Proliferation of NG2 Cells

after Focal Cerebral Ischemia

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1.   Zusammenfassung

Schlaganfälle gehören zu den häufigsten Ursachen für schwere Behinderungen und Todesfälle in Deutschland. Die zu Grunde liegende zerebrale Ischämie schädigt Nerven- und Gliazellen. Durch hirneigene Stamm- und Vorläuferzellen könnte geschädigtes Gehirngewebe regeneriert werden, wie zum Beispiel Oligodendrocyten aus so genannten NG2 Glia Zellen. Ziel der vorliegenden Studie war die Untersuchung der Proliferation von NG2 Glia Zellen nach fokaler zerebraler Ischämie.

Erwachsene, männliche Hooded Wistar Ratten wurden einem Endothelin-1 induzierten Arteria Cerebri Media Verschluss unterzogen (n=15) oder der Kontrollgruppe ohne Ischämie zugeteilt (n=6). Die Versuchstiere wurden 1, 3, 7 und 14 Tage nach fokaler Ischämie, bzw. nach Injektion von Kochsalzlösung in der Kontrollgruppe, eingeschläfert. Vorher erhielten sie jeweils über 24 Stunden Injektionen mit BrdU, um proliferierende Zellen zu markieren. In koronaren Hirnschnitten wurden NG2 und BrdU als Marker für proliferierende Zellen durch eine Fluoreszenzdoppelimmunfärbung sichtbar gemacht. BrdU positive Zellen und NG2/BrdU doppelt markierte Zellen wurden in der Penumbra und auf der entsprechenden gegenüberliegenden Seite quantifiziert.

Die statistische Analyse zeigte einen signifikanten Anstieg von NG2/BrdU doppelt markierten Zellen in der Penumbra 7 Tage nach der Ischämie im Vergleich zur gegenüberliegenden Seite (p<0.001) und im Vergleich zu den anderen Zeitpunkten (p<0.001). 1, 3 und 14 Tage nach der Ischämie zeigte sich kein signifikanter Unterschied bezüglich der Anzahl der NG2/BrdU doppelt markierten Zellen zwischen den untersuchten Zeitpunkten. Auch zwischen der Penumbra und der kontralateralen Seite ergab sich zu diesen Zeitpunkten kein Unterschied. Auf der kontralateral zur Penumbra liegenden Seite zeigte sich keine statistisch signifikante Veränderung der NG2/BrdU Zellzahl zwischen den verschieden Zeitpunkten. Für die Gesamtheit der BrdU markierten Zellen ergaben sich die gleichen Ergebnisse.

Diese Studie zeigte, dass NG2 Zellen in der Penumbra 7 Tage nach fokaler Ischämie vermehrt proliferieren. Aufgrund dieser zeitlichen Verzögerung könnten auch späte Therapien diesen Mechanismus positiv beeinflussen und somit eine Regeneration von NG2 Zellen und im Folgenden von Oligodendrocyten und der Myelinscheide bewirken.

2.   Abbreviations

ATP   adenosintriphosphate

BDNF   brain derived neurotrophic factor

BINCs   Brain Iba1$^+$/NG2$^+$ cells

BrdU   Bromodeoxyuridine

CNS   central nervous system

ET-1   endothelin-1

FGF-2   fibroblast growth factor 2

Iba1   ionized calcium-binding adaptor molecule 1

IgG   immune globuline G

MCA   middle cerebral artery

MCAo   middle cerebral artery occlusion

MRI   magnetic resonance imaging

NG2   nerve glial antigen 2

PBS   phosphate buffered saline

PDGF   platelet derived growth factor

PET   positron emission tomography

ROS   reactive oxygen species

SVZ   subventricular zone

3. Introduction

3.1. Pathobiology of stroke

Stroke is one of the leading causes of death in developed countries. In Germany approximately 165 000 people suffer a stroke each year and 40% of these patients die within the first year (Geschäftstelle des IZPH, 2003). Most of the surviving patients suffer from a permanent impairment, often requiring nursing home care (Ward et al., 2005). The number of strokes is projected to double by the year 2050 (Kolominski-Rabas and Heuschmann, 2002) because the incidence of cerebrovascular disease increases with age and there is a demographic trend towards an aging population.

A stroke is clinically defined as an abrupt onset of a neurological deficit lasting more than 24 hours, which is attributable to a focal vascular cause such as obstruction of a supplying artery, intracerebral haemorrhage or subarachnoid haemorrhage (Smith et al., 2005). In the present study, the term stroke will always refer to the event of ischemic infarction which is the most common cause (Kolominski-Rabas and Heuschmann, 2002).

Perfusion of an obstructed artery can be restored by clot lysis through administration of recombinant tissue plasminogen activator (rt-PA) within 2-3 hours (Deutsche Gesellschaft für Neurologie, 2004). However, due to strict inclusion criteria less than 5% of patients can benefit from this treatment due to the increased risk of haemorrhage beyond 3 hours (Gropen et al., 2006). Even patients who undergo successful clot lysis suffer from brain damage and the lesion size can still increase after lysis (Delgado-Mederos et al., 2007).

The obstruction of an intracranial artery causes the loss of function of the ischemic areas within minutes (Dirnagl et al., 1999). Animal studies have shown that areas with blood flow of less than 20% of the normal rate become irreversibly damaged within minutes and constitute the ischemic core (Ginsberg, 2003). Tissue surrounding the core region of infarction is referred to as the ischemic penumbra or peri-infarct region. It suffers from impaired cerebral blood flow of approximately 20-40% of the normal rate (Ginsberg, 2003). In humans similar observations have been made (Heiss et al., 2001a) and the penumbra has been shown to exist in MRI (magnetic

resonance imaging) and PET studies (Fisher and Garcia, 1996; Heiss, 2001b). The penumbra will eventually infarct unless reperfusion is initiated and/or neuroprotective therapy is administered (Schaller et al., 2005). Therefore, the main goal of any acute stroke therapy is to salvage the ischemic penumbra (Hossmann et al., 1994).

Brain tissue depends almost exclusively on oxidative phosphorylation of glucose for energy production in the form of adenosintriphosphate (ATP) (Lee et al., 2000). With the lack of glucose and oxygen both grey and white matter can not produce enough ATP to sustain their basal and functional metabolism (Saeed et al., 2007). With progression of energy depletion, cells can no longer sustain the physiological ion gradients required for cell integrity (Siesjö, 1992). The resultant increase in intracellular sodium concentration leads to an osmotic influx of water with consequent swelling, or even rupture, of the cells (Dirnagl et al., 1999). Furthermore, the brain's signalling systems and intrinsic cascades, which are induced under ischemic conditions, play a critical role in the high vulnerability of brain tissue to ischemia (Dirnagl et al., 1999; Lee et al., 2000).

The process of tissue damage from massively released excitatory neurotransmitters is known as excitotoxicity. Under ischemic conditions glutamate, which is the major excitatory neurotransmitter in the brain, is released in massive quantities from depolarising neurons (Lee et al., 2000). The accumulation of extracellular glutamate leads to stimulation of post-synaptic glutamate receptors. This results in calcium and sodium influx and potassium efflux in the affected cells. The energy depleted ischemic cells are not able to balance out these ionic changes, and as a result undergo cell death (Lipton and Rosenberg, 1994).

High levels of extracellular potassium and glutamate cause wave-like spreading depolarisations from the ischemic core towards the penumbra, known as peri-infarct depolarisations (Dirnagl et al., 1999). This process makes the surrounding cells even more vulnerable to energy depletion (Gidö et al., 1997) resulting in an increase in the infarct area (Mies et al., 1993).

Ischemia and reperfusion lead to an inflammatory reaction in the ischemic brain region. This is marked by activation, proliferation and accumulation of macrophages, microglia and leucocytes (Barone and Feuerstein, 1999) and expression of inflammatory mediators (Iadecola and Alexander, 2001). This post ischemic inflammation exacerbates initial levels of damage, for example through microvascular obstruction by neutrophils and production of toxic mediators by activated inflammatory cells (Dirnagl et al., 1999).

Levels of reactive oxygen species (ROS) become markedly increased during ischemia and after reperfusion (Saeed et al., 2007). The excessive amounts of ROS damage the tissue through lipid peroxidation, induction of inflammatory mediators and damage to DNA and mitochondria (Koroshetz and Moskowitz, 1996; Saeed et al., 2007).

Depending on the severity of the ischemia, cell death occurs either through necrosis or apoptosis. Necrosis is the fulminant cell death accompanied by cell membrane disruption. Apoptosis is the mechanism of programmed cell death (Wyllie, 1997). In the ischemic core, necrosis is the predominant mechanism, while in the penumbra apoptosis is thought to predominate (Dirnagl et al., 1999). Post ischemic changes in brain tissue are time dependent. Excitotoxicity, peri-infarct depolarisation, inflammation and apoptosis evolve over minutes to days after infarction (Dirnagl et al., 1999).

Oligodendrocytes are responsible for myelination of axons in the central nervous system (CNS). Death of oligodendrocytes leads to demyelination which results in slower nerve conduction and even conduction failure (Bunge, 1968). *In vitro* studies have shown that high levels of glutamate cause oligodendrocyte cell death (Tekkök and Goldberg, 2001). Additionally, they are highly susceptible to oxidative stress due to their high lipid content and lower expression of endogenous antioxidants (Juurlink, 1997).

3.2.   NG2 cells and regeneration of brain tissue

The adult brain contains neuronal stem cells and progenitor cells which can regenerate neurons, oligodendrocytes and astrocytes (Felling and Levison, 2003). After injury, these cells proliferate and migrate into the lesion (Jin et al., 2003; Nakagomi et al., 2009). If it were possible to stimulate this process, lost brain tissue could be replaced and hopefully the devastating effects of stroke could be reversed. The capacity for neuronal replacement is rather limited, however, remyelination and regeneration of oligodendrocytes works effectively (Franklin, 2002). It has been shown that in the early postnatal period oligodendrocytes are generated from progenitor cells, which express the surface molecule NG2 (nerve glial antigen 2; Fig. 1; Reyonolds and Hardy, 1997; Dawson et al., 2000). A large number of these NG2 cells remain abundant in the adult rodent and human brain (Chang et al., 2000; Nishiyama et al., 1997). They make up about 8-9% of the total cell population in white matter and 2-3% in grey matter and are considered the fifth major cell type besides neurons, oligodendrocytes, astrocytes and microglia (Dawson et al., 2003).The cells have small cell bodies (10-15µm) and extend various processes. In grey matter the processes have a mainly radial orientation in contrast to a rather longitudinal orientation in white matter (Dawson et al., 2003). The cells were first described as "light" and "medium oligodendrocytes" according to their appearance in electron microscopy studies (Mori and Leblond, 1971). Later the term oligodendrocyte progenitor cells was used because the cells maintain their ability to divide even in the adult CNS (Dawson et al., 2000; Horner et al., 2000) and can give rise to new oligodendrocytes (Gensert et al., 1997; Tanaka et al., 2003; Nishiyama et al., 2009; Dimou et al., 2008). It is widely accepted that perinatal NG2 cells serve as oligodendrocyte progenitor cells, however, in the mature brain not all NG2 positive cells regenerate oligodendrocytes but instead might serve other functions (Nishiyama et al., 2009; Dimou et al., 2008). Therefore, Nishiyma et al. (2002) have introduced the term polydendrocytes to reflect the multifunctional role and the close relationship to the oligodendroglial lineage. In the present study the general term NG2 cells is used. It has been suggested that NG2 cells play a role in signal transmission (Bergles et al., 2000; Butt et al., 1999; Ong et al., 1999) and are involved in the immune response since NG2 cells react to viral infection of motor neurons (Levine et al., 1998). *In vitro,* NG2 cells can develop into astrocytes (Raff et al., 1983).
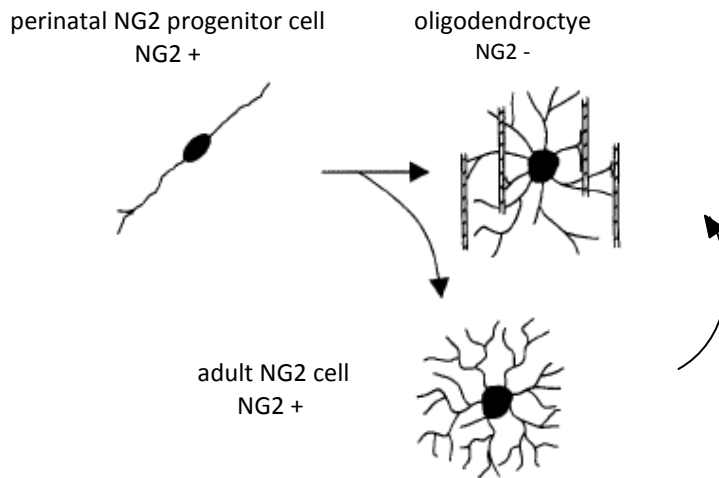
perinatal NG2 progenitor cell
NG2 +

oligodendroctye
NG2 -

adult NG2 cell
NG2 +

Fig. 1 *:* Illustration of stages of oligodendrocyte and NG2 cell differentiation. Perinatal NG2 cells are thought to differentiate into oligodendrocytes or remain in the adult brain as adult NG2 cells which can give rise to oligodendrocytes later or fulfil other function (modified from Dawson et al., 2000).

Outside the CNS NG2 is also expressed by embryonic and adult chondrocytes (Midwood and Salter, 1998), melanoma cells (Burg et al., 1998), vascular smooth muscle cells outside the CNS and endothelial cells of microvessels in the CNS (Grako and Stallcup, 1995). The latter are easily distinguished from NG2 glia cells cells through differences in cell shape and cell arrangement. Therefore, in the brain, NG2 has been regarded as an unequivocal marker for this new type of glia cells at the time when this study was conducted (Dawson et al., 2003; Polito and Reynolds, 2005). However, recently macrophages in the infarct core after focal brain ischemia have been found to express NG2 as well (Matsumoto et al., 2008).

NG2 cells react similarly to different kinds of injury: NG2 immunoreactivity increases and the cells appear to have shorter and thicker processes (Levine et al., 2001). These changes have been described in reaction to stab wounds (Hampton et al., 2004), experimental allergic encephalitis (Di Bello et al., 1999), viral infection of motor neurons (Levine et al., 1998) and ischemic injury (Tanaka et al., 2001 and 2003). Di Bello et al. (1999) showed that an increase in numbers of NG2 cells after injury is triggered by demyelination and not just injury by itself.

In vitro, NG2 cells are more susceptible to oxidative stress than astrocytes and mature oligodendrocytes (Baud et al., 2004; Husain and Juurlink, 1995). This is due to their low content of endogenous antioxidants such as glutathione (Thornburne and Juurlink, 1996). Furthermore they undergo apoptosis when exposed to high levels of a glutamate agonist (McDonald et al., 1998). Hence, these cells are vulnerable to the pathological conditions occurring under ischemic stroke, such as excitotoxicity and oxidative stress. Tanaka et al. (2003) have shown that NG2 cells disappear from the infarct core in ischemic stroke. However, in the peri-infarct area their number increases and this is associated with restoration of mature oligodendrocytes and myelination in this area (Tanaka et al., 2003). This suggests that NG2 cells, which survive the ischemia, can restore oligodendrocytes (Sozmen et al., 2009; Tanaka et al., 2003). There is also evidence that NG2 cells secrete neurotrophic factors such as brain derived neurotrophic factor (BDNF) when being stimulated by neurotransmitters (Tanaka et al., 2009). Hence NG2 cells might be able to create a favourable environment for tissue regeneration. This makes NG2 cells an interesting target to restore function of brain tissue after injury. *In vitro* studies have shown that platelet derived growth factor (PDGF), fibroblast growth factor 2 (FGF-2) and glial growth factor 2 enhance proliferation of NG2 cells in rodent and human tissue (Frost et al., 2003; Ibarrola et al., 1996; Wilson et al., 2003). Therefore, there is potential for the use of exogenous agents to enhance NG2 cell proliferation with the aim of restoring function following stroke.

3.3. The Endothelin-1 model of MCA occlusion

The Endothelin-1 (ET-1) method, which was chosen as a stroke model in this study, allows reversible middle cerebral artery occlusion in conscious rats and causes reliable ischemic brain damage (Sharkey et al., 1993; Macrae et al., 1993; Callaway et al., 1999). It has the clear advantage of timely separation of the induction of stroke from the surgical procedure so that the effect of anaesthetics is minimised. This is of importance because anaesthetics are known to be neuroprotective and enhance neurogenesis (Clarkson, 2006; Engelhard et al., 2007; Koerner and Bambrink, 2006), reduce infarct volume and have an effect on gene expression (Kapinya et al., 2002).

3.4.   Rational and aim of the present study

The aim of the present study was to determine the time course of the proliferation of newly derived NG2 cells following focal stroke using a middle cerebral artery occlusion model in conscious rats. Specifically, the aim was to identify the peak time of proliferation and hence a suitable time point after MCAo for future evaluation of the effects of neuroprotective treatments on NG2 cell proliferation. At the time of the present study, the only study to have shown an increase in the number of NG2 cells in the penumbra after ischemia did not utilise markers for new cell formation and hence could not conclude unequivocally that this increase was due to an increase in newly proliferating NG2 cells (Tanaka et al., 2003). Therefore, the present study employed double label immunohistochemistry to detect co-localisation of new cells and NG2 cells.

4.   Materials and methods

The experimental data was obtained in the laboratory of the Brain Injury and Repair group at the Howard Florey Institute (now named Florey Neuroscience Institutes), Melbourne, Australia. All procedures were performed in compliance with the ethical code of the National Health and Medical Research Council (Australia) under the approval of Howard Florey Institute Animal Ethics Committee (Application number 05-007).

4.1.   Surgical procedures and middle cerebral artery occlusion

Male adult Hooded Wistar rats (Adelaide University, South Australia), aged 8- 10 weeks and weighing between 260g and 320g, were divided into a middle cerebral artery occlusion (MCAo) group (n = 15) and a sham stroke group (n= 6). MCAo animals were sacrificed at 1 day (n=4), 3 days (n=3), 7 days (n=4) and 14 days (n= 4) post stroke. The sham stroke animals were killed at the same time points, namely 1 day (n=2), 3 days (n=1), 7 days (n=1) and 14 days (n=2) after sham stroke.

Middle cerebral artery occlusion was induced in conscious rats using the ET-1 method as described by Callaway et al. (1999). Rats were anesthetised with a mixture of 4% isoflurane, 48% oxygen and 48% air using an anaesthetic apparatus (CIG, Australia) and placed in a stereotaxic frame (Kopf® Instruments, USA). An incision was made in the scalp in anterior-posterior direction and held open with metal retractors. The junction between the sagittal suture and the coronal suture known as bregma was identified and was used as reference point. A small hole was drilled (Volverex G drill from NSK, Japan) into the skull at 0.2mm anterior, -5.9mm lateral and -5.2mm ventral from bregma and a cannula was inserted into the piriform cortex 2mm dorsal to the right middle cerebral artery according to the stereotaxic atlas for the rat (Paxinos and Watson, 1986). This was based on the method by Sharkey et al. (1993) and modified for this rat strain (Callaway et al., 1999). The cannula was secured in position with a small round plastic plate and acrylic denture cement (Vertex Dental, Netherlands) before closing the scalp with sutures. Sham stroke animals had the cannula inserted in the same way. Following surgical

procedures, animals were housed individually for 5 days with free access to food and water to allow recovery and to minimize any effects of the anaesthetic before MCAo.

Stroke animals were subjected to MCAo by administration of the long acting vasoconstrictor ET-1 (American Pepitde Co, USA) at a concentration of 20pmol per µl normal saline solution (0.9% saline solution: 9g NaCl in 1 L of distilled water). The solution was administered with a 30-gauge injector which protruded 2mm beyond the end of the previously implanted guide cannula. The injector was connected to a polythene tubing and ET-1 was administered at a rate of 20 pmol per µl per minute using a Hamilton syringe. Animals were given between 3 and 8 µl of ET-1 solution, equivalent to 60 – 160 pmol of ET-1 until symptoms of stroke occurred. Sham stroke animals were given 5 µl saline solution. During the operation rats were placed in a transparent plastic box for observation of behaviour during ET-1 administration.

Circling in the direction contralateral to the occlusion, clenching or failure to extend the contralateral forelimb and grooming were used as markers of a stroke occurring. The severity was determined as described by Roulston et al. (2008). Animals were rated on a scale of 0 (no stroke related behaviour) to 5 (severe stroke, loss of righting reflex). The severity of stroke is correlated with ratings of behaviors occurring during stroke, with high ratings being associated with more severe damage (Roulston et al., 2008). Animals which rated 0 were excluded from the study.

Body temperature is known to influence the degree of stroke-induced brain damage (Nagel et al., 2008). Low temperatures have been shown to be neuroprotective while high temperatures increase the ischemic damage (Reith et al., 1996; Nagel et al., 2008). Therefore, animals whose temperatures reached 40°C during and for 3 hours following stroke induction were excluded from the study. Rectal temperature was measured at 0, 30 or 60 and 180 minutes after induction of stroke. One animal in the 7 days group died soon after the induction of stroke and was excluded from further investigation.

4.2. BrdU Administration

Bromodeoxyuridine (BrdU) is an analogue of thymidine and is incorporated into newly synthesized DNA such as in dividing cells. All animals received 3 intraperitoneal injections of BrdU (Sigma-Aldrich, USA) at 4 hour intervals (50mg /kg, dissolved in saline) beginning 24 hours prior to sacrifice. Therefore, the quantified proliferation at each time point reflects a 24 hour proliferation period. Cell migration can be considered negligible during such a short time period, so that BrdU labeled cells represent locally proliferating cells.

4.3. Tissue preparation

At the given time points after stroke (1 day, 3 days, 7 days and 14 days), rats were anaesthetized with phenobarbitone (Merck, Germany; 80mg/kg, intraperitoneal) and the chest cavity was opened up. The rats were transcardially perfused with PBS at pH 7.4 (phosphate buffered saline solution: 34.5g NaH_2PO_4 x $H_2$0 (Merck, Germany), 35.5g Na_2HPO_4 (Merck, Germany) and 45.0g NaCl (UNIVAR®, Ajax Finechem, NZ) were diluted in 5L distilled water (distilled water system from Milipore, USA) and titrated to pH 7.4 by adding NaOH (BDH AnalaR®, Merck, Germany)) to remove blood. Subsequently, they were perfused with 400 ml of 4% PFA (Merck, Germany) as the fixative. Brains were then removed and placed in 4% PFA for no longer than 4 hours and stored in 30% sucrose solution (30g sucrose (Sigma®, USA) in 100ml PBS) at 4°C until sectioning.

Before sectioning brains were frozen over solid carbon dioxide (-80°C). Then they were cut into 60μm thick coronal sections with a cryostat (Cryostat CM 1900, Leica Microsystems, Germany). The 60μm thick sections were collected with a brush and placed into a 12-well tissue culture plate (Nunclon*TM* Surface, NUNC™, Denmark) filled with PBS. Each well contained a series of adjacent brain sections.

For long-term storage of brain sections, the PBS was replaced by cryoprotectant solution (50ml glycerol (UNIVAR®, Ajax Finechem, NZ) and 50ml ethylene glycol (Sigma®, USA) were diluted in 100ml PBS). Using this method the sections could be stored at −20°C until further processing.

4.4.   BrdU/NG2 double immunofluorescence staining of free floating sections

In order to determine the number of proliferating NG2 cells within a 24 hour period at 1, 3, 7 and 14 days post stroke, double immunofluorescent studies for BrdU and NG2 were performed. Free-floating brain sections were removed from the cryoprotectant solution and 2 sections each were placed into one well of a 24 well plate (NUNC™, Denmark) filled with PBS. Sections were washed 3 times for 10 minutes each in PBS. In detail, the solution was removed with a pipette tip (Gilson, USA), which was attached to a vacuum system (Boeco, Australia), while the sections were held back with a soft brush. The well was then filled with PBS and the plate was placed on a rocking platform mixer (Ratek Instruments, Australia) to ensure constant movement. This procedure was always carried out when the solution in the wells needed to be changed. For effective staining of BrdU, the DNA needs to be denatured to expose BrdU to the antibodies. Therefore, the sections were incubated with 2M HCl (33ml of 6M HCl (BDH AnalaR®, Merck, Germany) were brought to 100ml with distilled water) for 30 minutes at 25°C, by placing the well plate in a water bath (MEMMERT, Germany). To neutralize the hydrochloric acid, the tissue was treated for 10 minutes with 0.1M Boric Acid (3.09g Boric Acid salt (Sigma®, USA) were diluted in 500ml distilled water and titrated to pH=8.5 with NaOH or HCl). The tissue was again washed 3 times with PBS and incubated for 1 hour at room temperature with blocking solution (2% Normal Goat Serum (Chemicon International®, USA), 0.1% Bovine Serum Albumin (Sigma®, USA) and 0.3% Triton X-100 (Sigma®, USA) in PBS). This was done to reduce nonspecific binding of the primary or secondary antibody. After removal of the blocking solution, the tissue was incubated for 48 hours at 4°C with concentrations of 1:400 rat anti-BrdU antibody (Accurate Chemicals, USA) and 1:200 rabbit anti-NG2 antibody (Chemicon International, USA) in 1% Normal Goat Serum, 0.1% Bovine Serum Albumin and 0.3% Triton X-100 in PBS. The solution with the primary antibody was removed and the tissue was washed with PBS before being incubated with 1:400 Alexa anti-rat 568 antibody (Molecular Probes, USA) and 1:500 Alexa anti-rabbit 488 antibody (Molecular Probes, USA) in PBS for 90 minutes at room temperature and in darkness. The sections were mounted onto slides (Menzel- Gläser, Germany) after 3 washes with PBS. This was done by placing the tissue in a 0.1% gelatin solution (0.1% gelatin (Merck, Germany) was added to 40-50°C warm water and let cool down under constant stirring)

and carefully dragging each section with a brush onto a slide. The slides were cover-slipped (Menzel- Gläser, Germany) after application of mounting medium for fluorescence (Vectashield®, Vector Laboratories, USA), sealed with nail polish and stored in darkness at 4°C.

4.5.   Cresyl violet stain

Adjacent sections were stained with cresyl violet to identify regions of ischemic damage. Cresyl violet is a dye which stains nuclei, Nissl bodies and neurons in histological tissue. Briefly, sections were removed from cryoprotectant solution, washed 3 times in PBS and mounted onto slides using freshly made 0.1% gelatine solution as described above. The sections were left at room temperature over night to dry. The next day the mounted sections were hydrated by subjecting them to declining ethanol (LabServ™, BIOLAB LTd., Australia) concentrations. Sections were placed into 100% ethanol for 5 minutes and then twice into 95% ethanol for 2 minutes each, followed by two successive 2 minute baths of 70% ethanol and finally 2 minutes in distilled water (Milipore, USA). Sections were then placed for 2 minutes into the cresyl violet solution (0.5% cresyl violet solution : 2.5g cresyl violet (Sigma®, USA) and 1.5ml glacial acetic (BDH AnalaR®, Merck, Germany) acid were added to 500ml distilled water). Afterwards the sections were placed into a basin with tap water in which a constant flow of fresh water replaced the cresyl violet solution over a period of 5 minutes. The sections were then placed for 2 minutes into acid formalin solution (20ml of 8% paraformaldehyde (Merck, Germany) and 1 ml glacial acetic acid were brought to 500ml with distilled water) and following this, washed again in a tap water basin as described above. In order to dehydrate the sections, they were subjected to increasing ethanol concentrations. They were placed two times for 3 minutes each time into 95% and 100% ethanol before being placed three times for 3 minutes into histolene solution (Grale Scientific, Australia). Sections were cover-slipped with DePex mounting medium Gurr® (Merck, Germany) and stored at room temperature.

4.6.   Cell counting

Cell counting was performed using either the fluorescence microscope Leica DM LB2 (Leica, Germany) with a camera from Optronics (USA) or an Olympus BX51 fluorescence microscope with camera (Olympus, Japan). Comparing a reference section with scale bar, it was shown that

these two microscopes were equal in resolution, magnification and area size so that either microscope yielded the same result.

The damaged area could be identified for each brain in cresyl violet stained sections and immunofluorescence sections. In cresyl violet stained sections injury could be identified by the following:

1. Disruption of the layer structure of the cortex
2. Breakdown of extracellular matrix in sections from 7 and 14 days after MCAo

On immunofluorescence sections the infarct core could easily be identified in some sections by disintegration of the extracellular matrix. However, in sections without such obvious indications, adjacent cresyl violet stained sections were used to identify the infarct area.

Observation of BrdU labeled cells in the subventricular zone (SVZ) was used as a positive control for successful BrdU staining because cell proliferation occurs in the SVZ in the injured and uninjured adult brain (Zhang et al., 2001). Successful NG2 staining was confirmed by observation of NG2 labeled cells in the uninjured areas of the brain in sham and stroke animals because NG2 labeled cells are abundant in the adult brain (Chang et al., 2000; Nishiyama et al., 1997). Section selected for cell counting were chosen from –0.92mm to +1.2mm of Bregma (Paxinos and Watson 1986). A 60µm thin section was taken from every brain for cell counting with a fluorescence microscope. The peri-infarct region was first identified and then 4 regions at 20 times magnification were chosen for counting. Two of these 4 regions lay directly next to the infarct core, while the other two regions were lateral to the first two regions (figure 2). The microscope was connected through a camera to a computer. Each of the 4 regions was divided into four subregions at 40 times magnification using the program Steroinvestigator (Micro bright field, USA). This program was used to control the microscope movements and to ensure that the different subregions did not overlap. The subregions were then counted at 40 times magnification using image analysis software (Picture Frame, Micro bright field, USA). Displayed

at any one time on the computer screen was an area of 0.065 mm². In total, an area of 1mm² or a volume of 0.6mm^3 was counted per brain section.
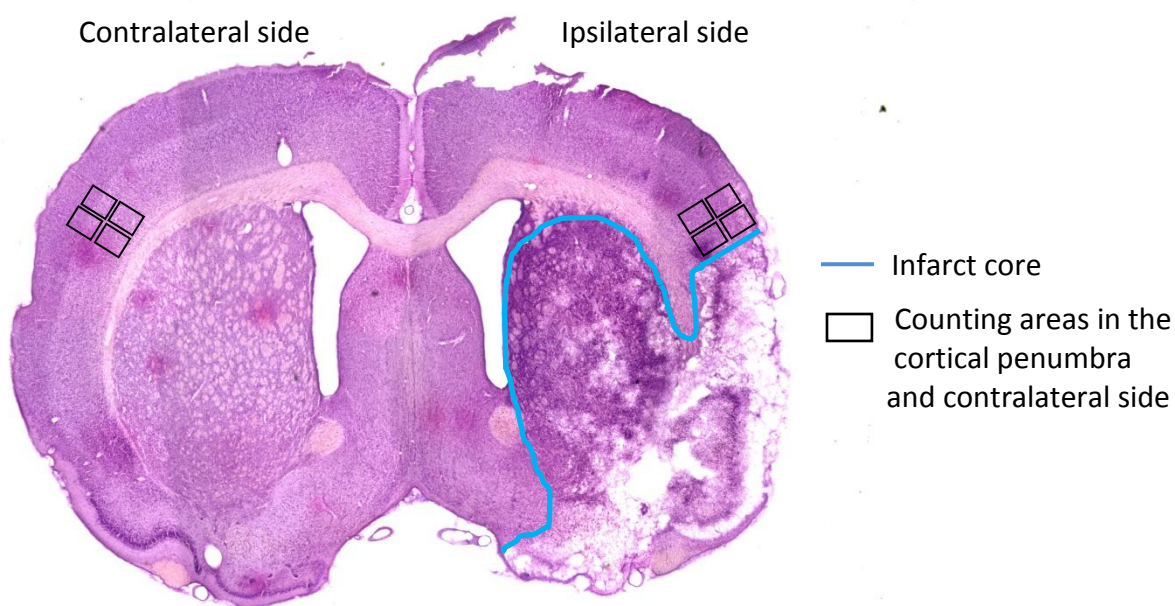

Fig. 2: Cresyl violet staining of a representative coronal section from 14 days after stroke. The infarct core is indicated by the blue line. 4 counting areas of 0.26mm^2 were chosen each in the penumbra and on the respective contralateral side. Two of these 4 regions lay directly next to the infarct core, while the other two regions were lateral to the first two regions. The regions were the subdivided to perform cell counting at 40x magnification.

BrdU immunopositive cells were visualized under the green filter of the microscope and appeared as red fluorescent nuclei. NG2 immunopositive cells were visualized under the blue filter and appeared as cells with fluorescent green membranes. The area was screened for BrdU positive labeled cells (red). By switching the filter NG2 staining was visualized and the cell could either be identified as a double labeled cell (green membrane surrounding the nucleus) or an only BrdU labeled cell (no green membrane matching the site of the BrdU cell). In areas of very high density of BrdU labeled cells, several pictures with different focus depth, taking into account the thickness of the sections, were taken for red and green fluorescence. The photos

were merged using Adobe Photoshop 7.0 (Adobe Systems Incorporated, USA) and counted as double labeled if a red nucleus was surrounded by a green membrane.

4.7. Statistical analysis

Comparisons of BrdU and NG2 co-labeled cells and BrdU only labeled cells were made between sections from groups of rats sacrificed at different time points after MCAo in both the ipsilateral and contralateral sides. Within time points, the ipsilateral side was compared to the contralateral side. Numbers of BrdU and NG2 co-labeled cells and BrdU only labeled cells were analysed using two-way repeated measures ANOVA and Tuckey's test for individual differences (Sigma Stat, Jandel Scientific Software, USA). The 2 way ANOVA was used because the design of the experiment contained two factors namely treatment (MCAo versus sham stroke) and time (1, 3, 7 and 14 days after MCAo). Repeated measures were used for comparisons between ipsilateral versus contralateral sides, since these were compared in the same animal. The ANOVA test only detects an overall difference between factors or within each factor. Therefore, the Tukey's test was used to determine individual differences between the groups. Tukey's test was chosen since it corrects for potential errors due to making multiple comparisons. P values < 0.05 were regarded as statistically significant. Additionally, for the 7 days group cell counting results between the counting areas close to the infarct core and the areas further away from the infarct were separately analysed using the paired Student's t-test (Graph Pad Prism 4.0, GraphPad Software, USA). A P value <0.05 was regarded as significant.

Body temperatures of stroke and sham stroke animals were statistically analysed using a one-way ANOVA followed by Tukey's post-test for each comparison. This test was chosen, because temperatures were not measured at exactly the same times in all groups. Within each group comparisons were made between temperatures measured over the first three hours after stroke using a one-way ANOVA test followed by Tukey's post test each time. Between groups comparisons were only made at 0 and 180 minutes because temperatures were measured at these times in all groups.

5. Results

5.1. Stroke severity ratings and body temperatures

One animal of the day 7 group died soon after MCAo and was excluded from the study. Stroke ratings of surviving rats differed from 1 to 3 according to the method described by Roulston et al. (2008) and are summarized in table 1.

Group	Stroke Rating				
	# 1	# 1-2	# 2	# 2-3	# 3
1 day group	2		1	1	
3 days group			3		
7 days group	1		1	2	
14 days group		1		2	1

Table 1*:* Numbers of animals by stroke rating per time group

After MCAo temperatures of animals remained under 40°C, so that no animal had to be excluded from the study for this reason. Temperatures before MCAo (0 minutes) and 180 minutes after MCAo did not differ significantly between groups (Table 2, P > 0.05, 1-way ANOVA and Tukey's post test for individual differences). Since temperatures were not measured in all groups at 30, 60 and 120 minutes, a statistical comparison could not be made between groups for these time points. Comparisons were made between temperatures at different times within each group. This showed a significant difference in temperature between 0 and 30 minutes after MCAo for the 14 days group (P < 0.05, 1-way ANOVA and Tukey's post test). At the other time points temperatures did not differ significantly (P > 0.05, 1-way ANOVA and Tukey's post test for individual differences) in the 14 days group. Respective the other groups, there were no significant differences between temperatures at any measured time point (P > 0.05, 1-way ANOVA and Tukey's post test).

Group	Time post Stroke				
	0 min	30min	60min	120min	180min
1 day group	37.03 ± 0.3	38.37 ± 1.2	TNT	TNT	37.5 ± 0.2
3 days group	37.1 ± 0.6	TNT	37.6 ± 0.7	37.23 ± 0.4	37.13 ± 0.3
7 days group	37.36 ± 0.9	37.7 ± 0.1	37.4 ± 0.53	37.14 ± 0.5	37.23 ± 0.5
14 days group	36.75 ± 0.3*	38.23 ± 0.6*	37.38 ± 0.5	TNT	36.9 ± 0.8

Table 2: Mean temperatures in °C ± standard deviation of animals from each group measured at different time points after MCAo. * P< 0.05 for temperature differences between 0 (pre- stroke value) and 30 minutes after MCAo in the 14 days group. TNT = temperature not taken

5.2.   NG2 cells and BrdU labeled cells in undamaged brain regions

NG2 cells could be found in all undamaged brain regions of sham stroke and stroke rats. This was used as a positive reference for successful NG2 staining. NG2 cells appeared as cells with small cell bodies and long processes. In the cortex, NG2 cells extended long and branched processes radially around the cell (Fig.3; A, B). In the corpus callosum, NG2 cells had an elongated shape and processes extended along the course of the white matter tract (Fig.3 ;C, D).
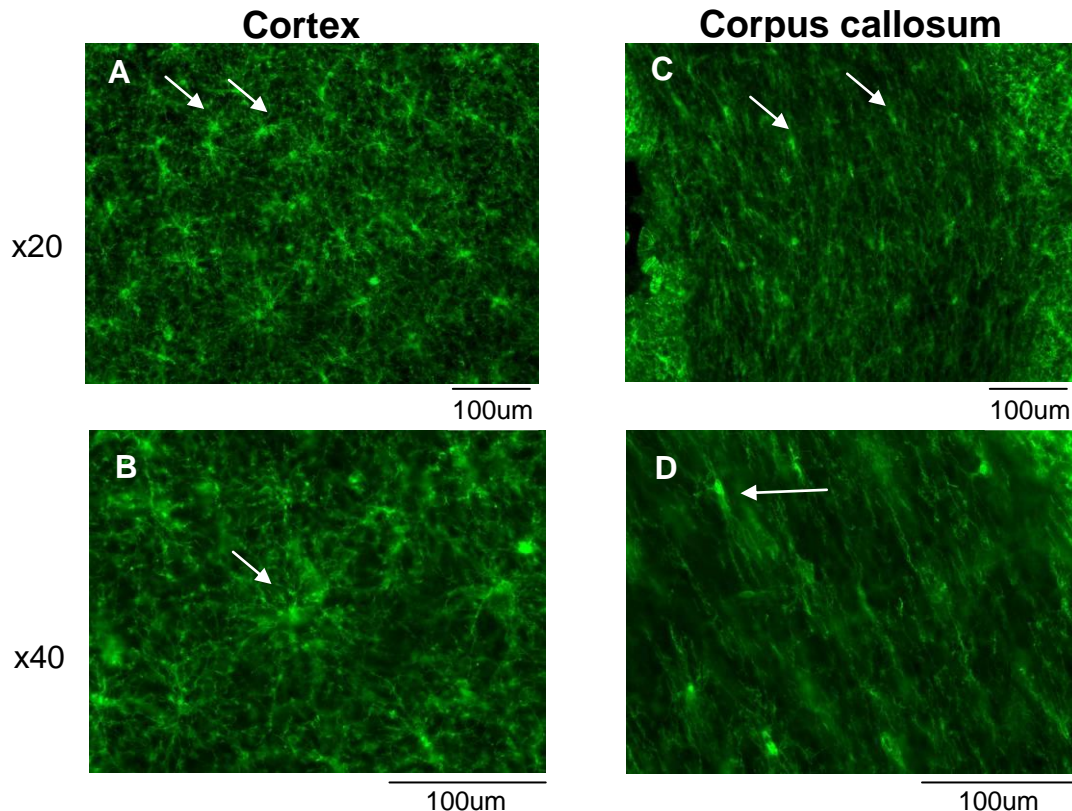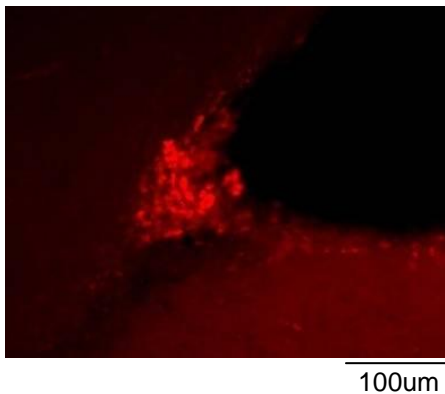

Fig. 3: Comparison of NG2 cells in the cortex (A, B) and the corpus callosum (C, D) in undamaged brain regions. Arrows point to some of the numerous NG2 cells. Note that NG2 cells in the corpus callosum appear elongated while those in the cortex extend processes radially around the cell (Photos taken at x20 and x40 magnification).

BrdU labeled nuclei were observed in large numbers in the subventricular zone (SVZ) in both stroke and sham stroke animals (Fig. 4). This was used as a positive reference for successful BrdU staining.

Fig. 4: BrdU labelled nuclei of proliferating cells (arrow) in the SVZ adjacent to the corpus callosum. (picture taken at x20 magnification)

5.3.   NG2 cells and BrdU labeled cells in the penumbra and in the contralateral side

Representative pictures of the penumbra from different time points after MCAo are shown at x20 magnification (Fig. 4) and at x40 magnification (Fig. 5). These pictures were taken from the counting areas as demonstrated in figure 2. One day after MCAo NG2 cells (A) were seen but no BrdU labeled nuclei (E), so that none of these NG2 cells were proliferating (I). On sections from 3 days after MCAo there could be seen some BrdU labeled cells (F, arrows) of which some were NG2/BrdU co-labeled (J, white arrows). At 7 days after MCAo numerous BrdU labeled cells were present (G, arrows) which were often NG2/BrdU co-labeled (K, white arrows). There were clearly more proliferating NG2 cells 7 days after MCAo than at the other time points after MCAo (I-L). Pictures from 14 days after MCAo show some BrdU labeled cells (H, arrows) and some NG2/BrdU co-labeled cells (L, white arrows), however in significantly smaller numbers than at 7 days after MCAo. At any time point NG2 cells could be seen in the penumbra (A-D).

Fig. 6 shows representative pictures of the penumbra and the corresponding contralateral side. On the contralateral side, proliferating NG2 cells (Fig. 6, E-H, arrow) were not numerous but were seen occasionally. There were no apparent differences in numbers of proliferating NG2 cells between different time points after MCAo on the contralateral side (Fig. 6, E-H). Seven days after MCAo the number of proliferating NG2 cells in the penumbra was much higher than on the contralateral side (Fig. 6, C and G). At 1, 3, and 14 days after MCAo there were no apparent differences in the number of proliferating NG2 cells between the penumbra and the contralateral side (Fig. 6, A, B, D, E, F, H).
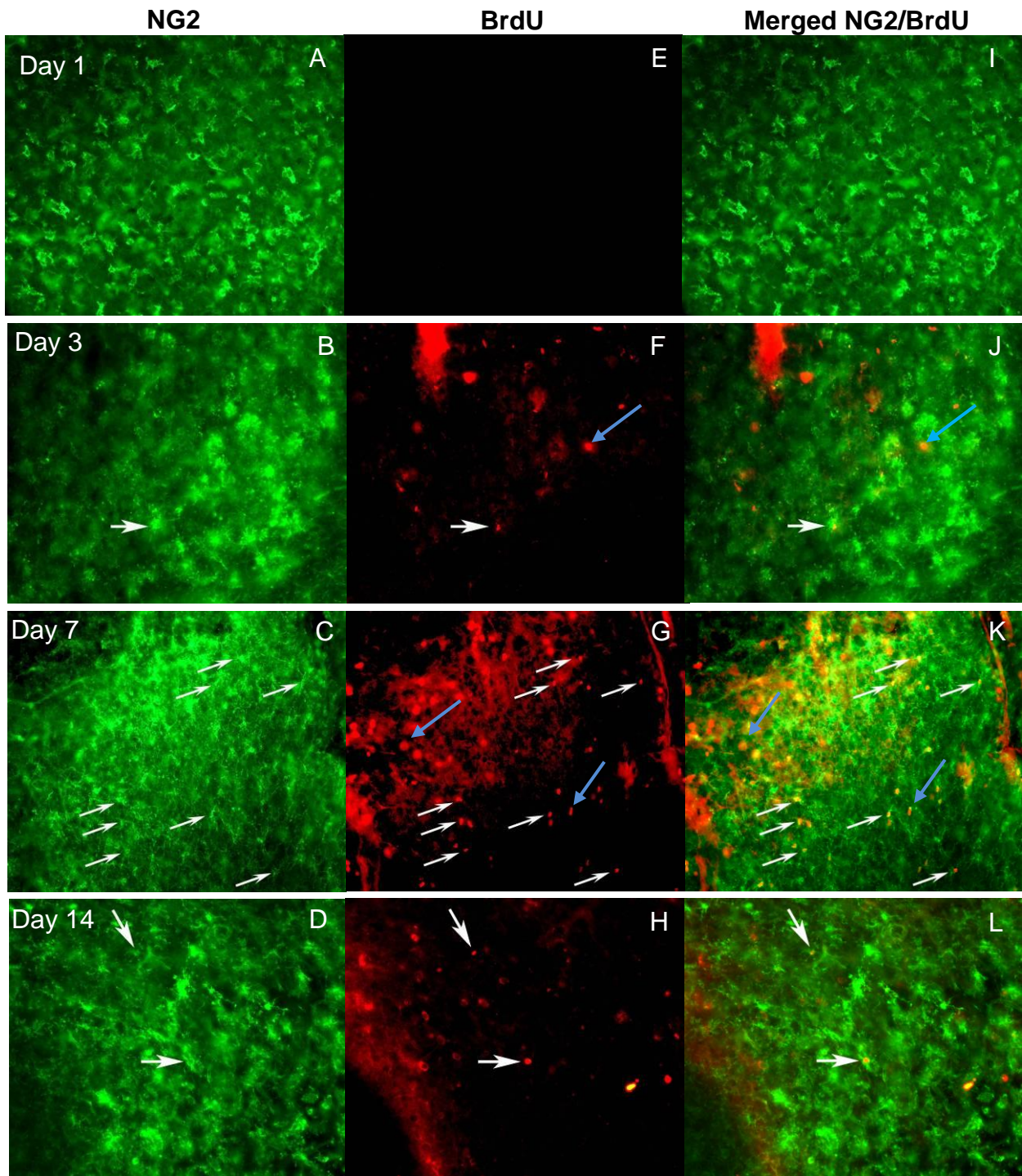
Fig. 5: Representative photos of the penumbra taken at x20 magnification.
NG2 staining (left column), BrdU staining (middle column) and merged pictures of NG2 and BrdU staining (right column). At this magnification co-localization of a strong green and red signal from NG2 and BrdU labelling respectively could be observed as an orange-yellow signal. Note the increase in numbers of proliferating NG2 cells (white arrows) 7 days after MCAo (K) compared with 1, 3, and 14 days after MCAo (I, J, L). Blue arrows indicate BrdU labelled cells which were not NG2/BrdU co-labelled.
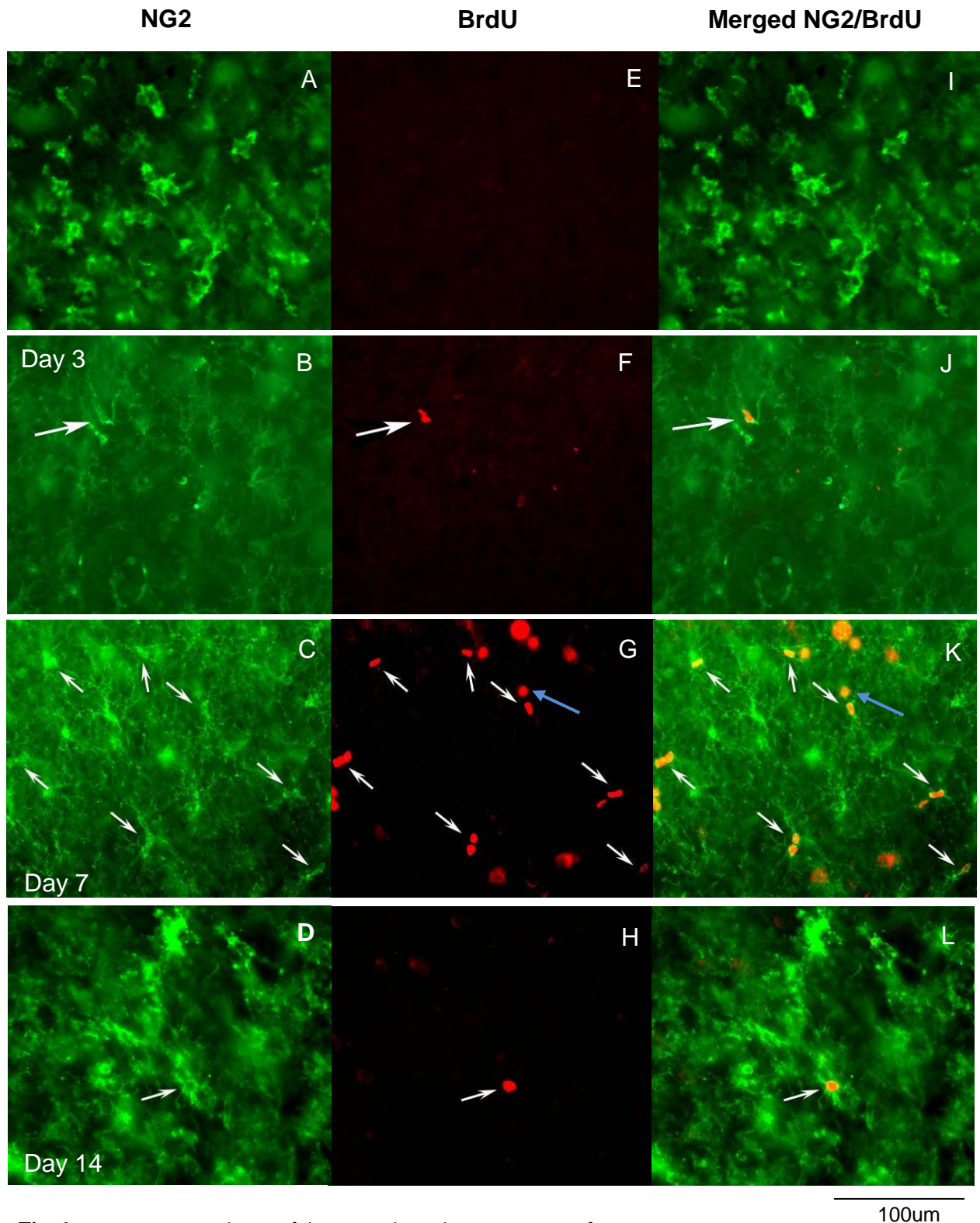
Fig. 6 : Representative photos of the penumbra taken at x40 magnification.
NG2 staining (left column), BrdU staining (middle column) and merged pictures of NG2 and BrdU staining (right column). White arrows indicate proliferating NG2 cells which had a red nucleus (BrdU positive) that was surrounded by a green membrane (NG2 positive). The blue arrows indicates a cell which was BrdU labelled but not NG2/BrdU co-labelled. Note the increase in numbers of proliferating NG2 cells 7 days after MCAo (K) compared with 1, 3, and 14 days after MCAo (I, J, L).

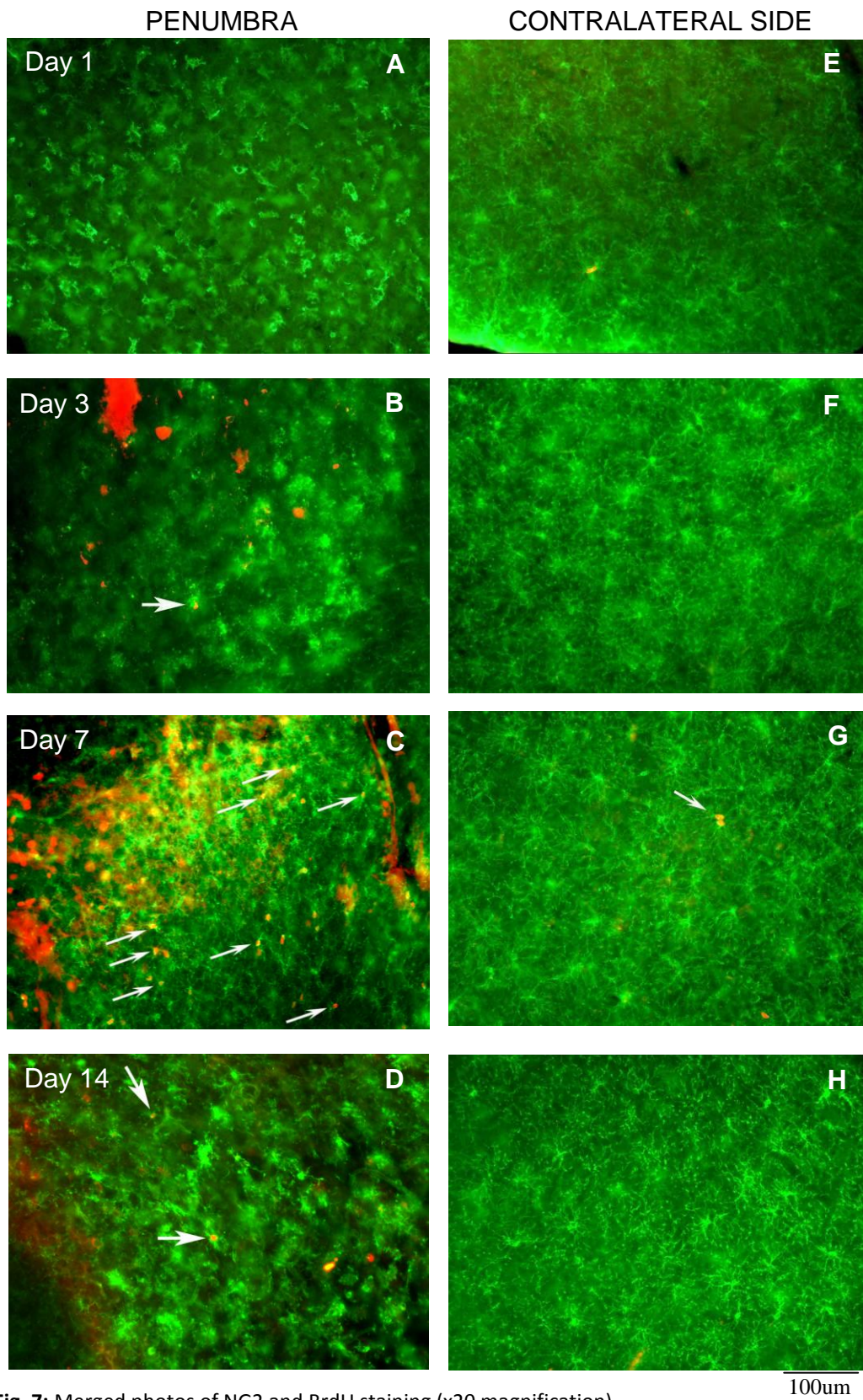PENUMBRA   CONTRALATERAL SIDE

Fig. 7: Merged photos of NG2 and BrdU staining (x20 magnification)
of the penumbra (left) and the contralateral side (right) from MCAo rats. Arrows indicate
proliferating NG2 cells. Seven days after MCAo more proliferating NG2 cells could be seen in
the penumbra than on the contralateral side (C, G).

5.4. Morphological changes of NG2 cells

Between 3 and 14 days after MCAo, though not yet at 1 day after MCAo, the area adjacent to the infarct core (also referred to as the inner penumbra) appeared as a belt like zone of up-regulated NG2 immuno-staining (Fig. 7, B-D, area 2). There was a clear delineation between the staining intensity of the inner penumbra and the surrounding outer penumbra (the area lateral to the inner penumbra) (Fig. 7, B-D, areas 2 and 3). The staining intensity of the outer penumbra was similar in intensity to uninjured tissue. In the inner penumbra NG2 cells displayed changes in shape when compared with either the outer penumbra or with the contralateral side. In the inner penumbra the processes of NG2 cells were shortened, scarcely branched and cell bodies appeared swollen as early as 1 day after MCAo (Fig. 8; E-H). NG2 cells in the outer penumbra appeared similar in shape to the contralateral side at every time point (Fig. 8; A-D, I-L).
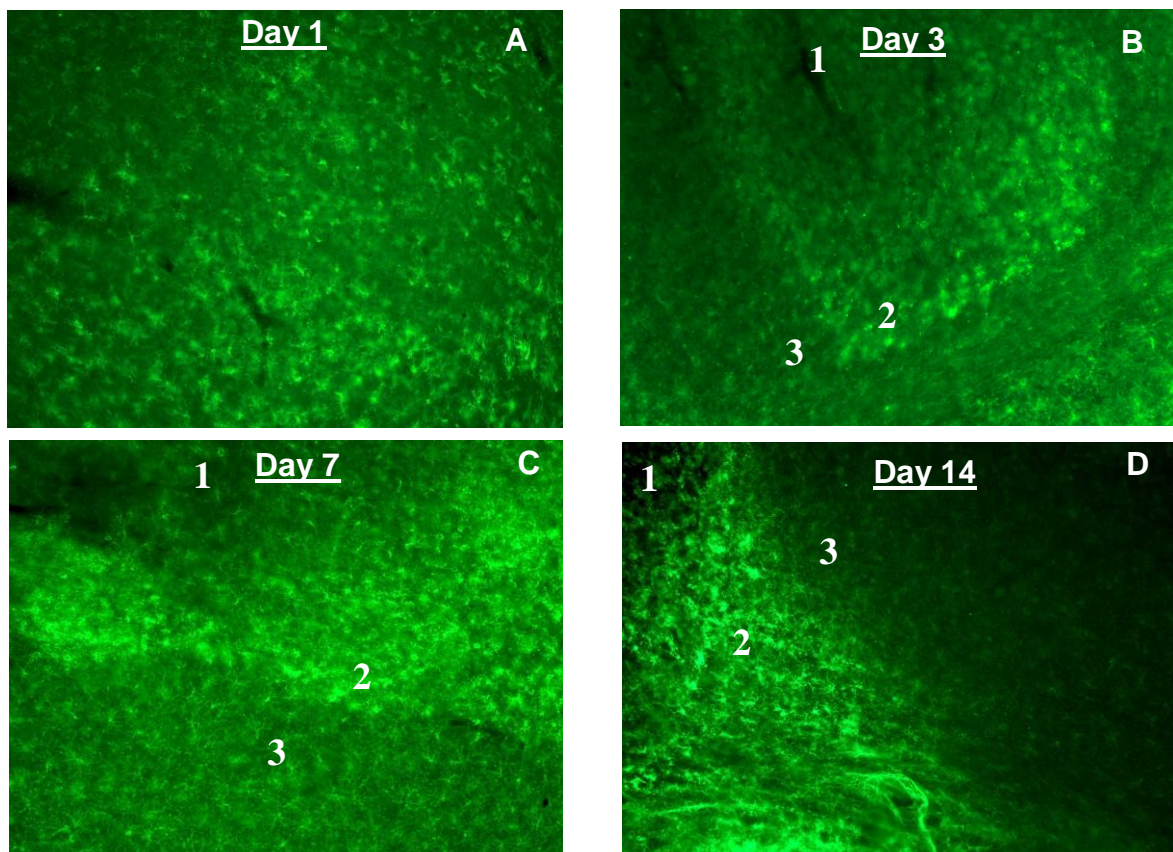

Fig. 8: (1) the infarct core, (2) the inner penumbra, (3) the outer penumbra. 3, 7 and 14 days after MCAo. There was up-regulation of NG2 immuno-reactivity in the inner penumbra 3, 7 and 14 days after MCAo. Note that these pictures were taken at x10 magnification and extend beyond the counting areas as indicated in figure 2.
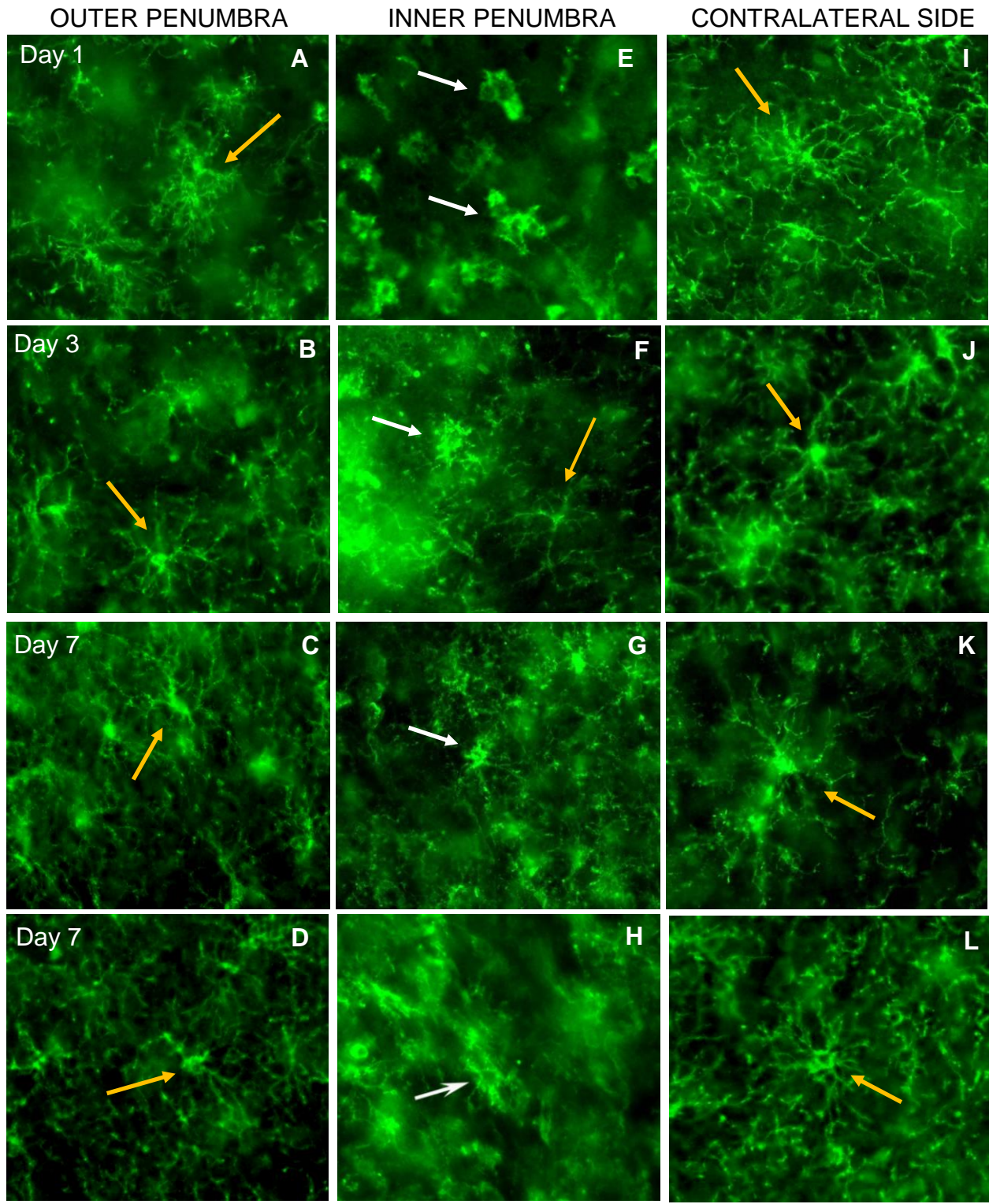
Fig. 9: NG2 staining of the outer penumbra (A-D), the inner penumbra (E-H) and the contralateral side (I-L) 1, 3, 7 and 14 days after MCAo. NG2 cells with morphological changes are indicated by white arrows, NG2 cells without morphological changes are indicated by orange arrows. Note that the morphological changes only occur in the inner penumbra and not in the outer penumbra or contralateral side. Picture F shows an NG2 cell in the area of up-regulation with morphological changes directly adjacent to an NG2 cell outside the area of up-regulation, which appeared normal in morphology. Pictures were taken at x40 magnification.

FigureFig. *9* shows a representative picture taken of an inner counting area (refer to methods figure 2) 7 days after MCAo. It illustrates that proliferation of NG2 cells could be seen in both the area of up-regulation (Fig. 9, A) and in the outer penumbra (Fig. 9, B). There was no obvious difference in the number of proliferating NG2 cells between the two areas. However, based on the preliminary experimental design, a statistical comparison could not be made between the inner and the outer penumbra.
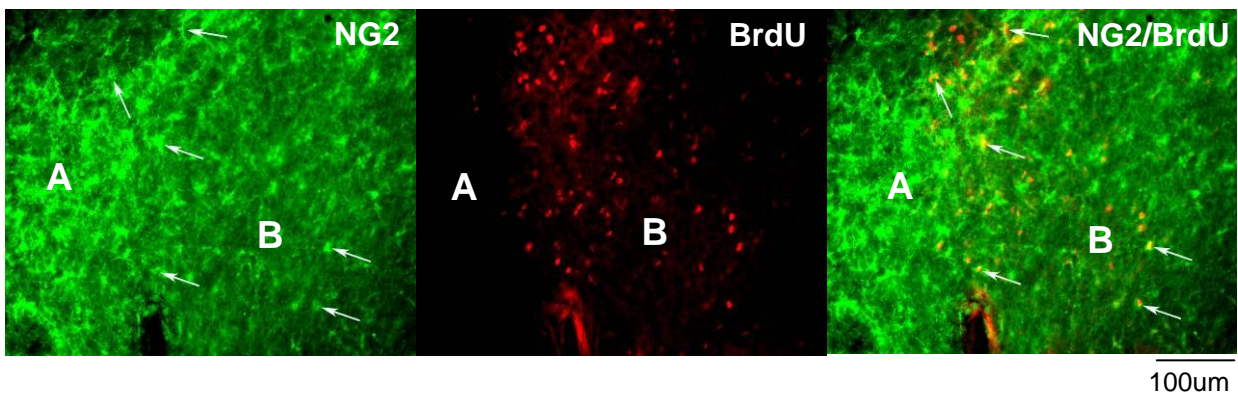

100um

Fig. 10: The inner penumbra with up-regulation of NG2 immuno-reactivity (A) and the outer penumbra (B) 7 days after MCAo. Left: NG2 immuno- staining, middle: BrdU staining, right: merged picture. Note that proliferating NG2 cells (arrows) can be seen in both the inner penumbra (A) and outer penumbra (B) (Photos taken at x20 magnification).

There were no differences in shape between dividing and non dividing NG2 cells within the same area as can be seen in Fig. 10. Dividing NG2 cells in the inner penumbra had short processes like their non-dividing neighbours. Dividing NG2 cells in the outer penumbra and dividing NG2 cells on the contralateral site both exhibited long and highly branched processes

| INNER PENUMBRA | OUTER PENUMBRA | CONTRALATERAL SIDE |

100um
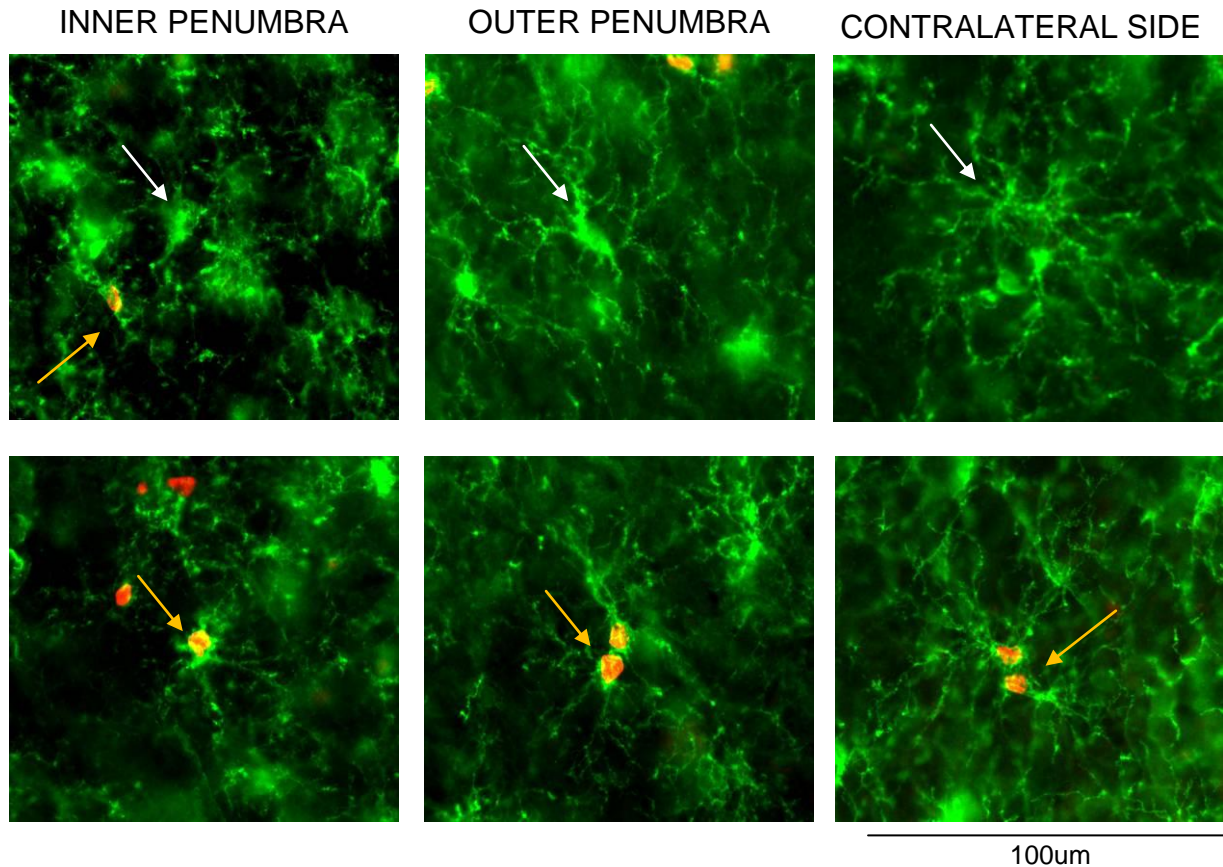
Fig. 11: Comparison of dividing and non-dividing NG2 cells 7 days after MCAo. The upper row shows non-dividing NG2 cells, the bottom row shows dividing NG2 cells in the inner penumbra (left), in the outer penumbra (middle) and on the contralateral side (right). Note that there was no apparent difference in shape between dividing (orange arrows) and non-dividing NG2 cells (white arrows) within the same area. (There are shown merged photos of NG2 and BrdU staining taken at x40 magnification)

5.5. NG2 cells and BrdU labeled cells in the infarct core

In the infarct core, NG2 cells changed markedly in morphology compared to the respective contralateral site. One day after MCAo, NG2 labeling of most NG2 cells was reduced to the cell body, giving them a dot like appearance. Only a few unchanged stellate shaped NG2 cells could be found in the infarct core (Fig.12). From day 3 onwards almost no NG2 labeled cells could be identified in the infarct core (Fig.13, F-H). Towards later time points the tissue in the infarct core disintegrated, leaving a pattern of holes (Fig.13, C, D, G, H). Background staining of the remaining tissue was so intense that it was impossible to distinguish between background staining and correct labeling of cells with anti NG2 antibodies as seen on the representative picture from sections from 7 and 14 days after MCAo (Fig.13, G, H). From day 3 onwards numerous BrdU labeled cells could be seen in the infarct core (Fig.13, B-D). However, it was impossible to identify if any of these cells were NG2/BrdU double labeled because of the high background staining in this area.

NG2 - infarct core   **NG2 - contralateral side**

100um
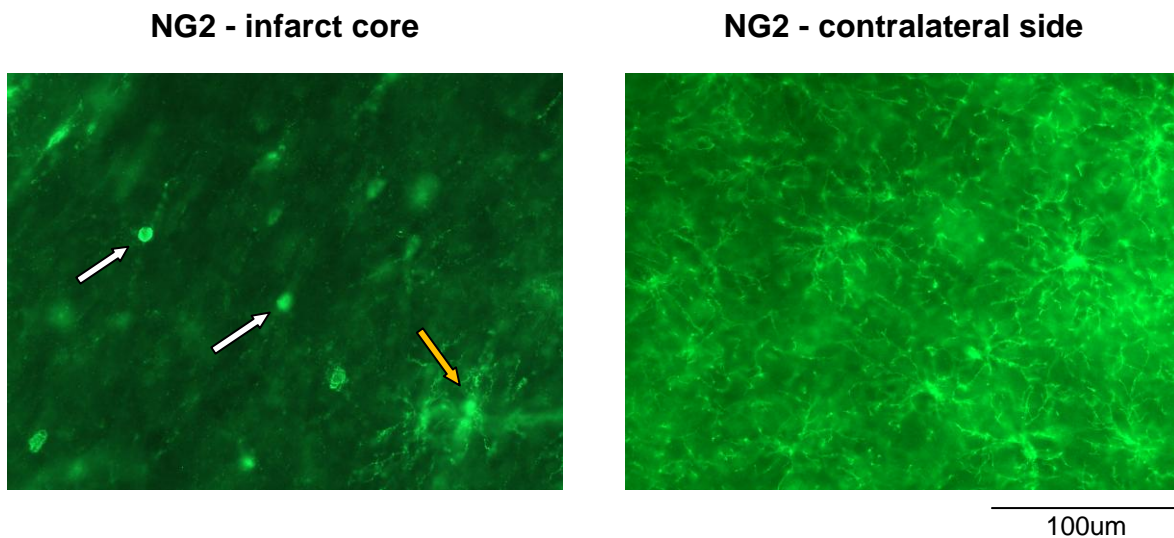
Fig. 12: Pictures of NG2 cells in the infarct core (left) and the respective contralateral area (right) 1 day after MCAo. White arrows indicate NG2 cells with a dot-like staining pattern. The orange arrow indicates one of the few remaining stellate shaped NG2 cells in the infarct core. (x40 magnification)

BrdU **NG2**
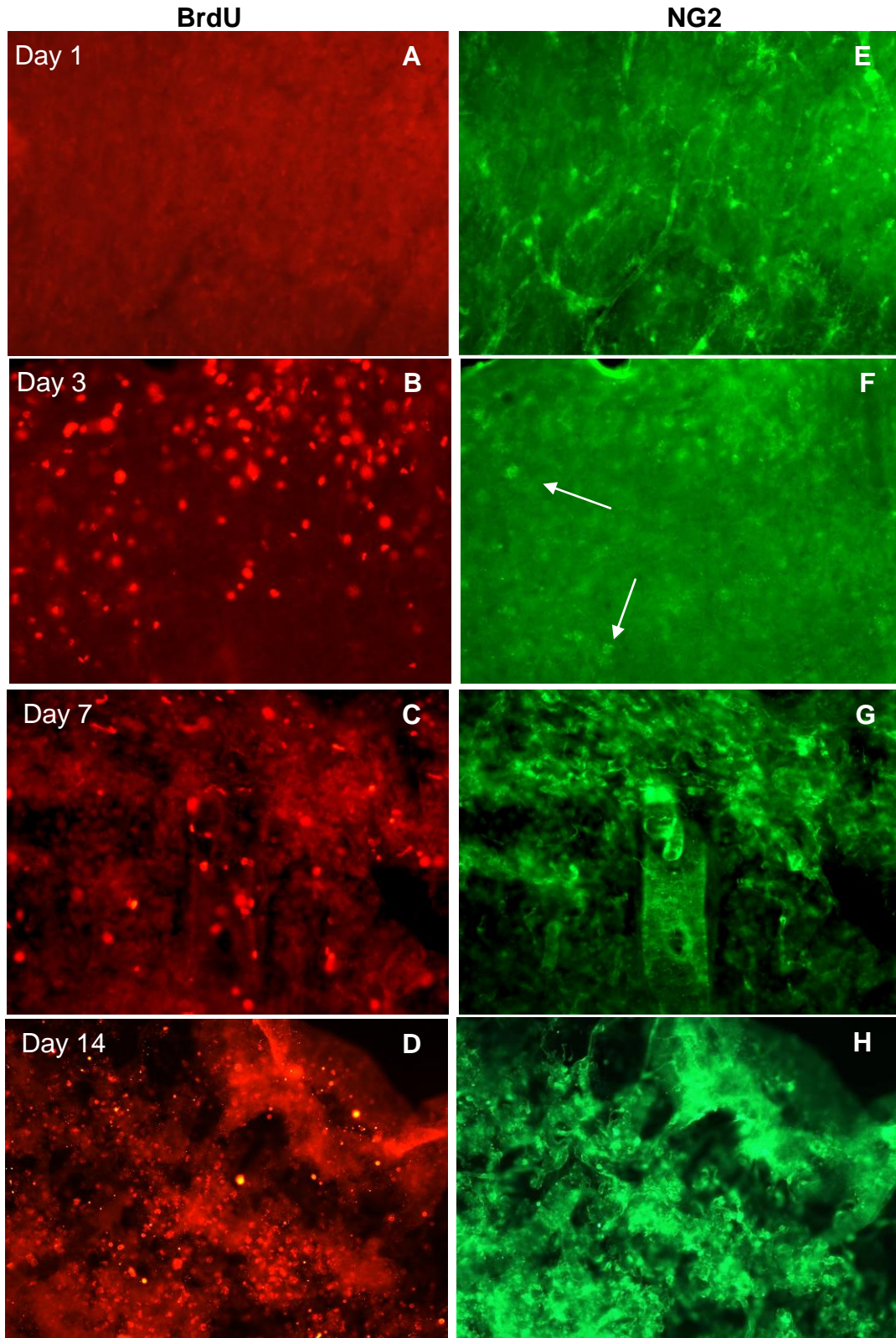
Fig. 13: Pictures of the infarct core 1, 3, 7 and 14 days after MCAo.
BrdU staining (left) and the corresponding NG2 staining (right). Numerous BrdU labelled cells were seen in the infarct core between 3 and 14 days after MCAo (B-D). 3 days after MCAo only few NG2 labelled cells could still be identified (F, arrows). 7 and 14 days after MCAo the tissue had disintegrated. The high background did not allow correct identification of NG2 cells (G,H). (Pictures were taken at x20 magnification).

5.6.   Cell counting results and statistical analysis

Sections taken from rats 1, 3, 7 and 14 days after MCAo were assessed for number of BrdU labeled cells and NG2/ BrdU co-labeled cells in the penumbra area and corresponding sites on the contralateral hemisphere and the same regions were compared with sham stroke animals. The statistical analyses revealed a significant increase in the number of NG2/ BrdU co-labeled cells in the ipsilateral (damaged) penumbra 7 days after MCAo compared with 1, 3 and 14 days after the insult (Fig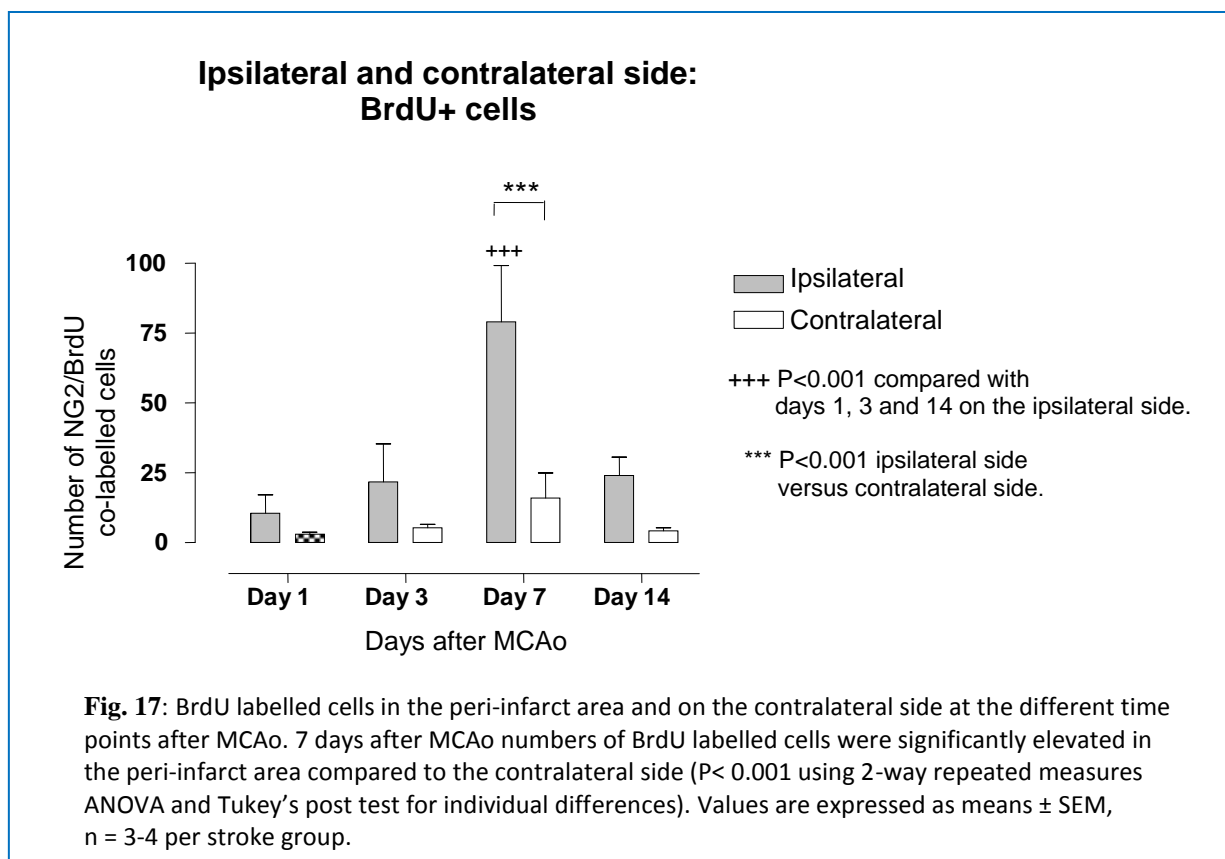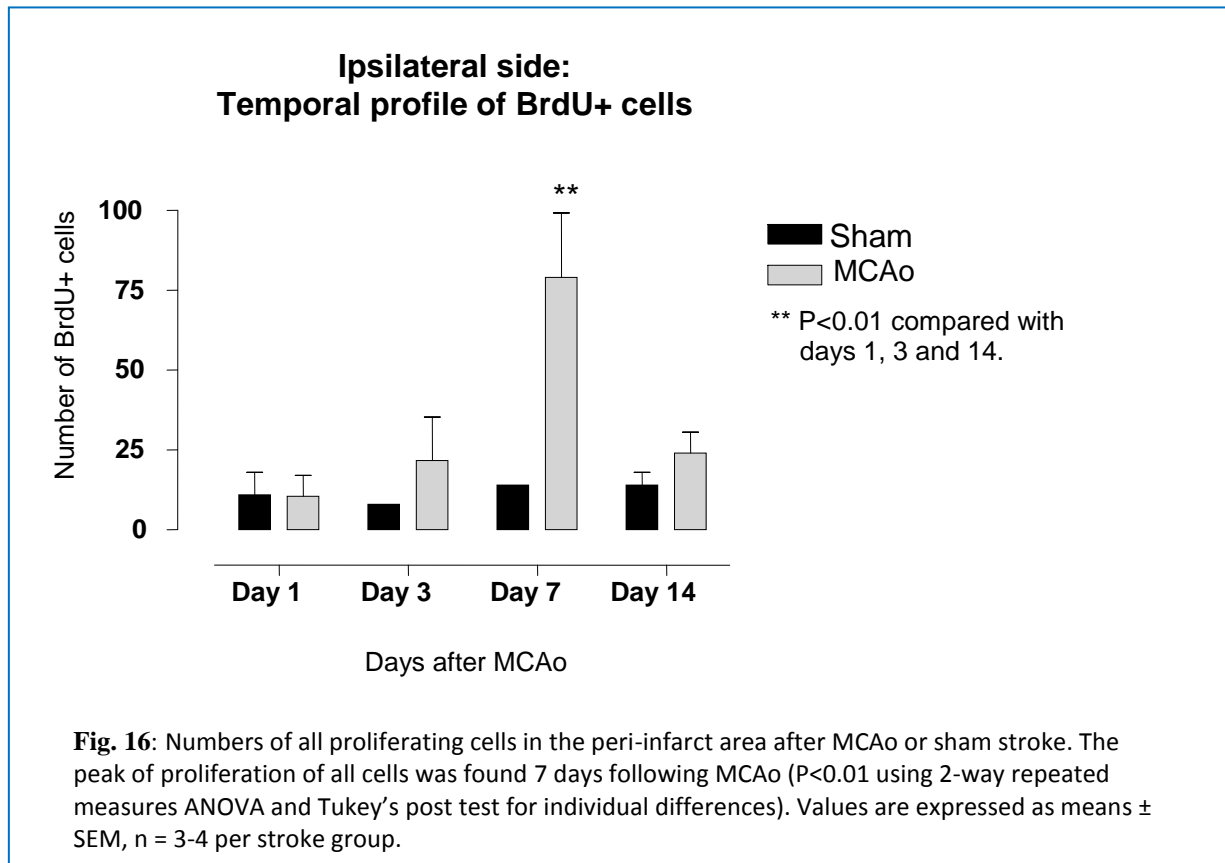. 13; P<0.001). There was no significant difference in number of NG2/ BrdU co-labeled cells between the sections from 1, 3 and 14 days after MCAo (Fig. 13). In sham animals, the number of NG2/BrdU co-labeled cells was negligible at any of the time points tested (Fig. 13). Seven days after MCAo the number of NG2/ BrdU co-labeled cells was also significantly higher in the ipsilateral cortex compared to the contralateral side (Fig. 13; P<0.001) where the number of co-labeled cells was negligible. In the sections from the other time points after MCAo numbers of NG2/ BrdU co-labeled cells did not differ significantly between the ipsilateral and the contralateral side (Fig. 13).

Results were similar for BrdU labelling, which is an indication of proliferation of all types of cells. There was a significant increase of BrdU labeled cells in the peri-infarct area in sections from 7 days after MCAo compared to sections from 1, 3 and 14 days after MCAo (Fig. 13; P<0.01) . There was no statistically significant difference in numbers of BrdU labeled cells between 1, 3 and 14 days after MCAo (Fig. 13). Seven days after MCAo, BrdU positive cells were also increased on the ipsilateral side compared to the contralateral side (Fig. 13; P<0.001). At the other time points there was a trend towards increased proliferation on the ipsilateral side compared to the contralateral side however differences were not statistically significant (Fig. 13).

In the contralateral hemisphere, numbers of NG2/BrdU co-labeled cells or numbers of BrdU labeled cells showed no statistically significant differences between different time points after MCAo and were not significantly different from sham animals (Fig. 13, Fig. 13).

Ipsilateral side:
Temporal profile of proliferating NG2 cells

Fig. 14: Numbers of proliferating NG2 cells in the peri-infarct area after MCAo or sham stroke. The peak of NG2 cell proliferation was found 7 days after MCAo (P<0.001 using 2-way repeated measures ANOVA and Tukey's post test for individual differences). Values are expressed as means ± SEM, n = 3-4 per stroke group.

Ipsilateral and contralateral side:
Proliferation of NG2 cells

Fig. 15: NG2 cell proliferation in the peri-infarct area and on the contralateral side at the different time points after MCAo. Seven days after MCAo proliferation of NG2 cells was significantly higher in the peri-infarct area compared to the contralateral side (P< 0.001 using 2-way repeated measures ANOVA and Tukey's post test for individual differences). Values are expressed as means ± SEM, n = 3-4 per stroke group.

Ipsilateral side:
Temporal profile of BrdU+ cells

Fig. 16: Numbers of all proliferating cells in the peri-infarct area after MCAo or sham stroke. The peak of proliferation of all cells was found 7 days following MCAo (P<0.01 using 2-way repeated measures ANOVA and Tukey's post test for individual differences). Values are expressed as means ± SEM, n = 3-4 per stroke group.

Ipsilateral and contralateral side:
BrdU+ cells

Fig. 17: BrdU labelled cells in the peri-infarct area and on the contralateral side at the different time points after MCAo. 7 days after MCAo numbers of BrdU labelled cells were significantly elevated in the peri-infarct area compared to the contralateral side (P< 0.001 using 2-way repeated measures ANOVA and Tukey's post test for individual differences). Values are expressed as means ± SEM, n = 3-4 per stroke group.
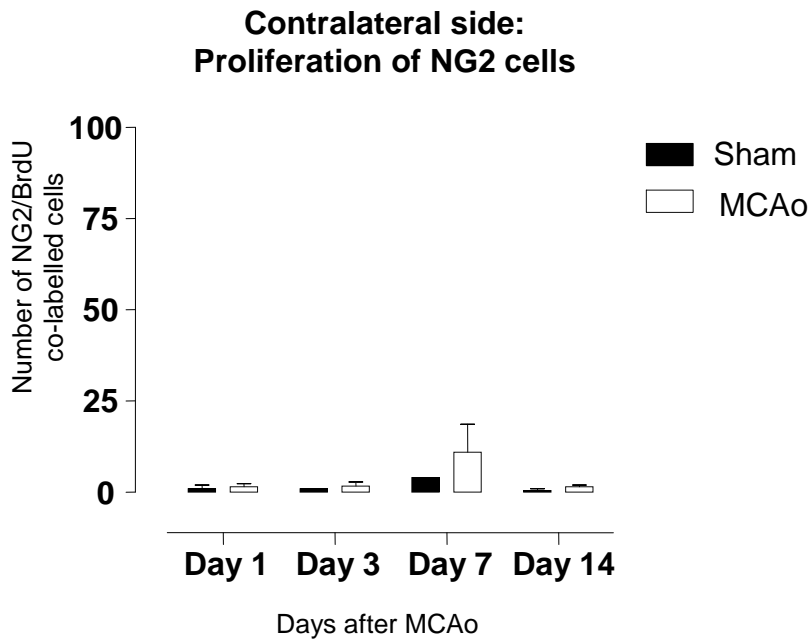
Fig. 18: Numbers of proliferating NG2 cells on the contralateral side after MCAo or sham stroke. On the contralateral side there were no significant differences in NG2 cell proliferation between different time points after MCAo (P>0.05 using 2-way repeated measures ANOVA and Tukey's post test for individual differences). Values are expressed as means ± SEM, n = 3-4 per stroke group.
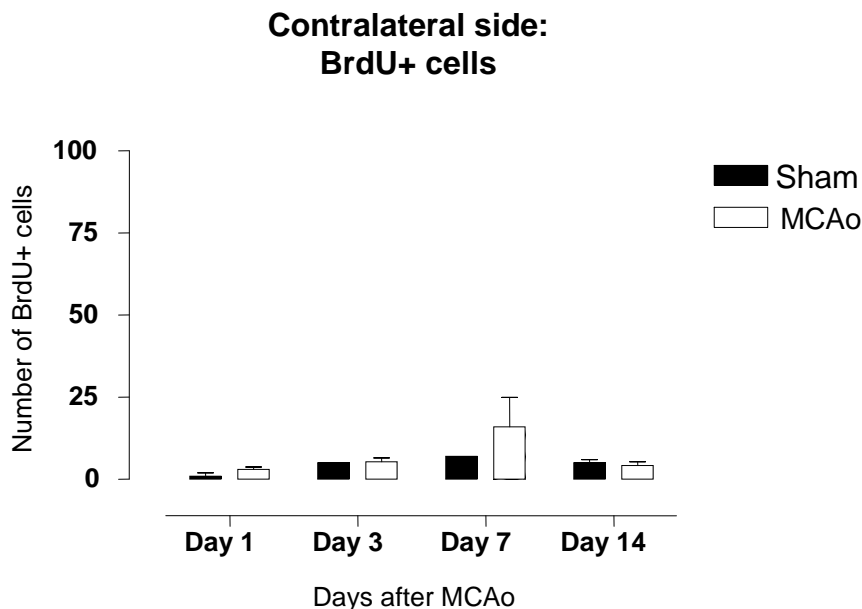

Fig. 19: BrdU labelled cells in the on the contralateral side after MCAo or sham stroke. There was no statistical significant difference in proliferation of all cells between different time points on the contralateral hemisphere (P>0.05 using 2-way repeated measures ANOVA and Tukey's post test for individual differences). Values are expressed as means ± SEM, n = 3-4 per stroke group.

6. Discussion

6.1. Proliferation of NG2 cells after ET-1 induced focal cerebral ischemia

The present study is the first study to directly demonstrate that NG2 cells increase proliferation in the penumbra after transient focal ischemia. At day 7 post stroke, NG2 cell proliferation was shown to be increased compared with days 1, 3 and 14, compared to sham stroke animals and compared to the uninjured contralateral side. At the other time points there was no statistically significant increase in proliferation. This highlights that proliferation of NG2 cells after stroke is a time dynamic process. Namely, levels of proliferating NG2 cells remain normal up to 3 days, peak around 7 days and decrease to normal levels by 14 days after MCAo. This is an important aspect which has to be considered in future studies that wish to evaluate the effect of interventions on NG2 cells after stroke. The finding of elevated NG2 cell proliferation 7 days after MCAo has also been confirmed by McRae and Callaway (2006, unpublished data). It is also in concordance with similar findings in a model of ET-1 induced lacunar white matter damage (Sozmen et al., 2009). Former studies have indirectly shown total numbers of NG2 cells to increase between 7 and 14 days after MCAo in the penumbra (Tanaka et al., 2001; 2003). However it remained unclear whether this increase in numbers was caused by NG2 cell proliferation or other processes such as migration of NG2 cells from distant areas. The present study, using co-labelling for NG2 cells and newly formed cells, together with the low migration ability of NG2 cells (Franklin et al., 1997) give evidence that the increase in NG2 cell number must be caused by local proliferation in the penumbra after MCAo. In the present study, proliferating NG2 cells were also observed in the area contralateral to the ischemic penumbra at any given time point after MCAo. The rate of NG2 cell proliferation did not differ significantly between different time points after MCAo or compared to sham stroke animals. This finding was also later corroborated by McRae and Callaway (2006, unpublished data). Therefore, it can be assumed that the number of proliferating NG2 cells in the area contralateral to the penumbra represents the physiological rate of NG2 cell proliferation and is unlikely to be induced by the ischemic damage.

The exact mechanisms which lead to an increase in NG2 cell proliferation have not yet been identified. Demyelination and growth factors could play a role. FGF-2 and PDGF have been

identified as mitogens for NG2 cells (Frost et al., 2003; Ibarrola et al., 1996; Wilson et al., 2003; Woodruff 2004; Zhao 2005) and these factors have also been found to be up-regulated in the penumbra after cerebral ischemia (Krupinski et al., 1997; Martinez et al., 2001). Identification of such factors which induce NG2 cell proliferation might also be used to enhance proliferation artificially. There is also evidence that demyelination is a trigger for NG2 cell proliferation (Di Bello et al. 1999) while the presence of myelin inhibits NG2 cells differentiation into oligodendrocytes (Kotter at al., 2006). Tanaka et al. (2003) showed a decrease in myelination in the cortical peri-infarct region between 2 and 7 days after MCAo. Hence, the time when proliferation of NG2 cells was increased in the present study, namely 7 days after MCAo, coincides with the time window when myelination was identified to be decreased. Tanaka et al. (2003) also showed that myelination was restored in the penumbra by 14 days after MCAo. At this time NG2 cell proliferation was no longer significantly elevated in the present study.

Recent studies also suggest that increased brain activity could act as a trigger for NG2 cell proliferation (Komitova et al. 2006). The close proximity of processes of NG2 cells to both nodes of Ranvier and synapses might enable them to detect brain activity (Butt et al., 1999; Ong et al., 1999). It has been shown that a subpopulation of NG2 positive cells react to neurotransmitter stimulation by producing brain derived neurotrophic factor (Tanaka et al., 2009). This suggests that increased brain activity can stimulate brain regeneration and that NG2 cells might play a key role in this process.

The present study also showed an increase in proliferating cells of all types in the penumbra 7 days after MCAo. Three and 14 days after MCAo there was a trend towards more proliferation in the peri-infarct area compared to the contralateral side but these differences did not prove statistically significant. Similarly to the present findings, Zhang et al. (2001) observed proliferation in the ipsilateral cortex distal to the ischemic lesion to peak 7 days after MCAo but also elevated proliferation 2 and 14 days after MCAo compared to the contralateral side. Most BrdU labeled cells 7 days after MCAo are proliferating NG2 cells, which constitute between 50-61% (95% CI interval calculated according to the modified Wald method) of all BrdU positive cells at this time. The remaining BrdU cells probably constitute astocytes and

microglia/macrophages which have been demonstrated to proliferate in the penumbra after MCAo (Hoehn et al., 2005; Komitova et al., 2006). In the area corresponding to the penumbra on the uninjured contralateral side of the brain, there were neither statistically significant changes in cell proliferation over the time course after MCAo nor compared to sham stroke animals. Note that this observation cannot be generalized but is restricted solely to the area contralateral to the penumbra. With respect to other brain regions, an increase in cell proliferation has been described in the SVZ contralateral to the injury side (Jin et al., 2001). Cell proliferation was not quantified in this region in the present study.

Cell proliferation and NG2 cell proliferation were not quantified in the infarct core in the present study. Nevertheless, some general observations could be made. As early as 1 day after MCAo, most NG2 cells in the infarct core changed markedly in morphology to cells displaying very few processes. Three days after MCAo almost no cells were identified as NG2 cells. Similarly, other studies reported the number of NG2 cells to be highly reduced in the infarct core already 2 days after MCAo and reported almost no cells one week after the infarct (Tanaka et al., 2003). However, it was obvious that there were many proliferating cells in the infarct core from 3 days after MCAo onwards. Most of these cells are probably proliferating immune cells, of which some might even express NG2 (Matsumoto et al. 2008). Seven days after the insult, the brain tissue in the infarct core disintegrated and displayed high background staining. Therefore the risk of falsely considering BrdU labeled cells as NG2/BrdU co-labeled cells was considered too great when using the methods of this study, so that quantification in this area was not undertaken.

The penumbra, which was investigated in this study, is not a homogenous zone. Instead, gene expression, protein synthesis and even selective cell death varies between different areas in the penumbra (Sharp et al., 2000). Therefore it is not surprising that reactions of NG2 cells to the ischemic injury vary between different areas of the penumbra. Between 3 and 14 days after MCAo, a belt-like zone of up-regulated NG2 immuno-staining (inner penumbra) was clearly apparent adjacent to the infarct core. NG2 cells in this area displayed strong NG2 staining around the cell body. Processes were short and scarcely branched. NG2 cells situated lateral to the area of up-regulation (outer penumbra) displayed the same normal morphology as NG2 cells

on the contralateral side. These observations raise the questions about the reason for these morphological differences. Among the discussed theories are morphology as an indicator for proliferation, morphological changes as a reaction to inflammation and that NG2-labeled cells with a different morphology may actually be a different cell type.

Several authors have suggested that the morphology of NG2 cells indicates their proliferative activity (Komitova et al., 2006; Tanaka et al., 2003; Zhao et al., 2005)**.** This theory could not be confirmed by the present study. Instead, the present observations indicated that morphology depends on the location but not on degree of proliferation. Dividing NG2 cells in the inner penumbra displayed the same morphological changes as their non dividing neighbor cells. Dividing NG2 cells in the outer penumbra had long and highly branched processes just as the other NG2 cells in that area. Morphological changes persisted 14 days after MCAo, but proliferation had returned to normal levels by then. It has been shown that NG2 cells display reactive changes but do not proliferate after viral infection (Levine et al., 1998). Therefore, using morphology as an indicator for proliferation is highly questionable. Several studies have given evidence that morphological changes of NG2 cells represent reactive changes to tissue inflammation (Di Bello et al., 1999; Levine et al., 1998; Nishiyama et al., 1997) and can be induced by injury related cytokines (Rhodes et al., 2006). Given the inflammatory environment after ischemia (Barone and Feuerstein, 1999), this could explain the morphological changes of some NG2 cells.

Recent studies have given evidence that at least some of those NG2-labeled cells with a different morphology might not be NG2 cells after all but a different cell type. In 2008, a study conducted by Matsumoto and colleagues identified NG2 expressing immune cells in the infarct core after focal brain ischemia (Matsumoto et al. 2008). These cells were termed BINCs (Brain ionized calcium-binding adaptor molecule 1 (Iba1)$^+$/NG2$^+$ cells) and are thought to be blood born macrophages. They occur in addition to NG2 cells which are NG2$^+$/ Iba$^-$. BINCs were described to have an amoeboid morphology. This also fits the description of NG2 cells with so-called reactive changes as observed in the inner penumbra in the present study. Therefore it is possible that some, however not all, NG2-labeled cells in the inner penumbra are BINCs.

When this study was conducted in 2005, it was widely accepted that NG2 characterised a new type of glia cells, referred to as oligodendrocyte progenitor cells, polydendrocytes or simply NG2 cells (Dawson et al., 2003, Nishiyama et al., 2002; Polito et al., 2005). The findings from Matsumoto et al. (2008) correct this view and raise questions about the validity of the results from the present study. Even though no marker for immune cells was used in this study, there is indirect evidence that the presence of BINCs did not have a great impact on this study. One has to note that the outer penumbra accounted for most of the total evaluated area. Here, NG2-labeled cells had the clear morphology of NG2 glia cells with long and highly branched processes unlike the amoeboid morphology of BINCs. Therefore, it is most likely that NG2-labeled cells in this area are true NG2 glia cells. Obviously this raises the question in which area of the penumbra NG2 cell proliferation mainly occurred. Unfortunately, a comparison between the inner and outer penumbra could not be made from this study design. However, a statistical comparison between the numbers of BrdU/NG2 co-labeled cells between the inner counting areas (refer to methods figure 2), which included the inner penumbra, and the outer counting areas from sections from 7 days after MCAo was possible. This did not show a statistically significant difference (P>0.05, paired Student's t-test) between the areas. This indicates that the noted increase in BrdU/NG2 labeled cells on day 7 also occurs in the outer penumbral areas where NG2 clearly labels NG2 cells. With respect to the inner penumbra, morphology is not a clear indicator to distinguish between NG2 cells with reactive changes and BINCs. However, Matsumoto et al. (2008) did not observe proliferation of Iba1$^+$ or BINCs in the peri-infarct area suggesting that the proliferating NG2$^+$ cells counted in the present study are likely to be true NG2 glia cells. Nevertheless, future studies need to employ double labelling for immune cells to unequivocally clarify this issue.

6.2.   Metholodological considerations and limitations of the present study

Body temperature has been identified to interfere with brain damage with low temperatures shown to be neuroprotective and high temperatures to increase ischemic damage (Reith et al., 1996; Nagel et al., 2008). In the present study there was a statistically significant temperature rise in the 14 days group 30 minutes after stroke compared to temperatures before stroke (P< 0.05, 1 way ANOVA and Tukey's post test for individual differences) which, however, normalized

within one hour. However, it is highly unlikely that temperature interfered with the study results because in the 1, 3 and 7 days groups temperatures did not differ significantly over the first 3 hours after MCAo (P> 0.05, 1 way ANOVA and Tukey's post test for individual differences). Furthermore temperatures between the different groups did not differ significantly before or 3 hours after stroke (P> 0.05, 1 way ANOVA and Tukey's post test for individual differences).
Based on stroke ratings there is no evidence that differences in stroke severity caused the increase in proliferation at day 7. On average stroke ratings in the 7 days group were a bit lower than in the 14 days group but a bit higher than in the 1 day group.

In some animals NG2 labelling was unsatisfactory in the outer cortical area. However, as penumbral proliferation of NG2 cells on day 7 was also increased compared to the contralateral side, this demonstrates that the increase cannot be due to an accidental difference in staining quality since the staining quality was equal between the ipsilateral and contralateral side. A possible cause for these differences in staining quality might be the storage in sucrose solution or the exposure to the fixative PFA. These were the only substances which had more contact with the outer brain areas than with the inner brain areas. However, neither possibility gives satisfactory explanations. It has been shown that fixation with PFA may diminish antibody binding capacity (Torres et al. 2006). However, this does not explain differences between different brains which were all subjected to the same fixation process. With regards to the sucrose solution, storage time was more variable. Unfortunately, the exact time was not monitored so that it cannot be analyzed if there was a correlation between staining quality and storage duration.

During the initial trials for this experiment, brain tissue was first exposed to a formamide and sodium-chloride-citrate solution at 65°C in order to expose the BrdU to the antibodies. Using this method, however, NG2 immuno-labeling was of poor intensity. It was realized that the NG2 staining tolerated exposure to HCL at 25°C, but no heating to 65°C. This observation can be explained by the nature of the NG2 molecule. It has been shown that the NG2 molecule contains a proteolytic cleavage side which leads to the formation of a extracellular fragment which remains associated with the transmembrane fragment by a strong non-covalent binding

(Nishiyama et al., 1995). This binding, however, gets destroyed by heating to 65°C but not by exposure to HCl (Nishiyama et al., 1995). It can therefore be assumed that the treatment with formamide at 65°C destroyed the non-covalent binding so that the extracellular NG2 fragment diffused into the surrounding tissue and was either not recognised by the antibody or caused a high background staining. In concordance with the study from Nishiyama et al. (1995) treatment with HCl at 25°C, as chosen for this study, allowed successful NG2 and BrdU labelling. However, one also has to note that even with this method background staining varied between animals and between antibody batches. It can be assumed that the existence of a cleavage site makes NG2 a fragile and sometimes difficult to stain molecule.

Limitations of the present study are firstly the relatively small number of rats in each group. Furthermore the variation between brains regarding NG2 immuno-labelling quality and/or stroke size increased the variation between cell count results making it difficult for small differences in cell numbers to achieve statistical significance. No information was gathered on the time dynamics between 7 and 14 days after MCAo, so that it cannot be known whether the peak of proliferation occurs exactly 7 days after MCAo. Since no direct labelling of myelination and oligodendrocytes was carried out in the present study, correlations between myelination and proliferation could not be directly demonstrated. Tanaka et al. (2003) indirectly demonstrated that increasing numbers of NG2 labeled cells correlate with restoration of oligodendrocytes in the cortical penumbra. However, after mechanical injury it was shown that cortical NG2 cells, unlike white matter NG2 cells, do not produce new oligodendrocytes (Dimou et al., 2008). Therefore, further research is needed to clarify this issue in cortical damage after stroke. The most important limitation, however, is the recently identified questionable specificity of NG2 as a marker only for NG2 glia cells (Matsumoto et al., 2008). Therefore, future studies should employ double labelling of cells with anti-NG2 and immune cells markers to exclude this confounding factor.

6.3.   Importance of the present study results and impact on future study design

Stroke has often lasting devastating effects on individuals. So far the central nervous system has been known for its limited repair capacity, so that there has been little hope that damage could get reversed. However, the discovery of stem cells in the brain, such as NG2 cells which could give rise to new oligodendrocytes, has raised hope that it might be possible to induce these cells to repair the injured tissue.

The present study was designed to quantify NG2 cell proliferation in the ET-1 model of MCAo over a two week period. It is the first study to have directly demonstrated that NG2 cells in the cortical penumbra increase proliferation after MCAo and that this proliferation peaks 7 days after MCAo. For future research this implies, that the expedient time for the evaluation of NG2 labeled cells after focal ischemia has to be chosen carefully. Influences on the proliferation of NG2-labeled cells should be evaluated at 7 days after MCAo when proliferation of NG2-labeled cells occurs at a high rate. Influences on total numbers of NG2 labeled cells should be evaluated 2 week after MCAo, when the peak of NG2 cell proliferation is over.

Since there is substantial evidence that oligodendrocytes regenerate from NG2 cells (Dawson et al., 2000; Levine et al., 2001; Polito and Reynolds, 2005; Sozmen et al., 2009; Tanaka et al., 2003; Watanabe 2004), the observation of proliferating NG2 cells following injury raises hope that oligodendrocytes can be restored after ischemia. Despite the low migration ability of NG2 cells, short migration distances from the area around a lesion into the lesion have been reported (Franklin et al., 1997; Watanabe at al., 2002). This raises hope that the elevated numbers of NG2 cells in the penumbra might not only help to repair injured tissue in the peri-infarct area but also in the infarct core. Hence, proliferation of NG2 cells might prove an important intrinsic repair mechanism of the post ischemic brain. On the one hand because of the possibility to restore Oligodendrocytes and on the other hand because of increasing evidence that these cells serve other important functions such as in the brain's signalling system (Nishiyama et al., 2009). Studies with growth factors have shown proof of principle that NG2 cell proliferation can be increased (Frost et al., 2003; Ibarrola et al., 1996; Wilson et al., 2003) and therefore it might also be possible to develop neuroprotective agents which can enhance proliferation of NG2 cells.

Many early clinical trials failed due to the late recruitment of patients (often 1 to 2 days following stroke) and administration of drugs for which the targeted deleterious process was already completed (Dirnagl et al., 1999). In this respect, the results from this study identify proliferation of NG2 cells as a promising target for stroke therapy since proliferation occurs with a delay of 1 week after ischemia so that even late intervention could influence NG2 cell proliferation and hence restoration of oligodendrocytes.

7.   Summary

Stroke is one of the leading causes of death and disability in Germany. The underlying cerebral ischemia damages neurons and glial cells. The identification of stem and progenitor cells in the brain raises hope that these cells could potentially be stimulated to repair brain damage. Regeneration of white matter can occur through NG2 cells, which can differentiate into myelin producing oligodendrocytes. The aim of the present study was to study proliferation of NG2 cells after cerebral ischemia.

Male adult Hooded Wistar rats were subjected to Endothelin-1 induced middle cerebral artery occlusion (MCAo) (n=15) or sham operation (n=6). Rats were given BrdU injections over 24 hours prior to being sacrificed at 1, 3, 7 and 14 days after MCAo or sham stroke. Brain sections were double labeled with anti-NG2 antibodies, a marker for NG2 cells, and anti-BrdU antibodies to identify newly proliferating cells. Double labeled NG2/BrdU cells and total BrdU labeled cells were quantified in the penumbra and in the corresponding region of the contralateral hemisphere.

The statistical analysis revealed a significant increase of NG2/BrdU co-labeled cells in the penumbra 7 days after MCAo compared with the contralateral side ($p<0.001$) and compared with 1, 3 and 14 days after MCAo ($p<0.001$). There were no statistically significant differences in the number of NG2/BrdU double-labeled cells between sections from the other time points. Neither were there statistically significant differences in NG2/BrdU co-labeled cells between the ipsilateral and the contralateral hemisphere at the other time points. On the contralateral side differences between NG2/BrdU co-labeled cells or BrdU labeled cells did not prove statistically significant between sections from different time points. The same results applied for total BrdU labeled cells.

The present study showed increased proliferation of NG2 cells in the penumbra 7 days after MCAo. Enhancement of proliferation of NG2 cells could lead to increased formation of oligodendrocytes and to regeneration of the myelin sheath. The observation that proliferation of NG2 cells peaks with a delay of 1 week after ischemic injury identifies this repair mechanism as a valuable target for late onset stroke therapies.

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