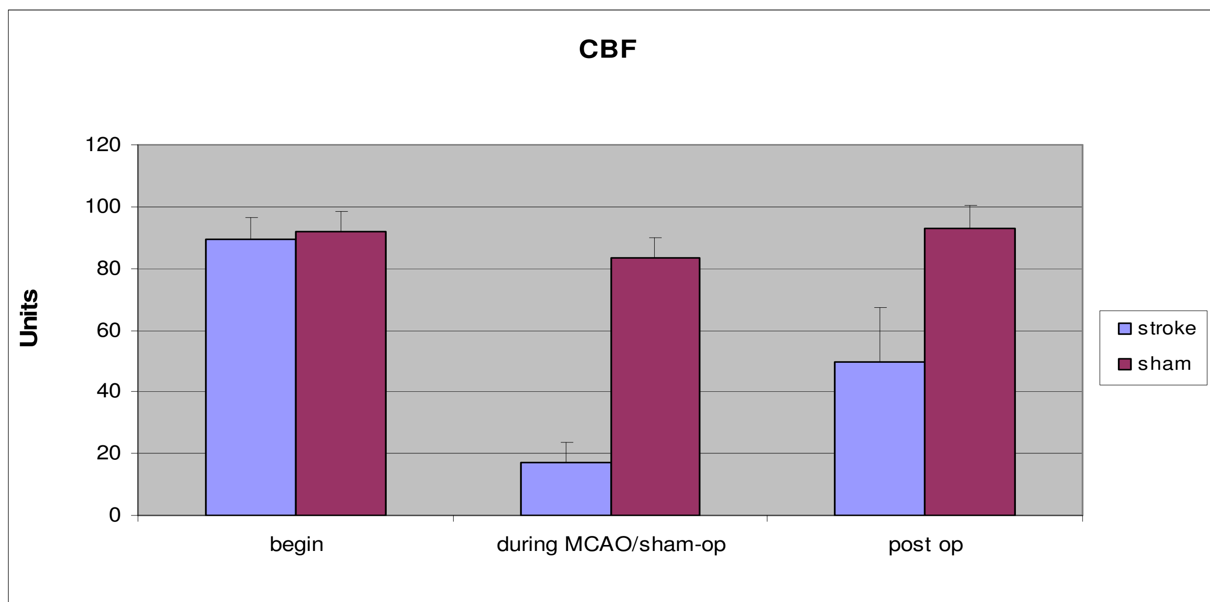
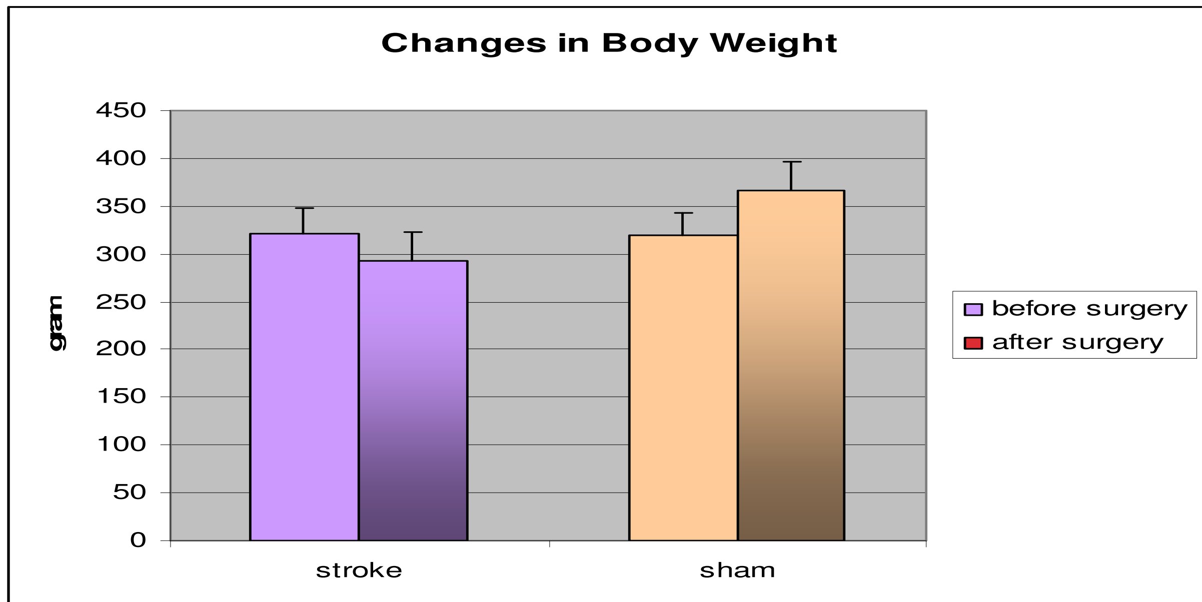


Figure 10: CBF in the sham group remained nearly constant during the surgery with a light drop under anaesthesia. In the stroke group there was a significant drop in CBF during the ischemia period (p<0.0001, n =9) and during the reperfusion period (p<0.0001, n =9).

4.1.2 Reduction in body weight

The animals were weighed before the surgery and on the days following. Those with stronger neurological deficits and presumably bigger infarction area moved less and showed reduced appetite and loss of weight.



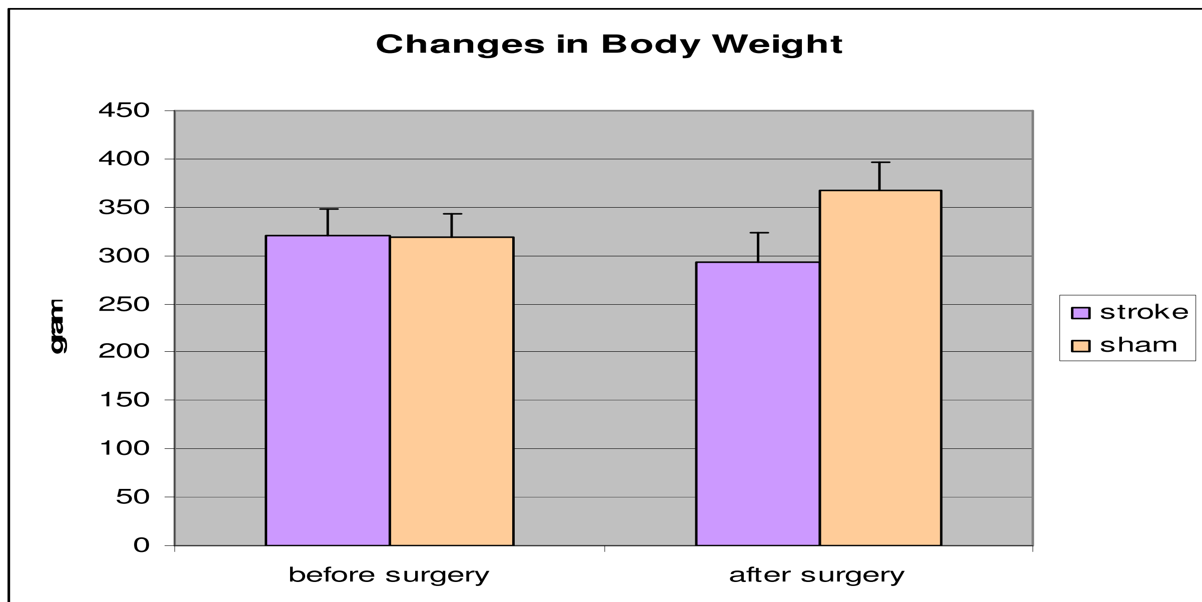


Figure 11: a) Slight, not significant weight loss in the stroke group before an after MCAO versus weight gain in the sham operated animals ($p>0.05$, $n=9$); b) shows significant difference between the weight of stroke and sham animals after surgery ($p<0.01$, $n=9$).

Accordingly, the sham operated animals had normal appetite and had no neurological deficits and gained weight as expected.

There was no significant difference between the weight gain and weight loss before or after MCAO in either of the two groups, whereas there was a significant difference when comparing the weight of the stroke animals after MCAO and the weight of the sham operated animals (see Fig. 11b).

4.1.3 Neurological deficit evaluation

Neurological findings were scored on an 18-point scale (Garcia *et al.*, 1995b) which includes six different parameters listed in Chapter 3 § 3.1.5

On the days after surgery all animals of the MCAO- group exhibited at least one of the neurological signs accompanying stroke: paralysis of anterior and/or posterior left paws, aggression, prostration, loss of body symmetry or no reaction to touch. Sham operated Rats had slight or no neurological signs.

Neurological evaluation, carried out after the grading scale developed by Garcia et al. (1995), showed a significant reduction of vital signs in the animals with cerebral infarct.

Animals	***Score points post MCAO/sham-OP (24hours)***
Sham	*17,56*
MCAO-operated	*12,33*

Table 7 Neurological score results, average.

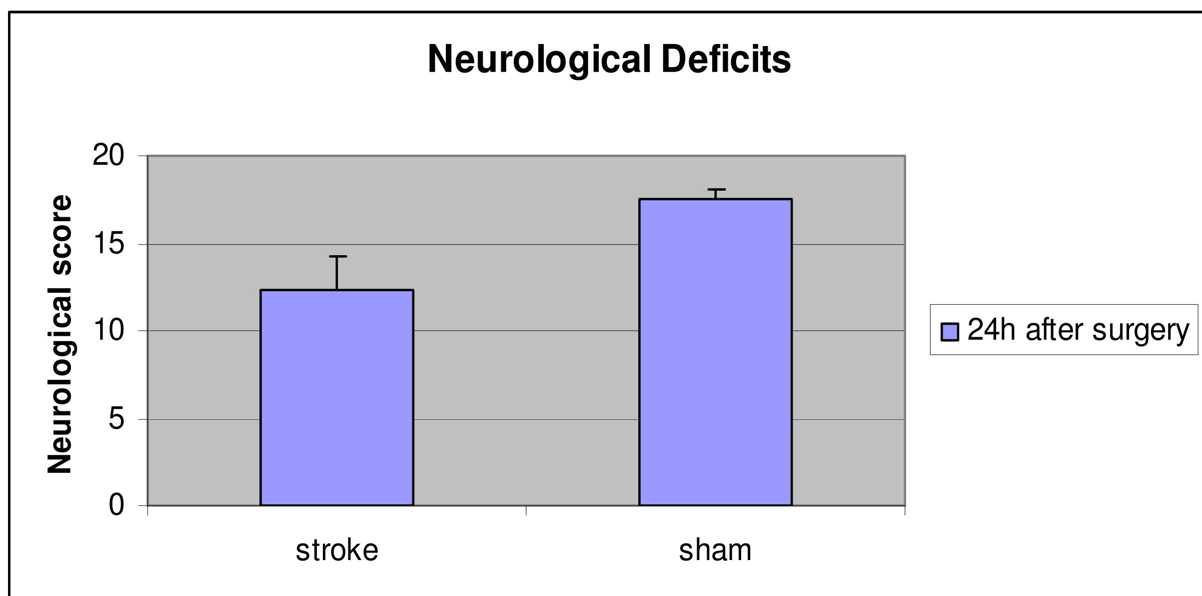


Figure 12: Score scale showing neurological deficits in the stroke group versus the sham group ($p<0.001$, $n=9$).

4.2 Infarction detection with the TTC staining method

There was a sharply demarcated infarct area in the brains of animals that underwent 90 min MCAO. (See Fig. 15). Unilateral tissue injury revealed a pale area in the ipsilateral hemisphere, located in the frontoparietal and temporal cortex and parts of the lateral segment of the caudate nucleus, owing to which it was easily distinguishable from the adjacent tissue (TTC staining method).

In cases in which it was not possible to advance the intraluminal catheter deep enough into the ACI, and correspondingly there was no significant drop in CBF, there was also a smaller or no infarction area. These animals were excluded from the study.

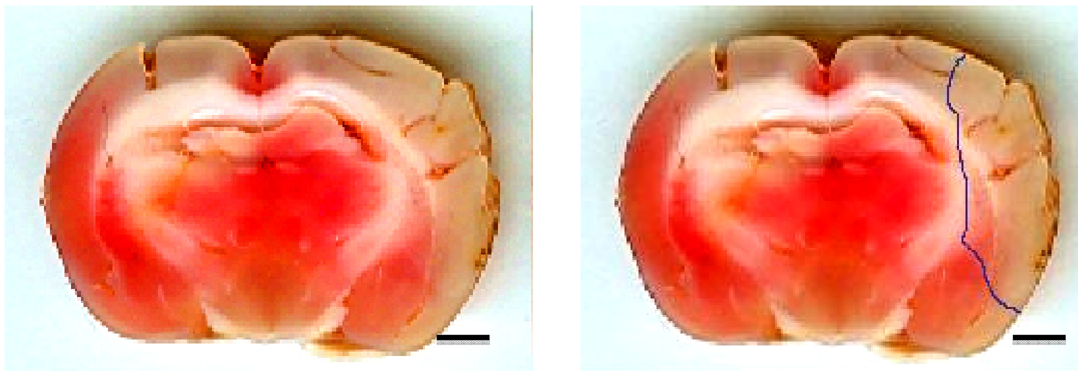


Image 3: TTC staining method of rat brain. The brain slice shows the grey-brownish ischemic area in the territory of the right MCA (stroke group). Scale bar 2 mm.

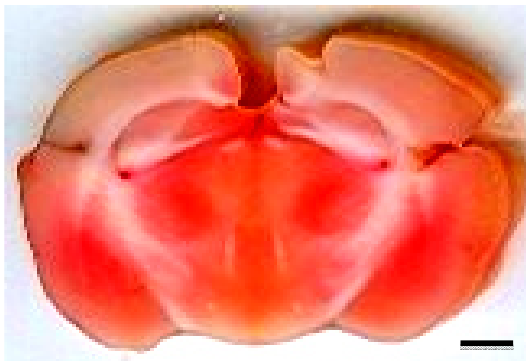


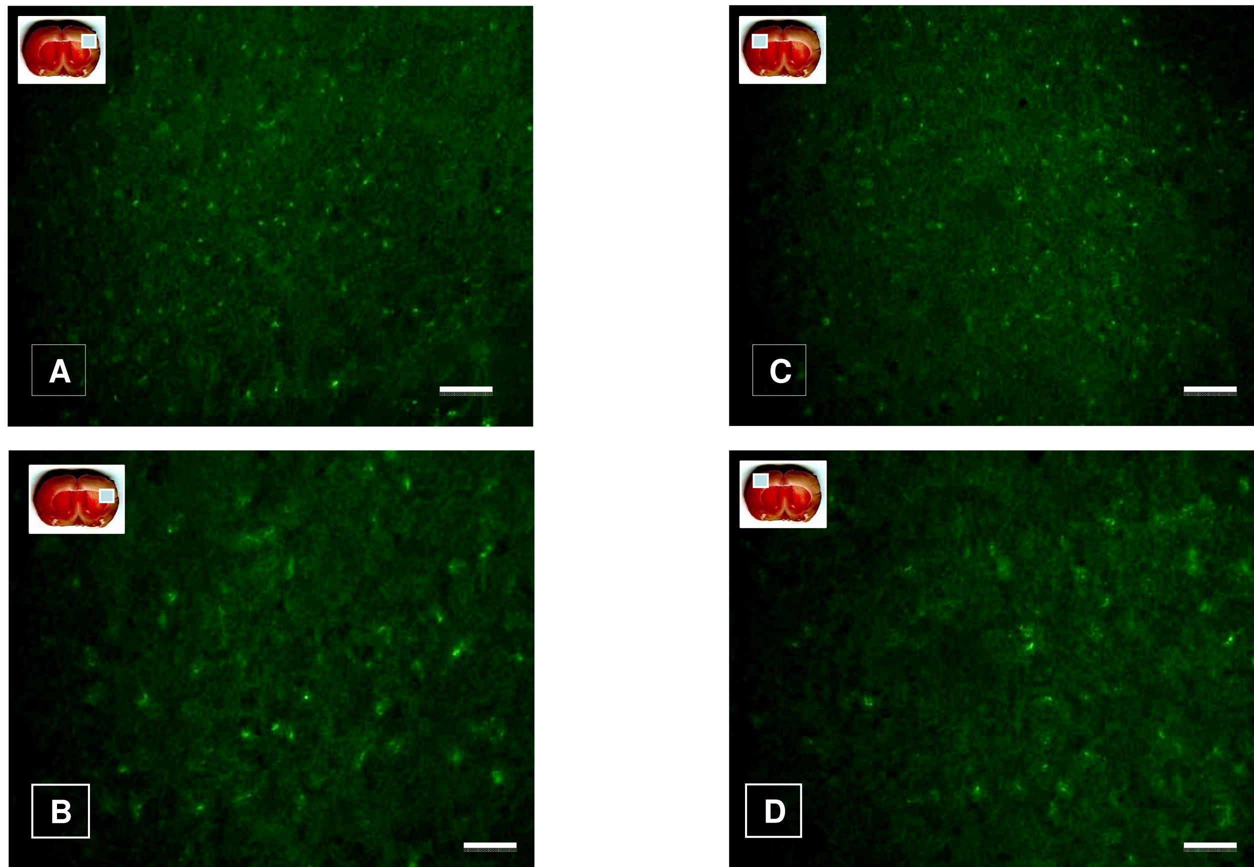
Image 4: TTC staining method of rat brain without detectable infarction area (sham group). Scale bar 2 mm.

4.3 Changes in the expression of Ang II receptors at histological, molecular and protein biochemical levels

First we made several immunofluorescence stainings of AT_1 and AT_2 receptor-positive cells in order to find their localisation and quantify any different expression.

4.3.1 No change of AT_1 receptor expression after MCAO

The number of AT_1 receptor immunostained cells, which were found predominantly in some brain regions such as the cerebral frontal cortex, cingulum and striatum, showed no significant fluctuation when the ischemic and contralateral hemisphere in stroke animals were compared.



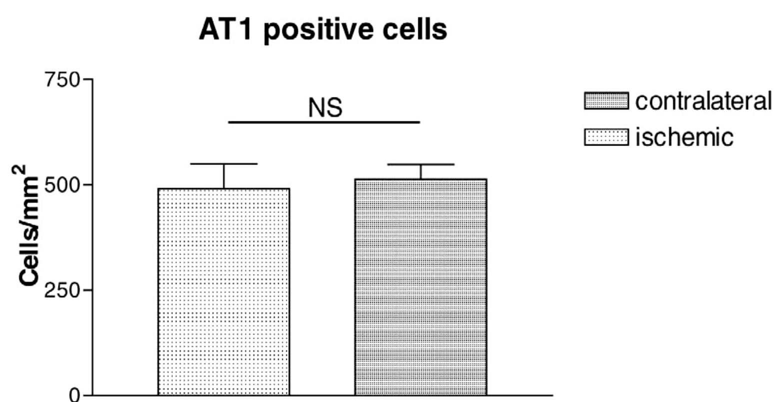


*Figure 13: Unchanged AT_1 receptor expression in brain cells: There was no difference in the number of AT_1 receptor-positive cells in ischemic (**A** and **B**) versus non-ischemic hemisphere (**C** and **D**). Scale bar 100 µm (**A** and **C**); 50 µm (**B** and **D**). Quantitative analysis was performed from digital images at x 100 using OpenLab imaging software. The resulting frame accounted 1.53 mm^2. AT_1-labelled cells for each sample were calculated as cell number per mm^2.*

These findings correlated well with the molecular results: there was no significant change in expression of cerebral AT_1 receptors either at mRNA or protein levels 2 days after MCAO (Figure A & B).

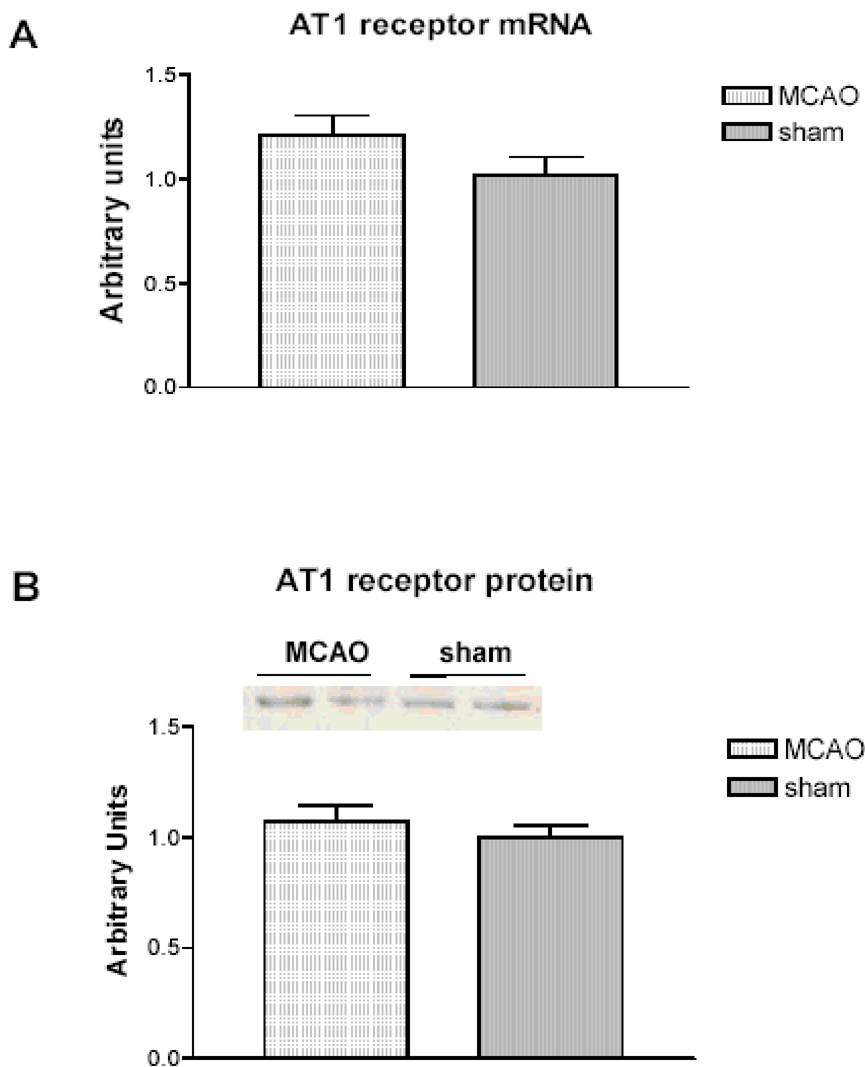
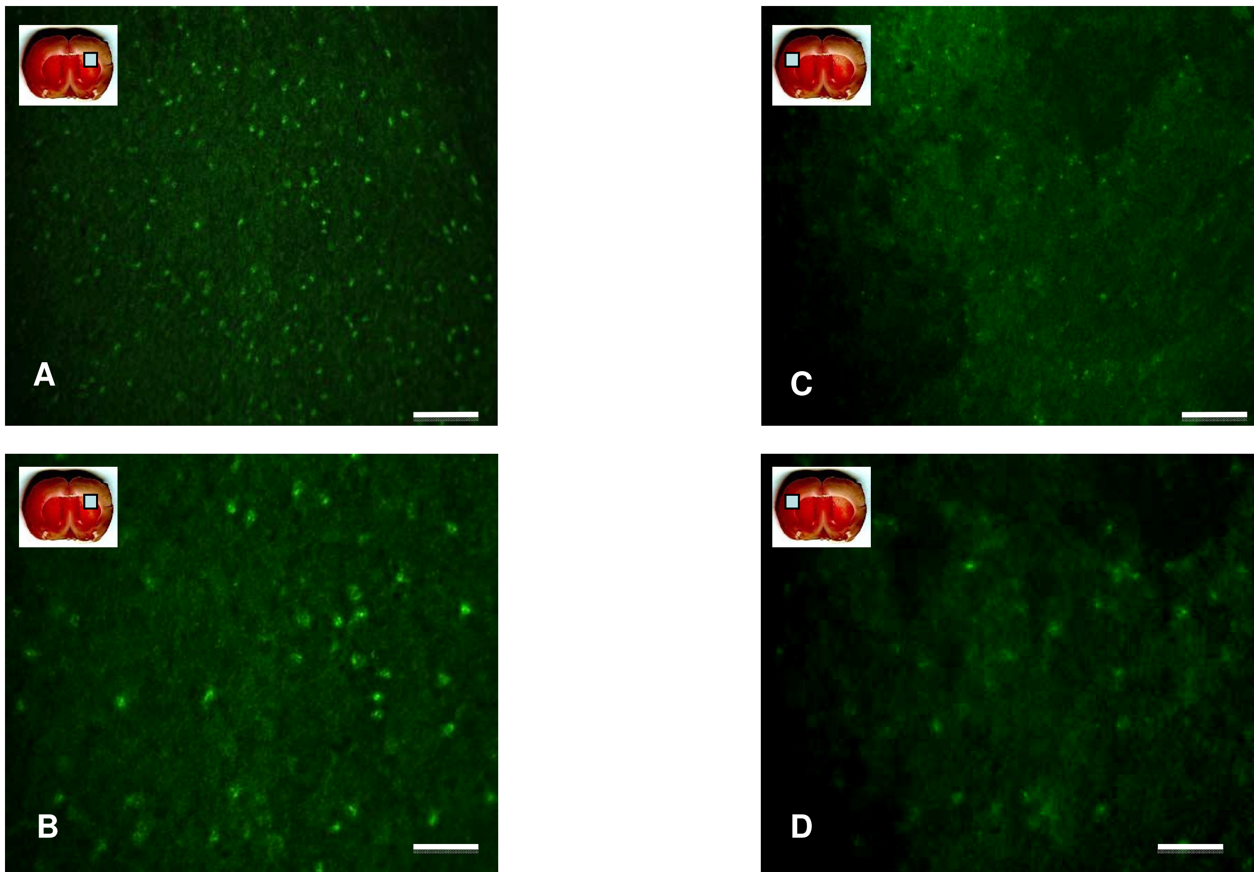


Figure 14: Unchanged expression of AT_1 receptor after focal cerebral ischemia: a) RT-PCR analysis showed no significant difference in cerebral AT_1 receptor expression between the stroke and sham groups; b) Western blot analysis. Densitometric analysis confirmed the result.

4.3.2 Upregulation of AT_2 receptor expression after MCAO

In contrast with the unchanged AT_1 receptor expression, the performed immunofluorescence staining on brain samples showed increased expression of AT_2 receptor-positive cells in the ischemic hemisphere when compared to the contralateral one (Figure A-E), and these were abundantly distributed in the brain regions around the infarct area, including the cerebral frontal cortex, piriform cortex, striatum and hippocampus.



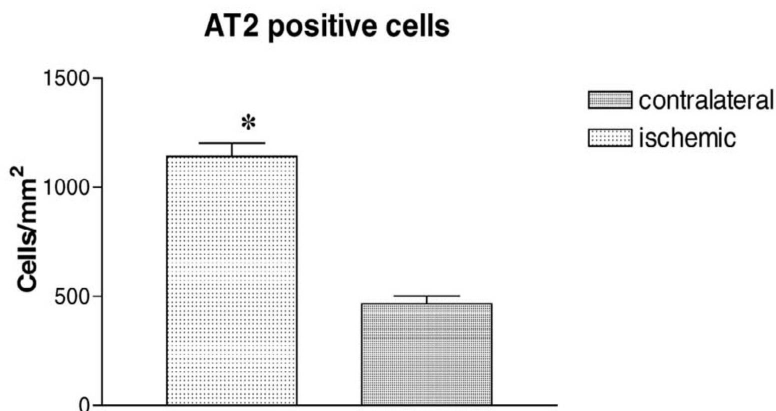


*Figure 15: Increased number of AT_2 receptor-positive cells after focal cerebral ischemia. Immunofluorescent labelling showed an increase in the number of AT_2 receptor-positive cells on the ischemic (**A** and **B**) when compared to the contralateral side (**C** and **D**). Scale bar 100 µm (**A** and **C**); 50 µm (**B** and **D**). Quantitative analysis was performed from digital images at x 100 using OpenLab imaging software. The resulting frame accounted 1.53 mm^2. AT_2-labelled cells for each sample were calculated as cell number per mm^2. * $p < 0.01$ versus contralateral hemisphere.*

Real-time RT-PCR and Western blot analysis confirmed a multifold increase in cerebral AT_2 receptor mRNA in the peri-infarct zone 48 hours after MCAO, in contrast to the unchanged AT_2 receptor expression in sham operated rats.

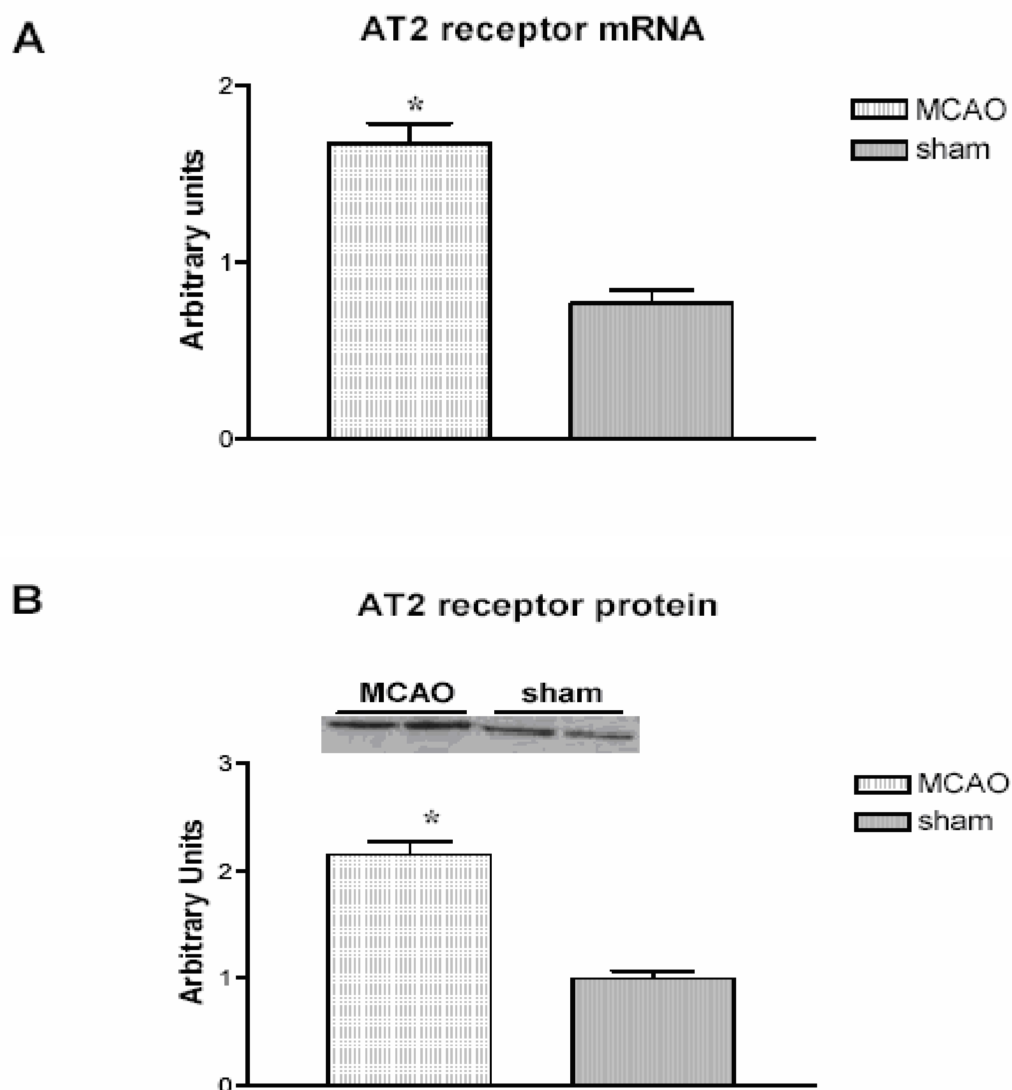


*Figure 16: Upregulation of AT_2 receptors after focal cerebral ischemia a) RT-PCR analysis revealed a 2.2-fold increase in AT_2 receptor expression 48 hrs after MCAO when compared to the sham operated rats; b) Western blot analysis of cerebral AT_2 receptor expression. Densitometry analysis confirmed the results (2.1-fold increase). * $p < 0.01$ versus sham control group.*

Another important finding was that the AT_2 positive cells looked morphologically like neurons.

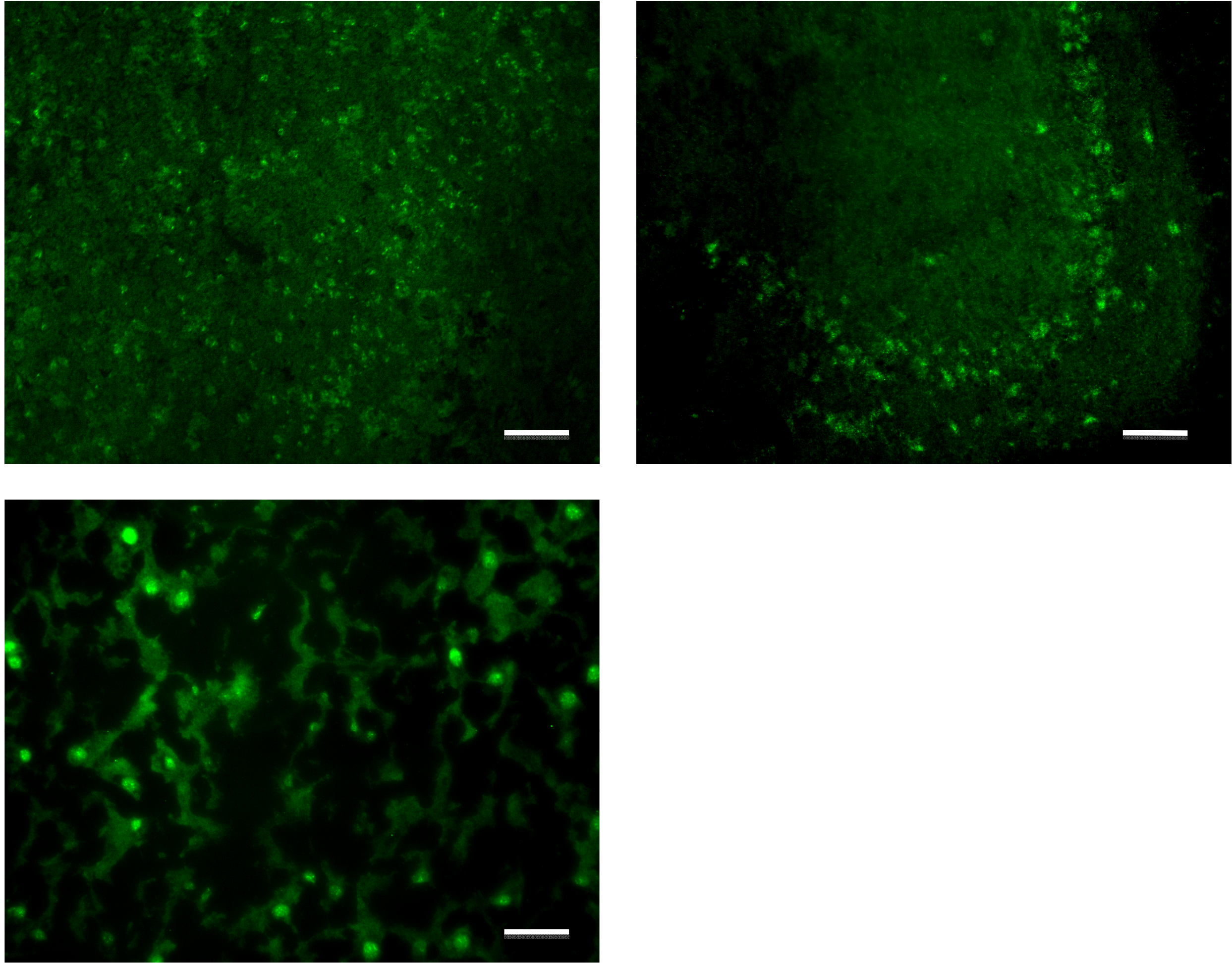


Image 5: FITC-labelled AT_2 positive cells in the striatal area. Scale bar 100 and 50 µm, respectively.

Therefore, we decided to perform some double immunofluorescence staining and used two neuron-specific markers – NeuN and MAP_2- to prove this (see 4.7.3).

4.4 Expression of neuronal and glial markers: NeuN, MAP_2, GFAP

We used several cell markers to identify some of the cell types in the brain: NeuN as a neuron-specific one, MAP_2 as another neuronal marker and GFAP as an astrocyte marker.

4.4.1 Expression of NeuN and MAP_2

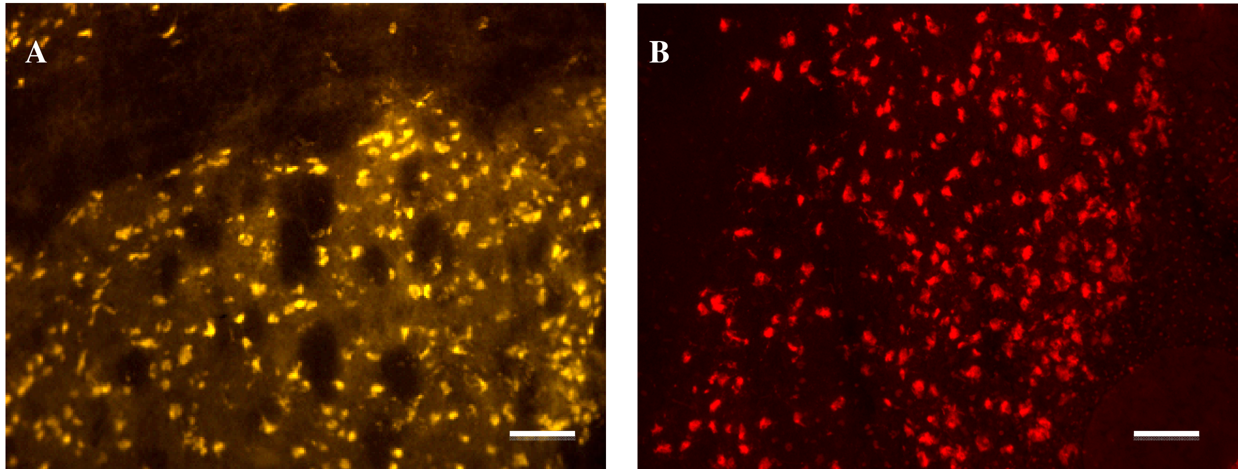


Image 6: Neuronal markers: Rhodamine (A) and Cy3 (B) fluorescent labelling with NeuN-marker showed intense positive signal of neuron-like cells in the cortex. Scale bar 50 µm.

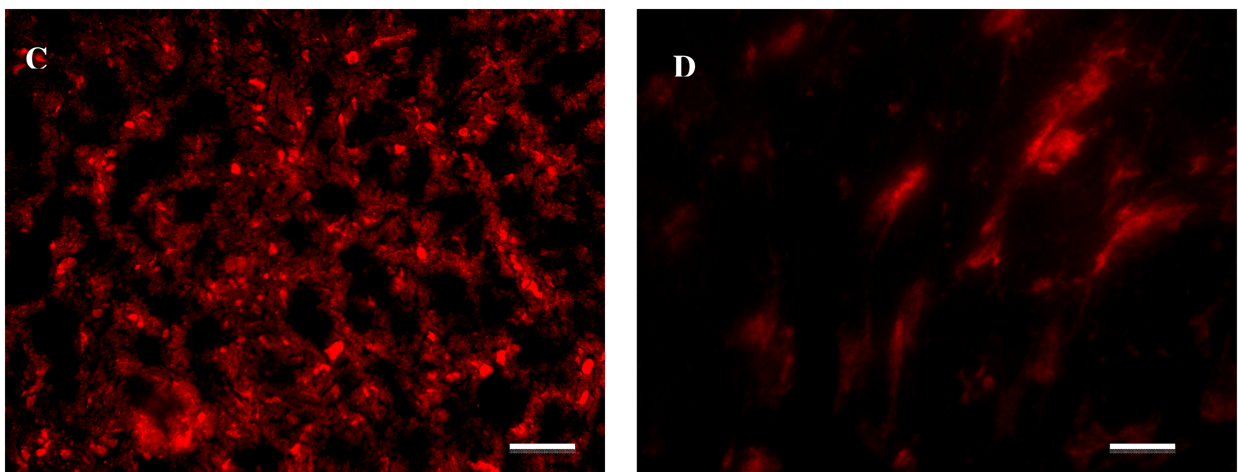


Image 7: Neuron Marker: Cy3-fluorescent labelling of cells with another neuron-specific marker, MAP_2 (C and D). Scale bar 50 and 25 µm, respectively.

Using the NeuN labelling, the slices revealed the neuron-typical nuclei.

A more specific marker- MAP_2- showed positively stained neurons in the ischemic hemisphere of a stroke animal. Some of the neurons showed visibly extended neurites as a sign of activation and growth (see also below 4.7.2.2.).

4.4.2 Expression of GFAP

After identifying neuron-typical cells, we tried to identify glial cells.

4.4.2.1 Identification

GFAP immunofluorescent labelling showed intense fluorescence of astrocyte-like cells throughout the brain parenchyma. The astrocytes derive its name from its starlike shape with multiple branching processes projecting out of the body and containing glial fibrillary protein. After injury (cerebral ischemia, e.g.) they first react with cytoplasmic swelling, later with hypertrophy and hyperplasia. (Robbins and Cotran, Pathologic Basis of Disease, 8th Edition, Saunders Elsevier, 2010. Chapter 28, p.1281)

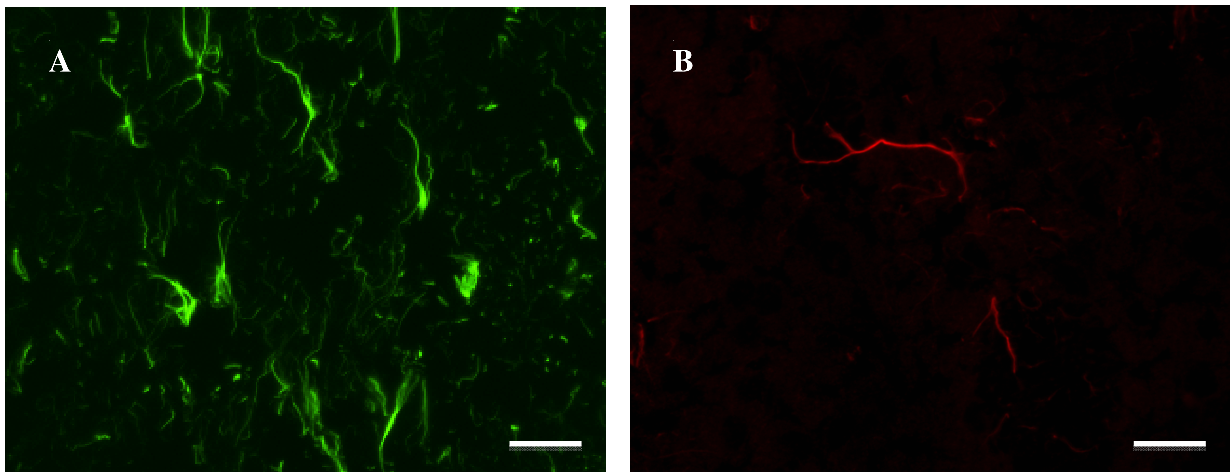


Image 8: A shows FITC-labelled GFAP-positive cells in an ischemic cerebral hemisphere. Note the prominent processes of the stained cells; scale bar 50 µm; B shows a greater magnification of Cy3-labelled GFAP-positive cells (scale bar 25 µm).

4.4.2.2 Quantification

Following MCAO the immunofluorescence showed a significantly increased number of GFAP-positive cells in both cerebral hemispheres, predominantly on the right side. The glial cells in the ischemic hemisphere were not only increased in number, but also showed more and thicker dendrites.

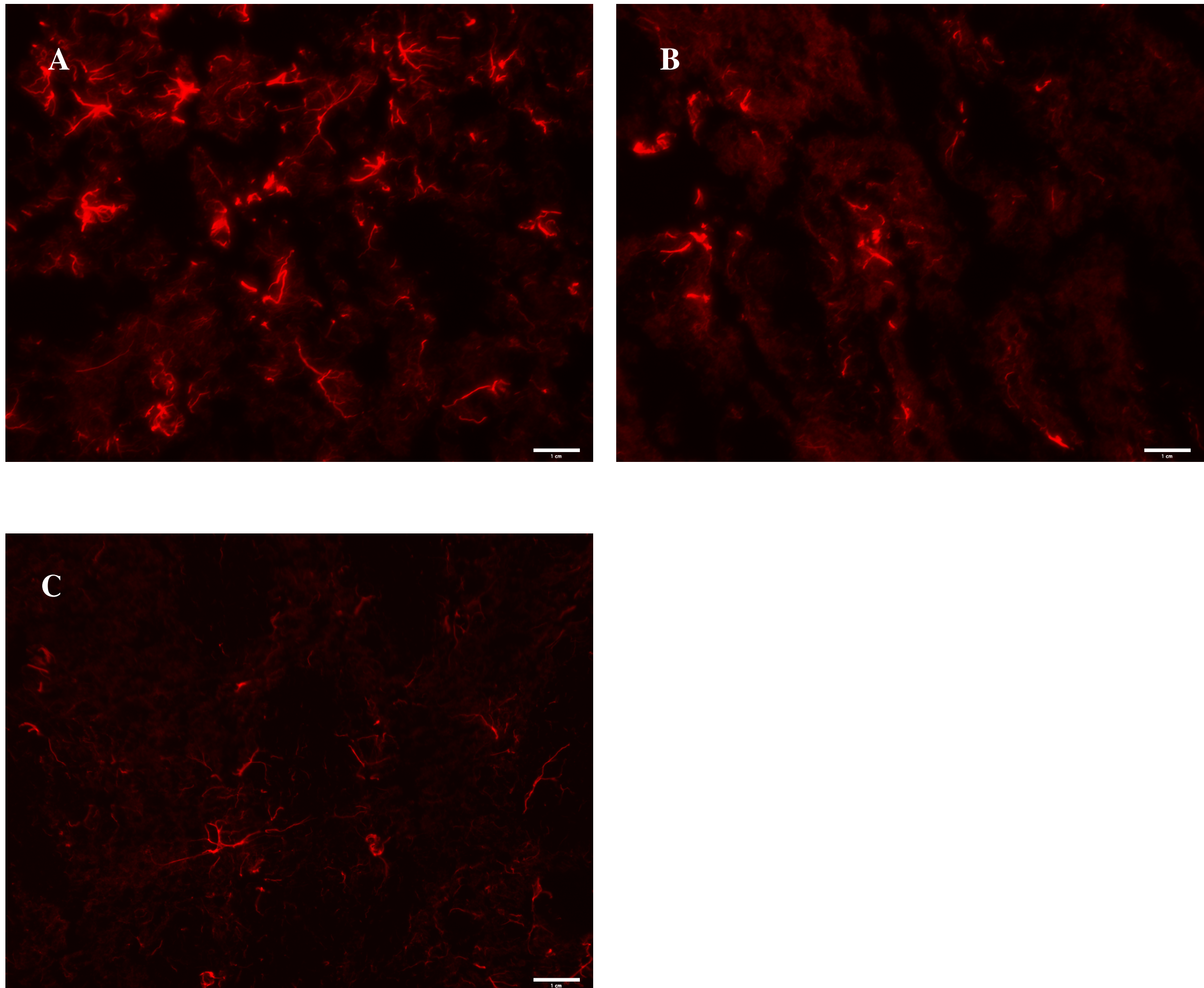


Image 9: Cy-3 labelled GFAP-cells in an ischemic (A) and contralateral, non-ischemic hemisphere (B) of a stroke animal. (C) shows normal astrocytes (with thin projections) in an animal from the sham group. Scale bar 50 µm.

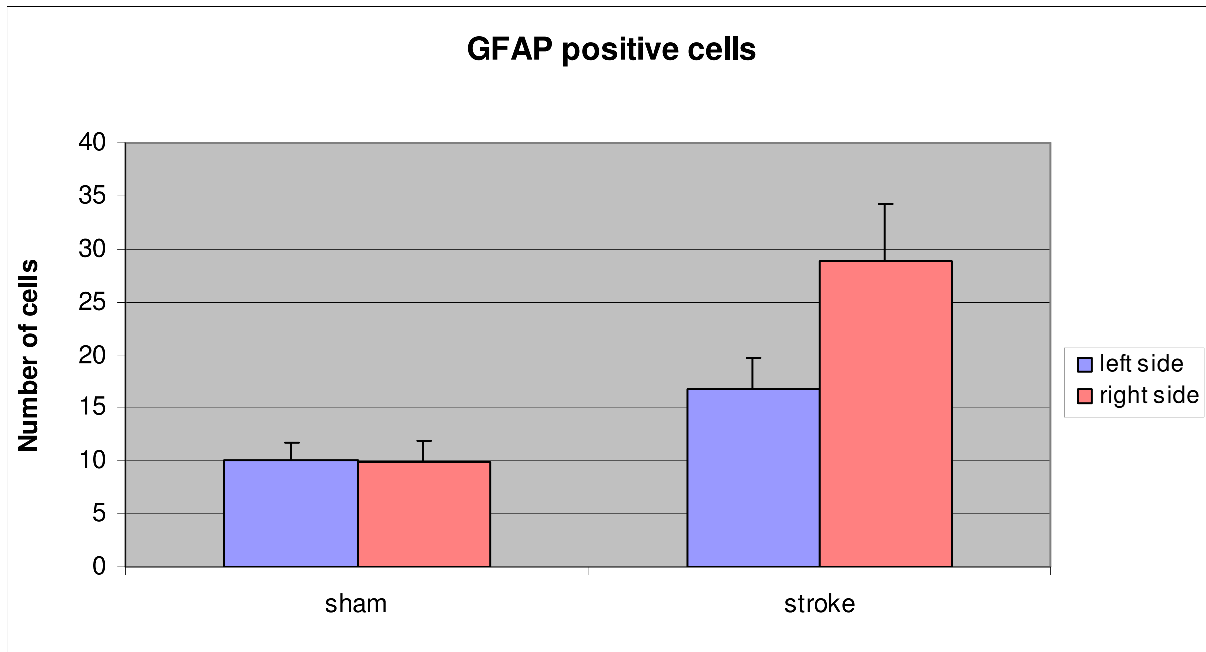


Figure 17: Increased number of GFAP-positive cells in animals from the stroke group. Quantitative analysis was performed from digital images at x 100 using OpenLab imaging software. The resulting frame area was 1.53 mm^2. GFAP-labelled cells for each sample were calculated as cell number per mm^2 (n=9).

The immunofluorescent labelling of astrocytes showed an increased expression of GFAP-positive cells in stroke-animals versus sham-operated animals: there was a significant difference of expression in both cerebral hemispheres ($p<0.001$), especially in the right one ($p<0.0001$).

4.5 Expression of inflammatory markers: ED1 and CD11b

When tissue suffers impaired oxygen supply eventually resulting in cell death, and after focal cerebral ischemia in most cases through necrosis, there is activation of glial cells as well as of inflammatory cells from the peripheral blood (neutrophils, macrophages) (Kumar V. *et al.*, 2010). These express on their surface markers such as ED1 and CD11b (see 3.3.3.3).

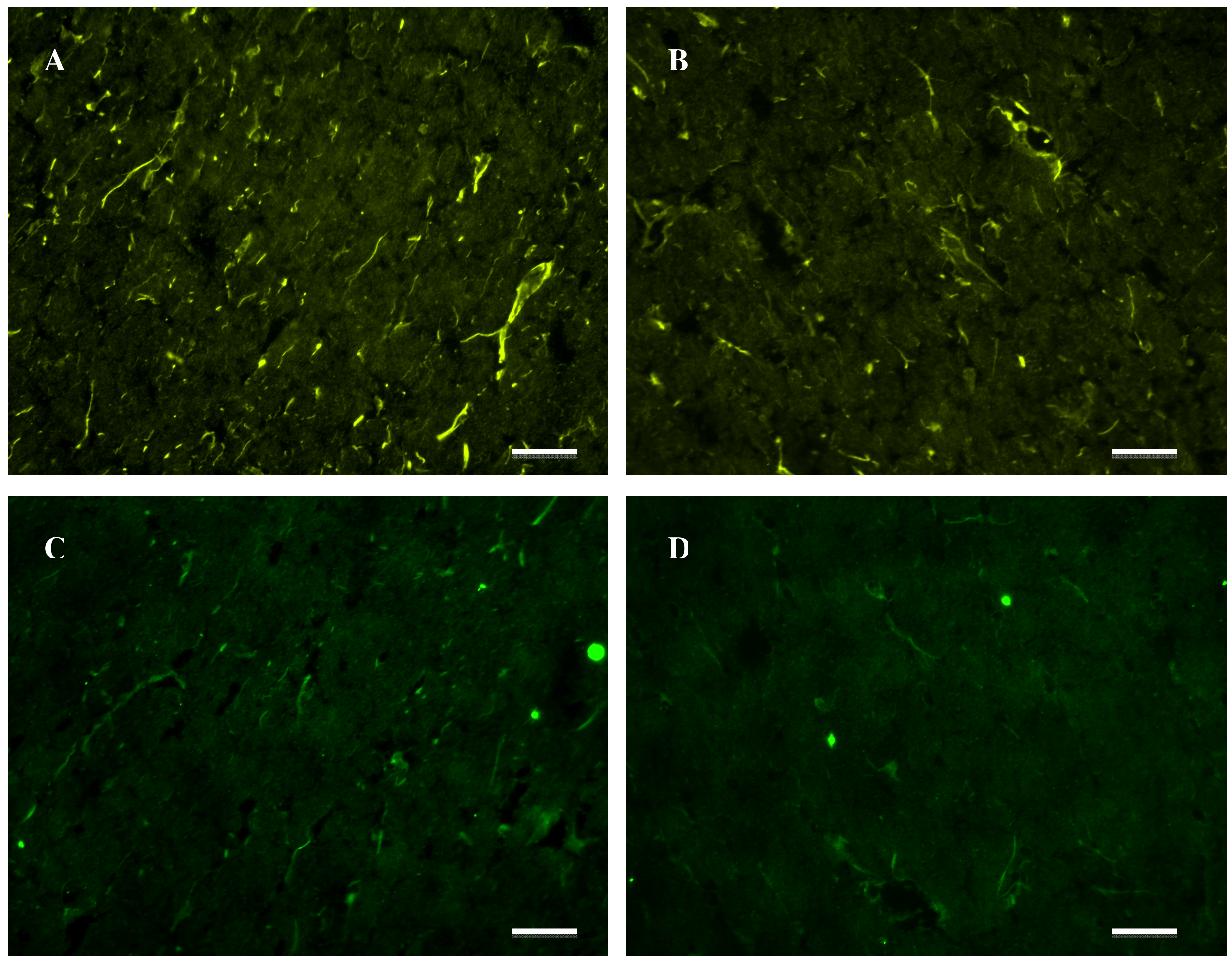


Image 10: CD11b-positive cells in the right (A) and left (B) cerebral hemisphere of a stroke animal (scale bar 50 µm). FITC-labelled ED1-positive cells on the right (C) and left (D) side of a stroke animal (Scale bar 100).

Notice the relatively increased number of CD11b- and ED1-positive cells in the ischemic hemisphere.

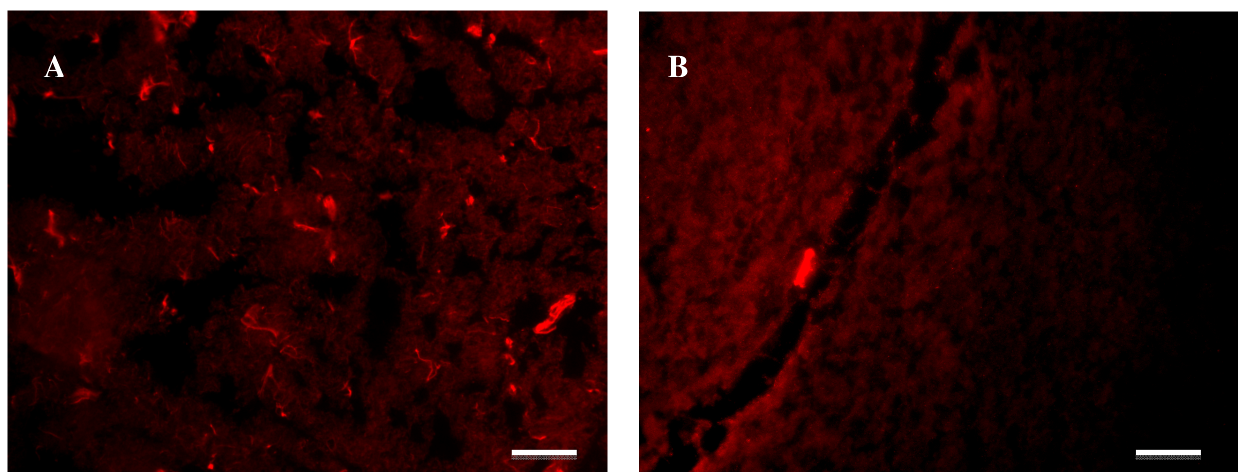


Image 11: Cy-3 Fluorescent labelling with Cy3-labelled ED1 marker in a stroke (A) and sham (B) animal, scale bar 100 µm.

Staining with ED1- marker showed increased number of ED1-expressing cells in the stroke group when compared to the sham one. The latter showed only few or no ED1-expressing cells.

The results showed us that in the ischemic hemisphere beside necrosis and apoptosis, inflammation took place as well.

4.6 Expression of apoptotic markers: cCasp-3

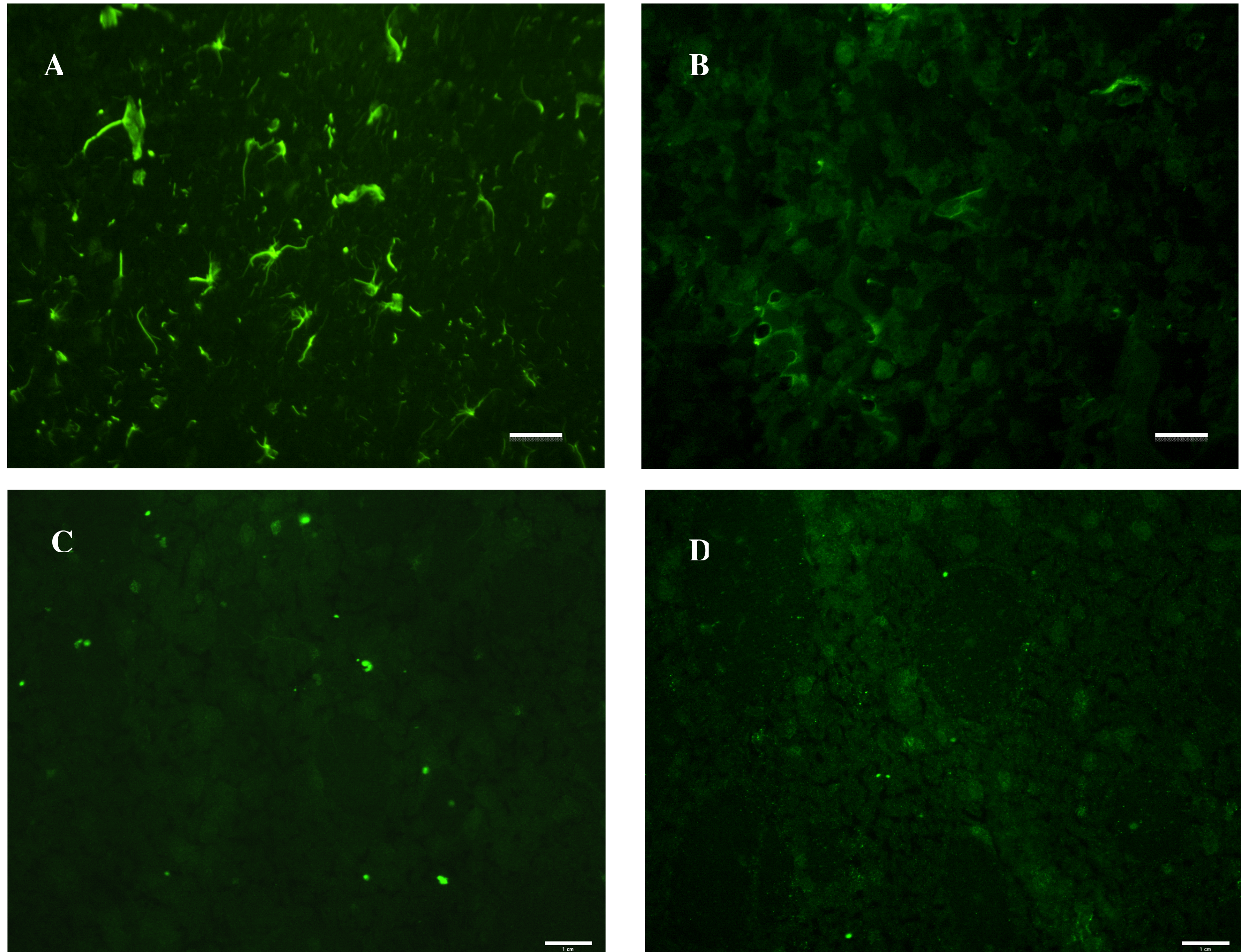


Image 12: Activated caspase-3 staining in the right (A) and left (B) cerebral hemisphere of a stroke animal and of a sham animal (C and D). Scale bar 50 µm.

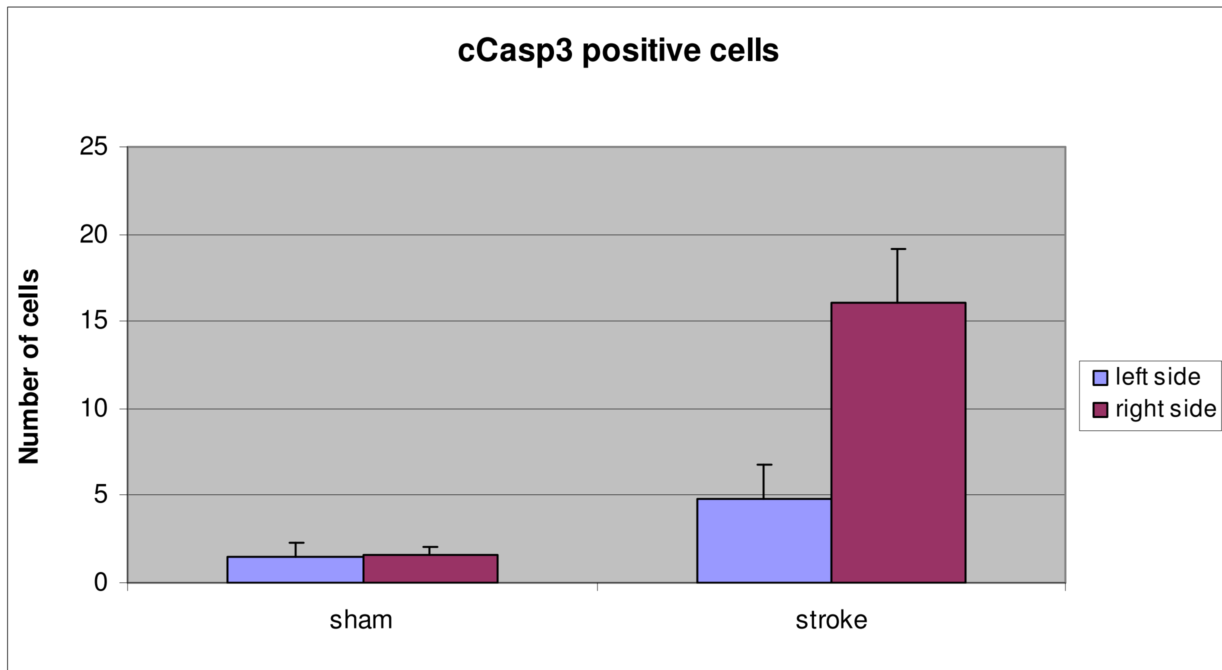


Figure 18: cCaspase-3 labelled cells were counted in the parietal cortex in three random and non-overlapping regions on each slice of a stroke or sham animal ($p < 0.001$, $n=9$).

Comparison between the sham and MCAO animals as well as between the left and right hemisphere of both groups showed that cCasp-3 labelled cells were significantly increased in number on the right side of the MCAO group, confirming the process of apoptosis after cell ischemia ($p<0.001$). In contrast, there were only few cCasp-3 positive cells in the brain parenchyma of sham animals.

4.7 Co-expressions

Undoubtedly, the next step was to identify the cell types, such as neurons or astrocytes, which happened to co-express either the AT_1 or the AT_2 receptor, or both, for example. Furthermore, by using many different markers in combination, we hoped to be able to say which of the two receptors mediates apoptosis, inflammation and regeneration.

4.7.1 Co-expression of AT_1 and GFAP

Double immunofluorescence showed an evident co-localisation of AT_1 receptor and GFAP, especially in the perivascular space.

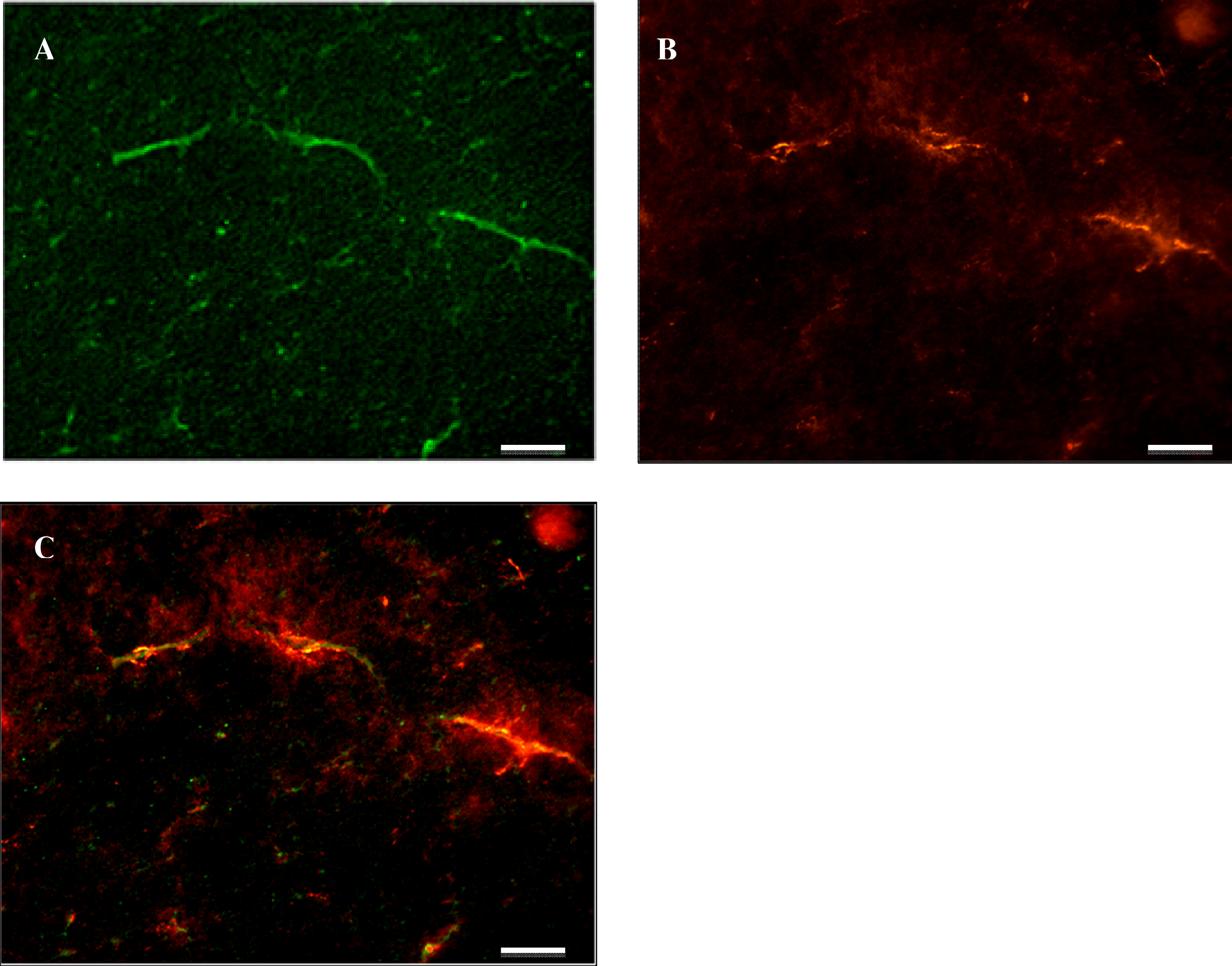
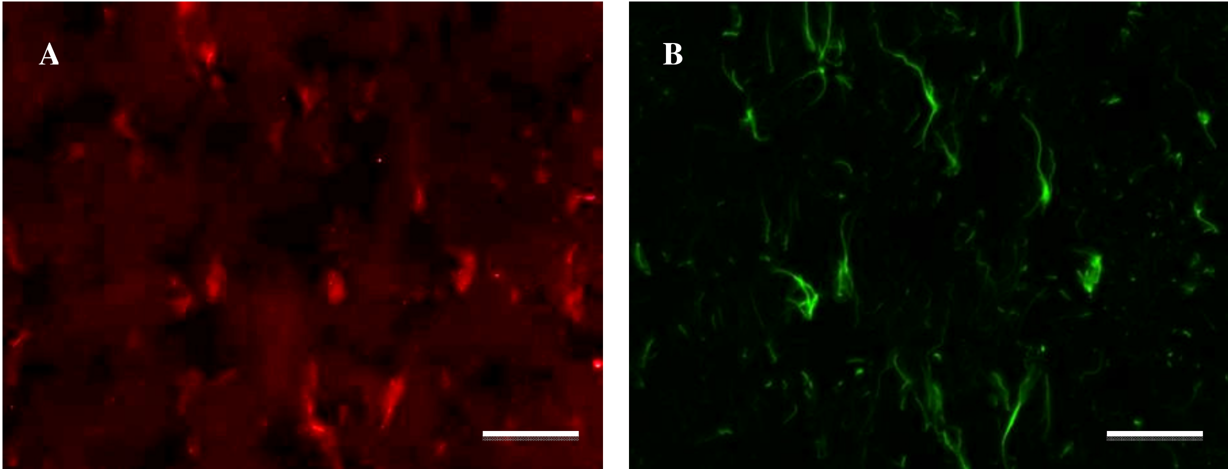


Image 13: FITC-labelled AT_1- receptor expressing cells (A) and Cy_3-labelled GFAP-positive glial cells (B) in the perivascular areas; (C) Co-localisation. Scale bar 50 µm.



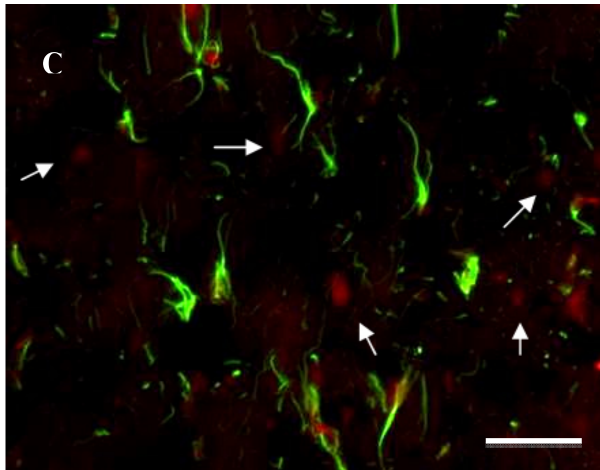
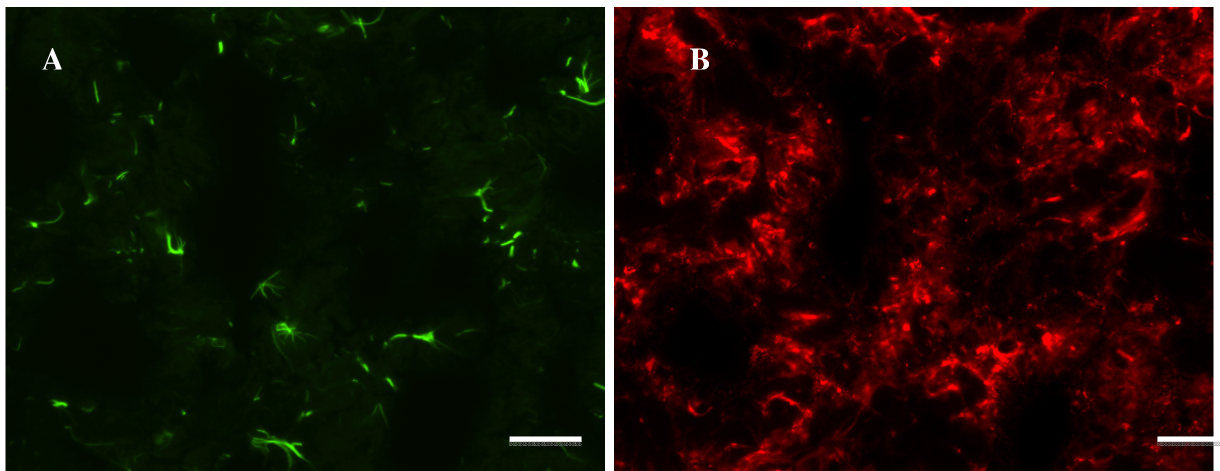


Image 14: Some of the AT_1 receptor-positive cells (A) co-localised with the GFAP-positive cells (B), co-localisation shown in (C). Few showed no co-localisation (arrows). Scale bar 50 µm.

This co-localisation was found in the infarct and periinfarct area of the right hemisphere. Some GFAP+ cells (especially the cell bodies) had also a specific signal for AT_1 receptor, while some AT_1+ cells showed no signal with the GFAP-specific marker.

4.7.2 No Co-expression of AT_1 and cCasp-3

Double immunofluorescence with the above mentioned apoptotic marker cCasp-3 and the *AT_1*-receptor specific marker showed no co-localisation.



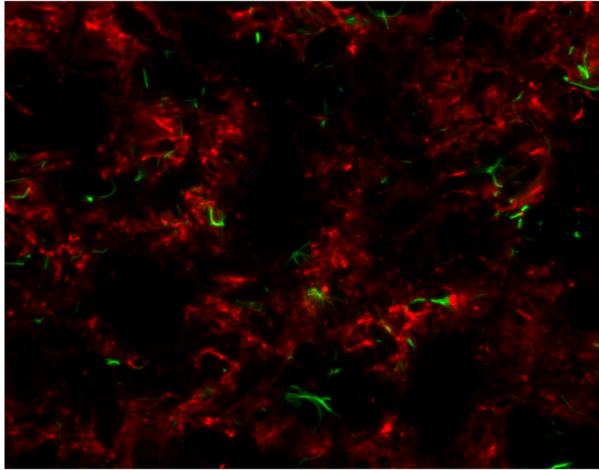


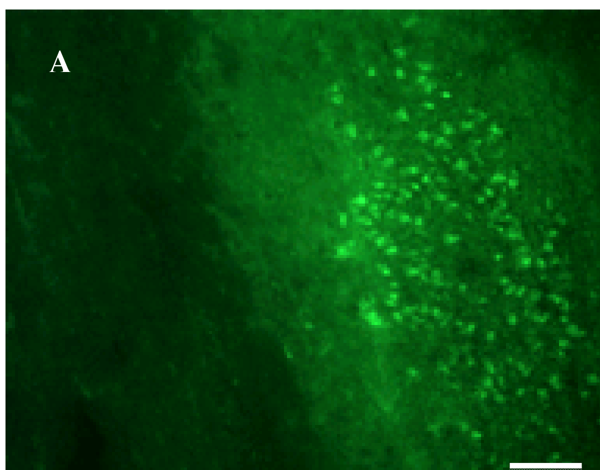
Image 15: FITC-labelled cCasp-3 expressing cells in the ischemic hemisphere of a stroke animal (A); double labelling with Cy_3-stain of AT_1- receptor-expressing cells (B) revealed no co-localisation of cCasp-3 and AT_1- receptor-positive cells (C). Scale bar 100 µm.

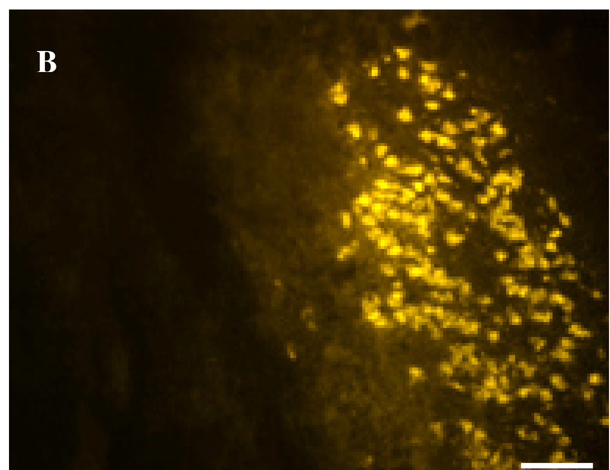
4.7.3 Co-expression of AT_2 and neuronal markers

We looked for a co-expression of AT_2-specific and the neuron-specific markers NeuN and MAP_2.

4.7.3.1 AT_2 and NeuN

Double immunofluorescence staining with the AT_2-specific and the neuron-specific marker NeuN showed an evident co-localisation signifying that AT_2 receptors were expressed by cortical neurons.





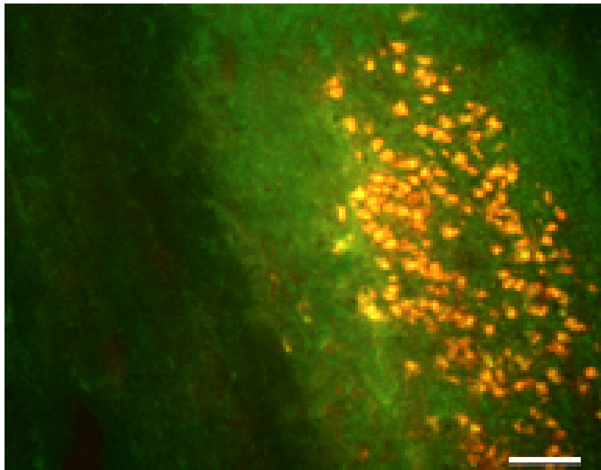
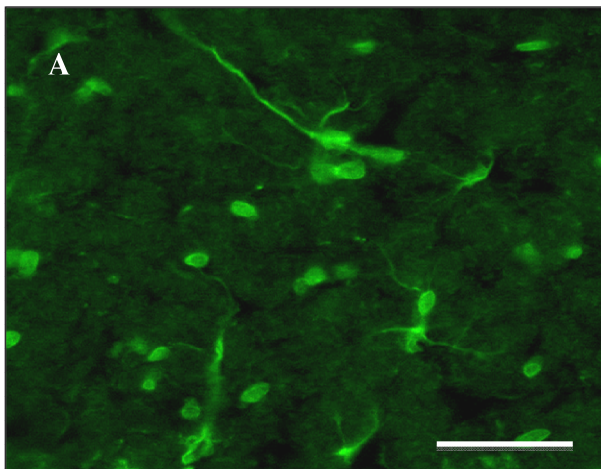
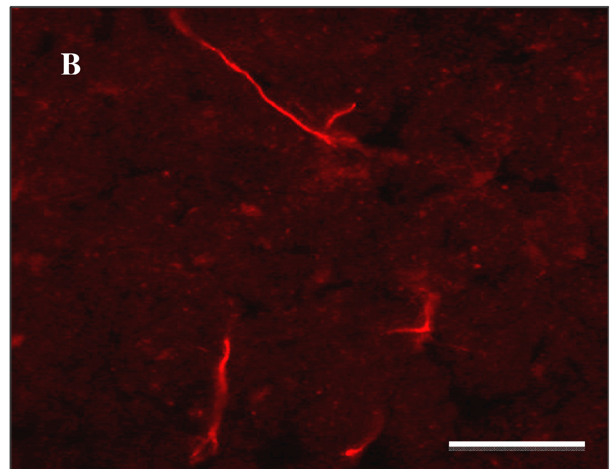


Image 16: FITC- labelled AT_2-receptors (A) and Cy_3-labelled NeuN-positive neurons (B) and their co-localisation *(C). Scale bar 100 µm.*

4.7.3.2 AT_2 and MAP_2

Co-localisation of AT_2-specific and the neuron-specific markers MAP_2 confirmed the NeuN-evidence that AT_2 receptors were located in neurons. Furthermore, there were noticeable long neurites to be found in the ischemic hemisphere when compared to the contralateral side.





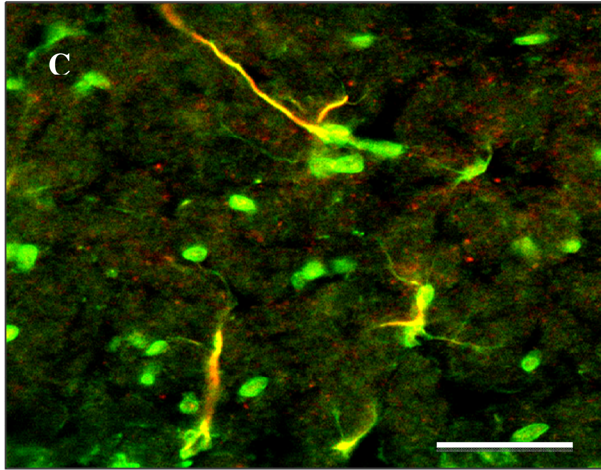
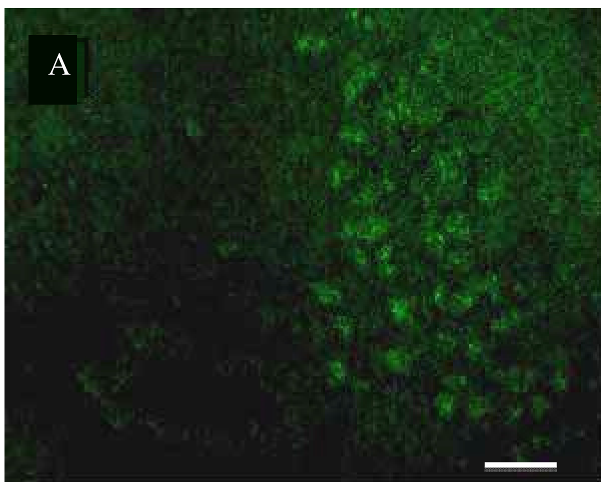


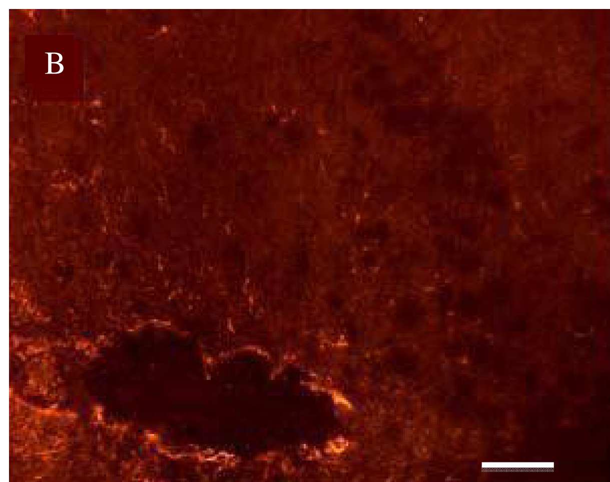
Image 17: FITC-labelled MAP_2-positive neurons (A) and AT_2-receptor-positive Cy3-labelled neurons (B) in the striatum neighbouring the ischemic area, and their co-localisation(C). Scale bar 25 µm.

The increased AT_2 receptor expression correlated with marked neurite outgrowth. Some neurons with strong AT_2 receptor expression showed longer and more intensely stained neurites compared to other neurons (stained only with MAP_2) expressing the AT_2 receptor weakly or not at all.

4.7.4 No co-expression of AT_2 and GFAP, or ED1

GFAP-positively stained glial cells were not co-localised with AT_2-expressing cells, in contrast to the co-expression with the AT_1 receptor. The AT_2-receptor expressing cells showed morphological likeness to neurons.





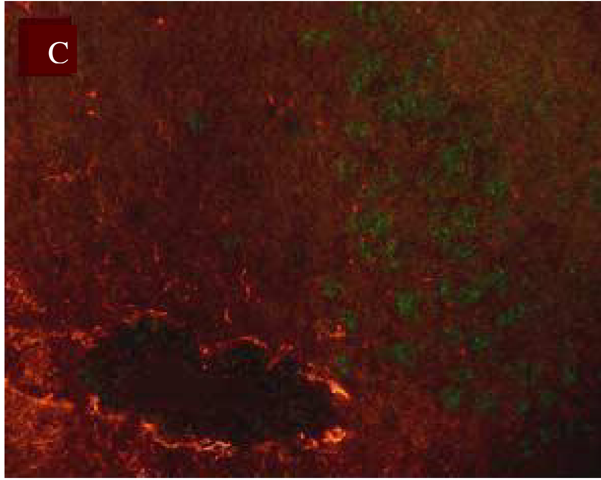


Image 18: FITC-stained AT_2-receptor expressing cells (A), Cy_3- labelled GFAP-expressing cells (B) and lack of co-localisation (C). Scale bar 50 µm.

Similarly, there was no co-localisation of the AT_2 receptor with the microglial marker ED1.

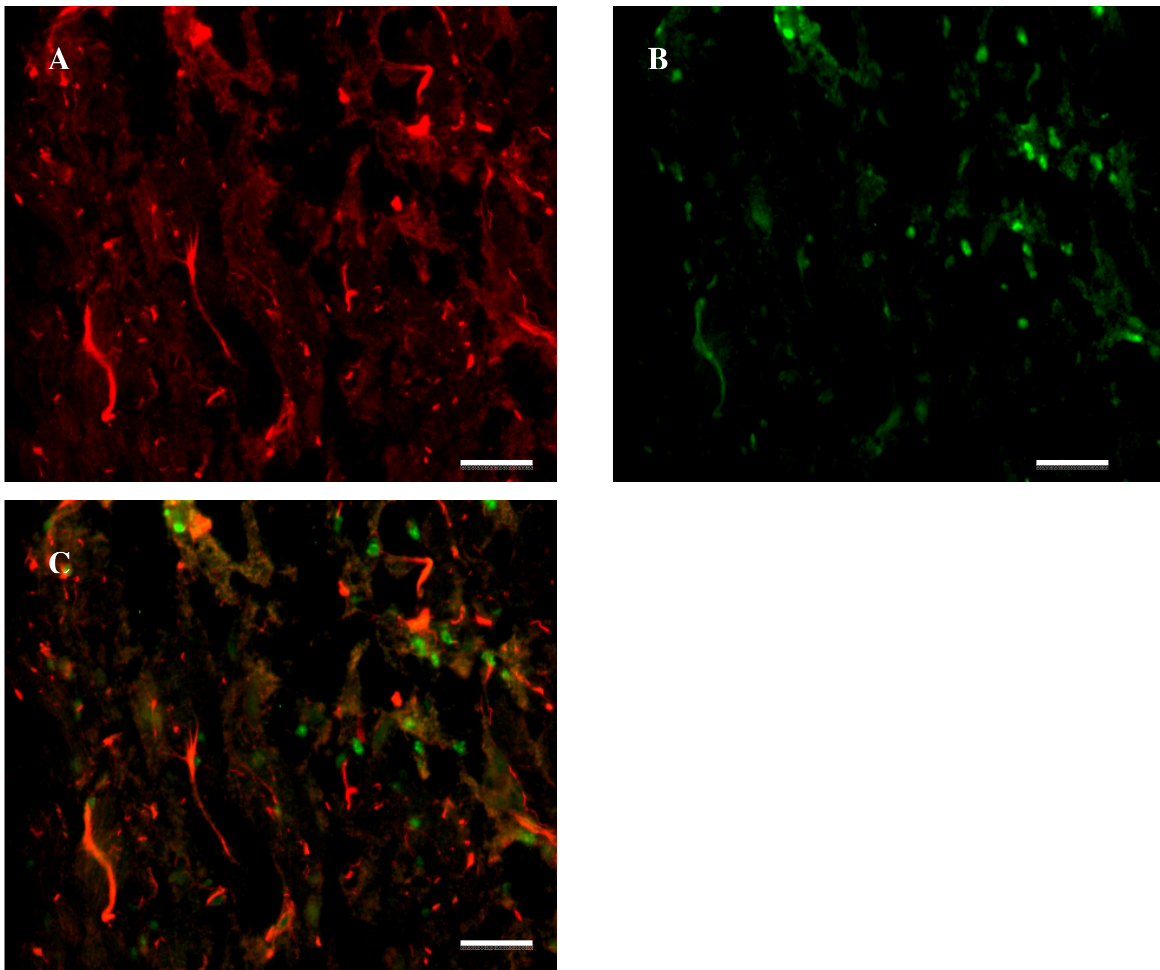


Image 19: Cy_3- labelled ED1-expressing cells (A) and FITC- labelled AT_2- receptor expressing cells (B). Lacking overlapping of fluorescent signal (C). Scale bar 50 µm.

4.7.5 MAP_2 and cCasp-3

Double immunofluorescence with cCasp-3 and MAP_2 showed partial co-expression of the two markers within the ischemic area. Some of MAP_2 positive (neuron-like) cells were also positive for cCasp-3 (thin arrows), while others were not (asterisk). This was readily explainable by the fact that some neurons were undergoing apoptosis while others were probably intact. However, there were also a few cCasp-3 positive cells, which showed projections typical of astrocytes (double arrows) and seemed to lie just around the MAP_2 positive cells.

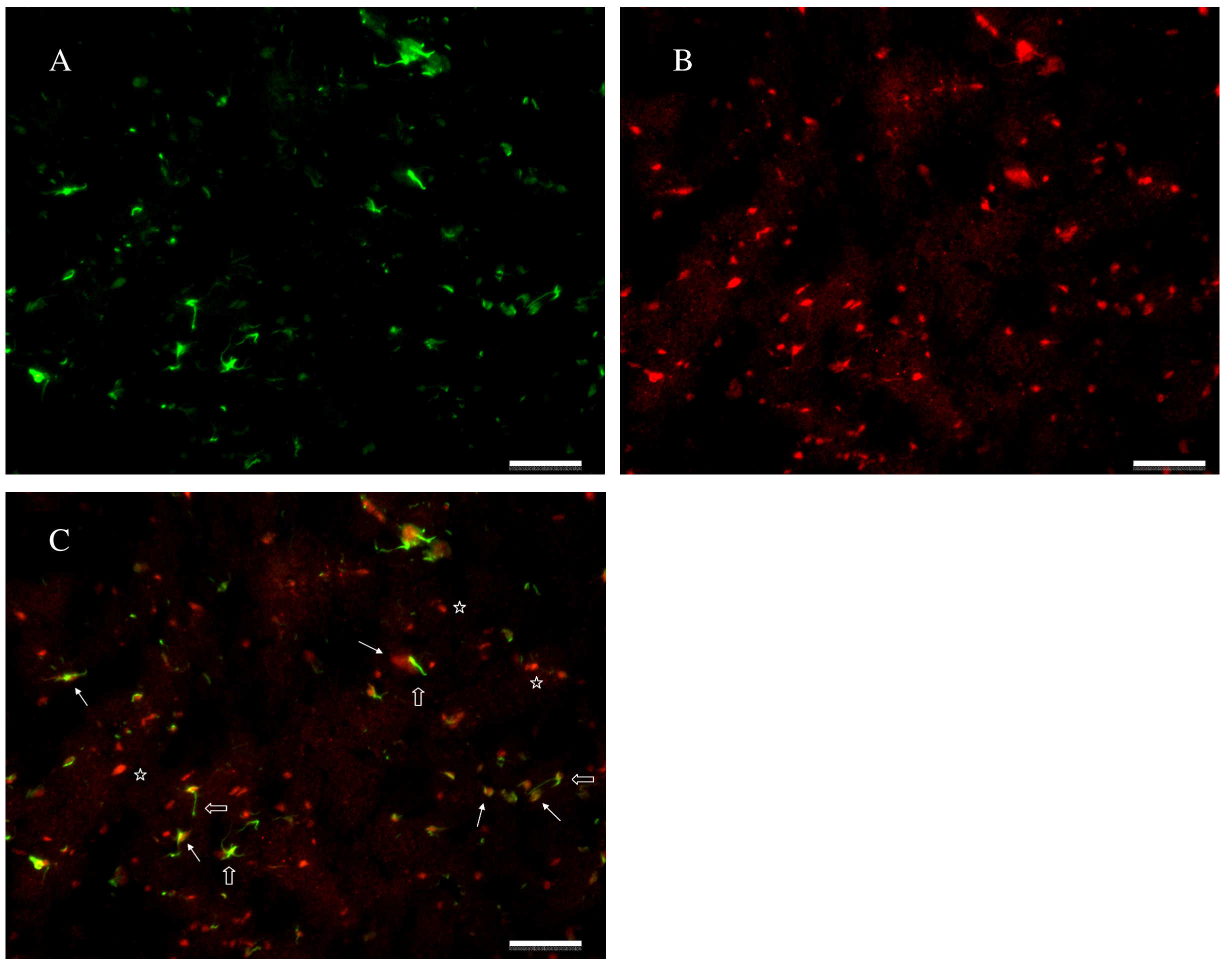


Image 20: Expression of FITC-labelled cCasp-3-positive cells (A) and MAP_2-positive, Cy_3-labelled cells (B) in the ischemic hemispheres of a stroke animal. C shows a partial co-localisation of the two markers. Scale bar 100 µm.

4.7.6 cCasp-3 and GFAP

It is well known that apoptosis, necrosis and inflammatory changes take place after focal cerebral ischemia. With double immunofluorescence stainings we looked for co-expressions of apoptotic and glial markers.

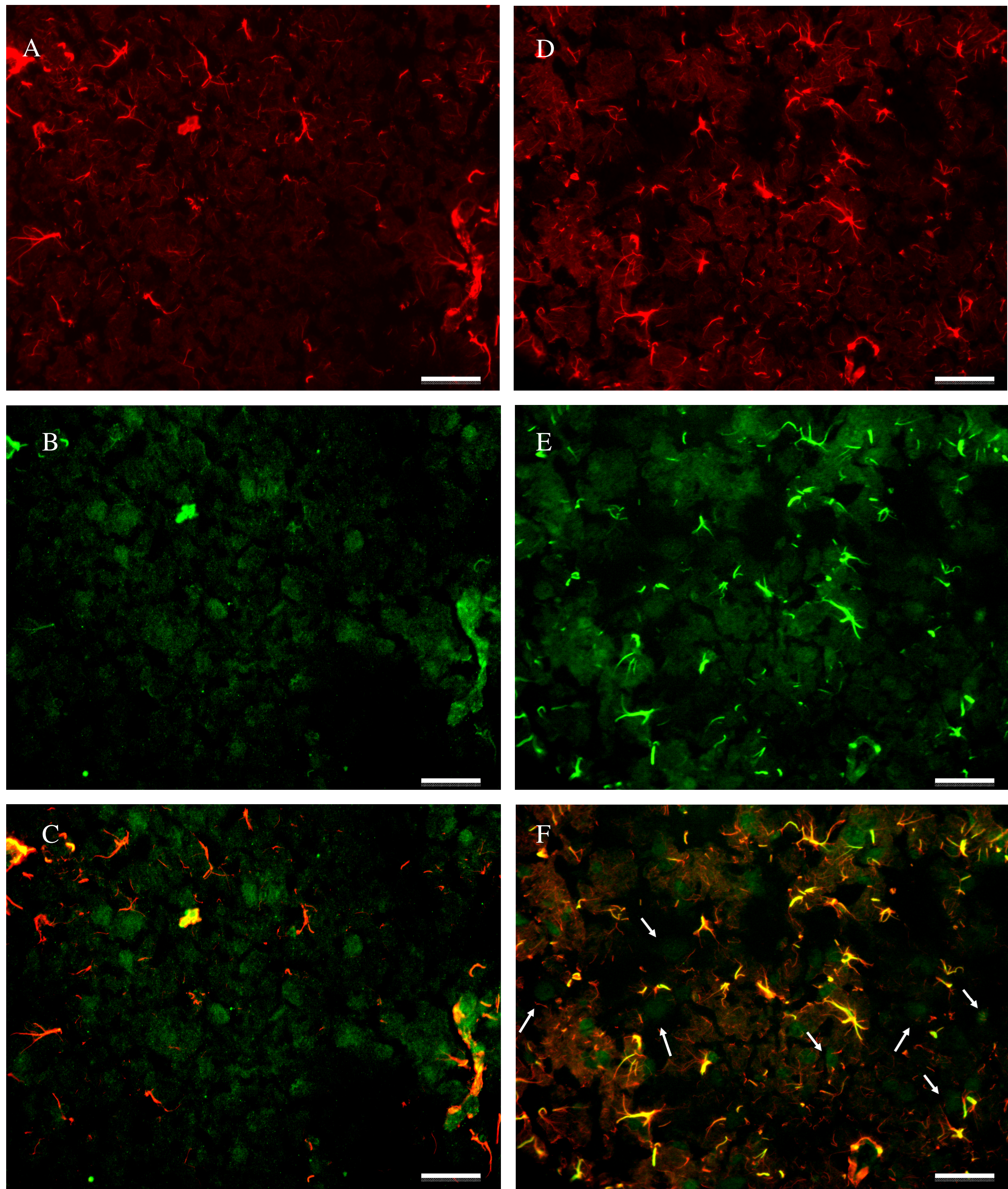


*Image 21: Expression of FITC-labelled cCasp**3**–expressing cells (B, E) and Cy3-labelled GFAP-positive cells (A, D) in the non-ischemic left hemisphere (A, B and C) and in the ischemic hemisphere of a stroke animal (D, E and F). Many of the GFAP-positive cells showed specific signal for cCasp-3, whereas some cCasp-3-positive cells with roundish configuration had no GFAP-specific signal (arrows in F). Scale bar 100 µm.*

Interestingly, many of the GFAP-positive cells in the infarct and periinfarct area had an intense cCasp-3 signal of the body and proximal projections. These GFAP-positive cells appeared larger and their projections seemed to be thickened. The distal projections had no cCasp-3 specific signal. Some other cells, which morphologically looked like neurons and were not positive for GFAP, showed a cCasp-3 specific signal. Especially in the left (non-ischemic) hemisphere, there were no cCasp-3-positive glia-like cells, but there were several cCasp-3-positive cells with neuron-like form.

5. Discussion

The RAS is well known for its function as a regulator of blood pressure and fluid and electrolyte balance with its major effective peptide Angiotensin II. Beside the systemic RAS, a lot of other tissues such as the heart, lungs, liver and the blood vessels have been shown to produce Ang II. The RAS was first known to act only in the periphery. Later RAS and its components were found in the brain and appeared to be autonomous. Several studies in the recent decades were able to show that inappropriate activity of RAS could be related to the occurrence of cerebral ischemia (Culman *et al.*, 2002). Some clinical trials, such as the LIFE trial, showed positive even preventive effect on stroke occurrence, independent of systemic blood pressure (Dahlof *et al.*, 2002). Furthermore, this endogenous and autonomous “brain- RAS” was shown to regulate cardiovascular processes in the body, and both neurons and astrocytes seemed to take part in these regulatory processes (Culman *et al.*, 2001; Ganong, 1984; Ganten *et al.*, 1978; Kumar *et al.*, 1988; Phillips, 1987; Raizada *et al.*, 1984; Unger *et al.*, 1988). Initially, little was known about the RAS pathways in the brain and the receptor subtypes which mediate its effects. Several research groups reported different, even partially antagonistic effects of AngII on different tissues, depending on the receptor subtypes involved: on the one hand, it initiated proliferation and hypertrophy, fibrosis and inflammation, and on the other hand, under certain conditions, it had anti-proliferative effects: it promoted apoptosis, or cell differentiation and regeneration (Unger, 1999). Then the AT_1 receptor was found to be abundant in the mature brain while the AT_2 receptor was highly expressed before and during the neonatal period, and its expression decreased speedily afterwards. Therefore the AT_1 receptor has been studied exhaustively while much less was known about the AT_2 receptor. The majority of the studies were done in vitro and both receptor subtypes seemed to play an important role in ischemic processes.

The proliferative effects mentioned─ cellular growth and hypertrophy, fibrosis and inflammation─ were found to be mediated via the AT_1 receptor subtype, whereas the AT_2 receptor was shown to have antagonistic effects and mediate apoptosis, neuronal regeneration and neurite outgrowth (Gallinat *et al.*, 1998; Lucius *et al.*, 1998; Shenoy *et al.*, 1999).

Before we started our experiments, there has been evidence of these effects in cell culture─ in primary cortical neurons, for example─ but up to that point there was little information on AT_2 expression and its effects in the brain parenchyma following MCAO in vivo. Our aim, therefore, was to visualize the different sites of expression of the AT_1 and AT_2 receptor subtypes, to specify which cells express these receptors and try to find a possible pathway or

explanation for their different distribution and effects as well as their possible correlations with the process of apoptosis and inflammation following cerebral ischemia.

Our construct comprised two groups: a “stroke” and a “sham” group; in the first one the animals underwent a transient MCAO that induced a focal cerebral ischemia, whereas the second group underwent only a superficial skin surgery. Then we performed several measurements and stainings as described above.

5.1 Infarct visualization

In order to visualize and prove a successful ischemia we used the TTC staining method. We performed no quantification but owing to the TTC staining, we could tell macroscopically whether the infarct area is small or big, or nonexistent. Some animals had wider arteries and the catheter had not probably fully prevented the blood supply in the MCA territory, so they showed a smaller infarction area. Few animals showed a steep drop in CBF during the ischemia period, which probably correlated with a very large infarction area, and they died shortly afterwards (<24hrs). Logically, animals with a bigger infarction who survived it had more neurological deficits than those with smaller ischemic lesions.

In addition, knowing where the infarction area was, we could later easily correlate this with different receptor expression and cell counting areas.

5.2 Expression of AT_1 and AT_2 receptors

Attempting to understand the role of these two angiotensin receptors we used immunohistological, immunofluorescence and double immunofluorescence stainings in order to visualize the expression of AT_1 and AT_2 receptors in different brain areas and their co-expression with other cell markers. After some difficulties with the interpretation of the immunohistological stainings, we proceeded with numerous immunofluorescence stainings, which showed good results. These results were confirmed in the Western Blot analysis and PCR.

5.2.1 Influence of focal cerebral ischemia on AT_1 expression.

We found no relevant difference in the expression of AT_1 receptors in the rat brain before or after a focal cerebral ischemia. On the other hand, clinical evidence of the positive effect of ARBs on the neurological outcome of patients with FCI led to the idea that the angiotensin-mediated effects may not due to a direct interaction with the AT_1 receptor but might rather be a result of AT_2 receptor activation. However, since in adult brain tissue the number of the AT_1 receptors by far outnumbers that of the AT_2 receptors, it is possible that no increase in number is

needed for a significant AT_1 receptor-mediated effect. In the future this effect can be further studied in experiments with a selective AT_2 receptor antagonist.

We could show that the AT_1 receptors showed co-localisation mainly with GFAP-positive cells, proving that AT_1 receptors were localised in astrocytes. This fact was consistent with the in vitro results of Sumners et al. (Sumners *et al.*, 1991). These AT_1 receptor-positive and GFAP-positive cells were predominantly localised in perivascular regions of the brain parenchyma (see §4.7.1), where the astrocytes' projections play an important role in maintaining the barrier function. So it is possible that the clinically proven positive effects of the AT_1 receptor antagonists are due not only to the reduction of the rate of apoptosis of the neuronal cells, but also to the prevention of injury on the astroglial cells and their foot processes. The last mentioned effect may reduce or even prevent the inflow of macromolecules and inflammatory cells from the periphery and the ensuing oedema. Decrease in inflammation and oedema on the other hand leads to a better neurological outcome.

We could not show a co-localisation of AT_1 receptors and neuronal markers (NeuN, MAP_2) but some of the AT_1-positive (and GFAP-negative) cells did appear morphologically neuron-like. Further stainings in this direction are required (see Image 13).

Interestingly, AT_1 receptors were not co-localised with cCasp-3 either, a marker of apoptosis, whereas cCasp-3 itself was expressed in many GFAP-positive cells in the infarct and periinfarct zone. This could be an indication that apoptosis is promoted by the glial cells with an indirect or no interaction of the AT_1 receptor. But since ischemia and apoptosis are strongly dynamic processes, further detailed studies are needed to rule out or prove such an interaction. Next possible step is to try a triple fluorescent staining with all three markers: AT_1-, cCasp-3- and GFAP- specific antibodies.

Furthermore, the expression of AT_1 receptors in astrocytes may be a hint about their role in the astrocyte-neuron interactions. There is increasing evidence that the astrocytes may be the pivot of brain metabolism, repair and degradation. Their foot processes at the capillaries are important for the functioning of the blood-brain-barrier. In their active state, such as we found after focal cerebral ischemia, where the glial cells show powerful ramifying projections (becoming "gemistocytic astrocytes", see Ch.28, p.1281 in (Kumar V. *et al.*, 1997) and Image 7 and 8 in this theses) and a clear increase in number, they might through secretion of neurotrophic factors and repair of the extracellular matrix, contribute to the neuroprotective and regenerative processes discussed below. On the other hand, astrocytes are highly sensitive to ischemic-hypoxic injury and when affected, their function as buffers and detoxifiers, and supporters of the

blood-brain-barrier fails. The permeability of the vessels increases and the barrier allows infiltration with macrophages from the periphery.

It is one of our future aims, therefore, to further examine the relation of AT_1 receptors, astrocytes and apoptotic markers by applying an AT_1 receptor antagonist and look for a positive or negative correlation between the expression of AT_1 receptor and cCasp3. In the setting of inhibited AT_1 receptors, increased cCasp3-positive astrocytes and smaller ischemic area/better neurological outcome, we could prove that there are other pathways of neuroprotection.

5.2.2 Influence of focal cerebral ischemia on AT_2 expression

AT_2 receptors could influence the outcome of cerebral ischemia in different ways.

5.2.2.1 Upregulation of AT_2 receptors and their exclusively neuronal localisation

Using immunofluorescence as well as Western blot analysis and PCR, we were able to show that in contrast to AT_1 receptor expression, AT_2 receptor expression after focal cerebral ischemia was markedly increased in the periischemic area and this mainly in regions involving motor and sensory functions: in the cerebral frontal cortex, piriformis cortex, hippocampus und striatum.

Furthermore, in the immunofluorescence stainings the AT_2 receptors were found exclusively in neurons and not in glial cells (again in contrast to the AT_1 receptors). All AT_2 receptor-positive cells were also positive with the NeuN-marker and had morphologically the typical form of neurons (See Image 5).

These two facts— the increased number of AT_2 receptors after FCI and their localisation in neurons— hinted at a possible protective function of the AT_2 receptor, though via yet unknown mechanisms. A strong support came from previous studies, which showed that blockade of central AT_1 receptors can reduce infarct size and improve neurological outcome as well as suppress stimulation of AP-1 transcription factors (Dai *et al.*, 1999), whereas the number of AT_1 receptor-positive cells remained the same or decreased, thus strengthening the idea that possible upregulation and activation of previously suppressed AT_2 receptors may play a major role in neuronal repair mechanisms. It is presumable that through blocking the AT_1 receptors, there is more AngII available for the AT_2 receptors and consequently there are stronger AT_2 receptor-mediated effects.

So in future studies we will try, by using both a central AT_1 and AT_2 receptor antagonist— by alternately blocking each one of them and comparing the outcome— to rule out changes promoted by the AT_2 receptor only.

5.2.2.2 Influence of AT_2 receptors on neuronal regeneration and repair

Once we discovered the neuronal localisation of AT_2 receptors, we looked for another specific marker. We found out that AT_2 receptors co-localised with MAP_2-positive neurons. Many of these MAP_2-positive neurons in the infarct and periinfarct area increased in number and showed an outgrowth of neurites as a sign of reparation and regeneration. Thus we postulated that this neuronal regeneration happens perhaps through the activation of AT_2 receptors.

More than a decade ago, Millan et al. (Millan *et al.*, 1991) suggested the role of the AT_2 receptors in neuronal plasticity and differentiation because of their over expression in foetal rat brain. After maturing of the brain, their numbers decrease quickly and remain low, except in cases of brain injury or other pathologic changes, when their number might rise again. Other studies have also supported the AT_2 receptor mediation of myelination and neuroregenerative responses of dorsal root ganglia (Gallinat *et al.*, 1998) and Unger et al. demonstrated the role of angiotensin II in neurite regeneration of retinal ganglion cells and dorsal root ganglia neurons in vitro, and the axonal regeneration of retinal ganglion cells after optic nerve crush in vivo (Lucius *et al.*, 1998; Reinecke *et al.*, 2003). Nio et al. showed the upregulation of AT_2 receptor expression in myocardium after acute MI, probably closely related to reparative mechanisms.

Likewise, after our in vivo- experiments, we could show via immunofluorescence that after an ischemic injury there is an increase in AT_2 receptor expression associated with activation of neurite outgrowth and proliferation in the adjacent area. Real-time RT-PCR and Western blot analysis confirmed a significant increase in cerebral AT_2 receptor mRNA in the peri-infarct zone, whereas in sham-operated rats there was no change of AT_2 receptor expression.

Given the analogies of the different studies and the considerable similarities in the results achieved, we believe that cerebral AT_2 receptors exert neuroprotective effects by supporting neuronal survival and neurite outgrowth following ischemia-induced neuronal injury. Whether the AT_2 receptors influence the cell metabolism directly (by influencing the toxic superoxide production, for example), or interact with the glial or inflammatory cells, or both, is still to be investigated. Attempting to understand the exact pathways of how this neuroprotection works is certainly a challenging aim for further studies (see also § 5.3.).

5.2.2.3 AT_2 receptors do not induce apoptosis directly

Bearing in mind the wide variety of studies showing that AT_2 receptors promote apoptosis, we tried to prove this fact in vivo by looking for co-localisation of AT_2 receptors and cCasp-3. Unfortunately, we could not show any co-expression of AT_2 receptors and cCasp-3 in neurons.

In the double immunofluorescence staining of MAP_2- and cCasp-3-positive cells there was a partial co-localisation of these markers, which meant that some neurons had signs of apoptosis while others seemed intact. This could be the case if there were no direct mediation of AT_2 receptors of apoptosis after FCI. There are probably other, more complicated, pathways for apoptosis induction (with an indirect influence or without the interaction of the AT_2 receptor), which are not possible to visualize with the stainings we applied.

Bearing in mind the positive results of AT_1 antagonists in patients with cerebral ischemia, it appears possible that the surplus of angiotensin during AT_1 receptor blockade activates the AT_2 receptors, which leads to neuronal regeneration and growth. Of course, since the AT_1-receptors are localised in astrocytes, and astrocytes are known for having both harmful and beneficial effects, it is possible that direct inhibition of the AT_1 receptor itself induces reduction of apoptosis. Certainly, a combination of both effects is also possible. What will then be the role of the AT_2 receptor, if it co-localises with cCasp-3?

5.3 Influence of FCI on inflammation

Following focal cerebral ischemia, there was an evident stimulation of inflammatory processes in the surrounding tissue and, though to a lesser extent, in the contralateral hemisphere. It is a long-known fact that resident microglia is quickly activated after ischemic injury and other inflammatory cells are recruited from the periphery (Stoll *et al.*, 1998).

Since there was an increase of ED1 and CD11b-positive cells in both the periischemic area as well as in the contralateral hemisphere, it can be assumed that there was activation of resident microglial cells as well as recruitment of cells from the blood stream going into both hemispheres.

The inflammatory markers did not co-localise with the NeunN-positive cells and the ED1 and CD11b-positive cells did not express AT_2 receptors, so we assume that there is no direct correlation or interdependence between the AT_2 receptor and the inflammatory cells. While in the periphery the anti-inflammatory effect of the AT_2 receptors has been shown (Culman *et al.*, 2002; Volpe *et al.*, 2003), it is to be further investigated whether and how the AT_2 receptors is involved in activating resident microglia or in recruiting inflammatory cells from the periphery.

It is known that during and after ischemia the affected cells produce cytokines and express adhesion molecules to recruit neutrophils from the periphery. Necrotic cells release molecules which evoke inflammatory processes (such as uric acid, ATP or HMGB-1) but even cells which aren't damaged but had suffered hypoxia are able to release a HIF-1α protein

(hypoxia induced factor-1α) and activate the transcription of multiple genes, among which is also the VEGF (inducing high vascular permeability) (Kumar V. *et al.*, 2010).

In our stainings some of the GFAP- (and AT_1-) positive cells were localised in the perivascular space. It can be presumed that their activation following hypoxic injury (via the AT_1 receptor, for example) could lead to weakening of the blood-brain-barrier and easier passage of inflammatory cells from the circulation into the CNS. This once again shows the important role of glial cells in pathologic processes in the brain. The reactively changed, activated glial cells (gemistocytic astrocytes) had more and thicker projections around NeuN-stained neurons. It remains unclear, whether the inflammation processes (closely related to phagocytosis) have a beneficial effect on intact or mildly damaged neurons and their survival— for example, via activation of astrocytes and secretion of certain (neurotrophic) factors- or a harmful one, or both. Usually inflammation is a complex, intermingled play with partly reparative and partly injurious components taking place either simultaneously or one after another. Lee et al. were able to demonstrate an NGF expression by reactive astrocytes under focal cerebral ischemia, which suggested a positive astrocyte-neuron interaction and support for neuronal survival (Lee *et al.*, 1998; Lee *et al.*, 1996).

Braun et al. showed that apoptotic neurons were located next to groups of inflammatory cells, which could be another hint of the influence of inflammation on apoptosis (Braun *et al.*, 1996). We were not able to mark apoptotic neurons with the cCasp-3-antibody, yet in further experiments we could try other markers of apoptosis.

The intrinsic supporting and salvaging mechanisms may not suffice to restore the greater part of the damaged cells even with smaller ischemic areas. The slowly developing processes in the penumbra correlating with worsening of neurological symptoms could be closely related to a progressing post-ischemic inflammation due to further neuronal death and astrogliosis. Thus prevention of inflammation could lead to a better neurological outcome in patients who had suffered a stroke.

5.4 Influence of FCI on apoptosis

In acute ischemia neurons die both by necrosis and apoptosis, depending on the severity and localisation of damage (membrane versus nucleolus and DNA, for example). Furthermore, there is an intrinsic (mitochondrial) and extrinsic (death receptor-initiated) pathway of apoptosis. The latter is closely related to the TNF- receptor family and the Fas ligand. In the initiation phase, some caspases become catalytically active (see also §1.4) and this is what we tried to show by using a specific immunofluorescent marker.

In the immunofluorescent staining of the brain of stroke animals, the apoptotic marker cCasp-3 was certainly increased in the right hemisphere after FCI (see §4.6., Fig. 18) but did not co-localise with the AT_1 receptor. Therefore the AT_1 receptor doesn't seem likely to be the direct mediator in astrocyte apoptosis following cerebral ischemia. We still have to find out whether the AT_2 receptor is expressed in apoptotic neurons or not. Since it is known that the AT_2 receptor promotes apoptosis in other tissues, it is highly probable that it would co-localise with apoptotic markers. On the other hand, due to its known regenerative and proliferative effects, it may be that the signal transduction is much more complex and that some AT_2 receptor-expressing cells co-express cCasp-3 and some do not.

First we were able to show that the AT_1 receptors were mainly localised in astrocytes. After focal cerebral ischemia there was a significant increase of astrocytes in the infarct and periinfarct area and these astrocytes looked activated. Even in the contralateral hemisphere there was an obvious increase in the number of reactive astrocytes. Since the astrocytes are known to be very active in cerebral metabolism and very sensitive to hypoxic-ischemic changes, it is possible that these cell changes are at least partly mediated by the AT_1 receptor.

In vivo experiments and several clinical studies (LIFE, SCOPE and ACCESS trials) have shown the positive role of ARBs (via blockade of AT_1 receptors) in reducing the incidence and preventing recurrent stroke as well as their beneficial effect on neurological outcome independently of blood pressure regulation. These findings indicate that an inhibition of the AT_1 receptor with an ARB under focal cerebral ischemia might have no direct influence on apoptosis; much more, the neuroprotective effects of the AT_1 receptor antagonist could be based on preventing astrocytes from becoming apoptotic and rather bringing them to secrete neurotrophic factors, for example. Or, as mentioned previously, the superfluous Angiotensin II (due to the blocked AT_1 receptors) is thus free to react with the increased in number and not blocked AT_2 receptors.

On the other hand, cCasp-3 was expressed in many of these hypertrophic GFAP+ cells (see §4.7.7) and correspondingly, cCasp-3 labelled cells were significantly increased in number in the right cerebral hemisphere of the stroke animals. This circumstance speaks for a close correlation between astrocytes and apoptosis.

Furthermore, the cCasp-3-positive cells were located in close proximity to MAP_2-positive neurons, so it is easy to presume that there is an interaction between these two cell groups.

However, other studies have shown that AT_1 receptors mediate apoptosis in various other tissues, such as the myocardium, blood vessels of rats and in in-vitro coronary endothelial cells (Diep *et al.*, 1999; Leri *et al.*, 1998; Li *et al.*, 1999). It is possible that an early stimulation of the

AT_1 receptor initiates apoptosis in astrocytes but is later no longer expressed in those cells, which then become cCasp-3-positive (for example, cells already in execution phase). Cell death is a dynamic processes lasting several days. Further studies─ with triple immunofluorescence or in vitro studies─ are needed for obtaining a better understanding of these interactions.

Second, we showed that the AT_2 receptors were expressed exclusively in neurons and those that survived the ischemic injury after MCAO (not in the central infarct zone) showed an outgrowth of neurites and signs of regeneration. Iwai at el. were able to show that the neuroprotective effect of Valsartan (AT_1 receptor antagonist) was weakened in AT_2 receptor deficient mice (Iwai *et al.*, 2004), which is good evidence for the important role of the AT_2 receptors in neuronal regeneration and tissue recovery after cerebral ischemia.

If we could find any co-localisation of AT_2 receptors and cCasp-3, we could prove a direct link between apoptosis and activation of AT_2 receptors. It may be that only some AT_2 receptor-positive cells express cCasp-3 too, whereas others, responsible for regeneration and repair, do not. Even more interesting then will be the question what other factors determine the which and when of these processes. Understanding them better will give us better opportunities for treatment.

So the neuroprotective effect of ARBs, as mentioned earlier, might be due to a surplus of angiotensin and its interaction with the increased expression of AT_2 receptor, or due to AT_1 receptor-mediated neuroprotection via activation of astrocytes and release of NGF (as suggested by Lee et al.), or by both. It is important to find out in future studies whether a selective AT_1 receptor antagonist reduces or increases the number of activated astrocytes and whether it increases the number of apoptotic cells.

5.5 Critique of the methods

It is to be taken into account that these are our first experiments to try to show the AT_2 receptor expression in the rat brain after MCAO and FCI (in vivo) using immunofluorescence. We had only a limited number of animals, that is to say on average about 7-10 animals per group. We are aware that to show a real statistical significance more animals per group are needed. We used our "preliminary" results for further studies. Furthermore, a lot of additional stainings are to be performed in order to be able to make more clear-cut decisions. Some of the stainings we performed were not taken into account, since they could not be evaluated (due to inappropriate concentration of 1st of 2nd antibodies, for example). In some of the stainings (cCasp-3 and GFAP, for example), some of the cCasp-3 positive cells looked like neurons. Triple immunofluorescent stainings are required to prove this. Another point is that the MCAO model

has as its disadvantage the variable size of infarction (due to varying collateral blood supply) (Stoll *et al.*, 1998). This could be obviated or taken into account during the evaluation by visualizing the infarction areas and comparing their sizes (via MRI, for example).

5.6 Future possibilities

To conclude, we hope that we were able to throw new light on the importance of the AT_2 receptor expression in the brain. We were able to show that there is no significant change in the expression of AT_1 receptor in the rat brain after MCAO but there is an upregulation of AT_2 receptor-positive cells in neurons of the (peri-) ischemic brain area along with signs of neuroregeneration. Since there is good clinical evidence for the positive effect of AT_1-receptor antagonists in patients with FCI, the next step is to perform experiments including a third group of animals, which would be treated with an ARB. We could then again quantify the expression of GFAP- and cCasp-3- positive cells and look for a correlation between the AT_1 receptor expression, inflammation and apoptosis. It is possible that beside neuroprotection and regeneration, the ARBs reduce inflammation and apoptosis, which on its own influences favourably the neuroregenerative process. In addition, we could use a selective AT_2-receptor antagonist or agonist to further specify the enigmatic pathways.

In our next studies and in further experiments we will try to mark AT_2-receptor positive neurons with the cCasp-3-antibody, and try other markers of apoptosis. Furthermore, in order to measure the volume of the infarction area of stroke animals and compare them with those of the ARB- treated animals, for example, an MRI of the rat brain could be performed, giving us a three dimensional view of the infarcted area.

We once again realized that brain damage and brain repair is a complex and dynamic process, in which the collaboration between neurons and astrocytes plays an important role, and where the decision to live or die (apoptosis versus regeneration) runs on a thin line. Further experiments using a selective AT_1-receptor antagonist and/or AT_2-receptor antagonist as well as an agonist are needed to further clarify the interactions between neurons, astrocytes, inflammatory cells and the AT_1/AT_2 receptor expression. New insights into the signalling mechanisms that take place in the neuronal and glial cells through a down- or upregulation of the AT_1 and AT_2 receptors and the ensuing neuroprotective effects could lead to faster and better therapy options and even provide prevention.

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Statement of Originality

I declare that this thesis has not been submitted in any form for another degree or diploma at any university. The achieved results and discussion in this thesis are my own work and information derived from the literature or unpublished work of others has been acknowledged in the text and a list of references provided.

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Information

The experimental part of this doctoral thesis took place at the Center for Cardiovascular Research from 2003 till 2005 under the supervision of Prof. Dr. Thomas Unger.

Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

List of Publications and Presentations

Thesis article: Li J, Culman J, Hörtnagl H, Zhao Y, Nadezhda G, Timm M, Blume A, Zimmermann M, Seidel K, Dirnagl U, Unger T. "*Angiotensin AT2 receptor protects against cerebral ischemia-induced neuronal injury*", FASEB Journal 21 Jan 2005;

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Li J, Hörtnagl H, Nadezhda G, Timm M, Zimmermann M, Unger T. "*Angiotensin AT2 receptors are exclusively upregulated in neurons and may support neuronal survival and neurite outgrowth after cerebral ischemia in rats*", Abstract: Hypertension. 2004; 44:570.

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