Aus der Abteilung für Molekulare Neurobiologie Labor
Leiter: Prof. Dr. Aurel Popa-Wagner
der Klinik und Poliklinik für Neurologie
Direktor: Prof. Dr. Christof Kessler
der Medizinischen Facultät der Ernst-Moritz-Arndt-Universität
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Ana-Maria Buga
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Dekan: Prof. Dr. H. Kroemer

1. Gutachter: Prof. Dr. Aurel Popa-Wagner

2. Gutachter: Prof. Dr. W. Schaebitz

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Zusammenfassung

Alte Ratten erholen sich schlecht nach einem einseitigen Schlaganfall, wohingegen junge Ratten eine bessere Erholung, möglicherweise mit Hilfe der contralateralen, gesunden Hemisphäre zeigen. Wir fragten uns, ob von der Regel abweichende, alters abhängige Veränderungen der transkriptionalen Aktivität im Gehirn alter Ratten als ein entscheidender Faktor der reduzierten funktionalen Erholung zu Grunde liegen könnten. Es wurden die Periinfarktregion und die dieser entsprechende contralaterale Region von 3 und 18 Monate alten Sprague Dawley Ratten analysiert. Unsere experimentalen Endpunkte waren cDNA Arrays, die Gene beinhalteten, die die Hypoxie-Signalgebung, den DNA-Verlust und Apoptose, die zelluläre Antwort auf die Schädigung, die axonale Schädigung und Regeneration, Dendritogenese und Neurogenese abbilden. In erster Linie konnten folgende transkriptionalen Ereignisse gefunden werden: (i) frühe Hochregulierung von DNA-Verlust und Down-Regulierung von anti-apoptotischer Gene in der Perininfarktregion alter Ratten nach dem Schlaganfall: (ii) beeinträchtigte Neurogenese, v. a. bei alten Ratten; (iii) beeinträchtigte Neurogenese im Bereich der nicht geschädigten contralateralen Hemisphäre bei alten und jungen Ratten zu jedem Zeitpunkt nach dem Schlaganfall; (iv) bei alten Ratten merkliche Hochregulation von mit Inflammation und Vernarbung assoziierter Gene. Diese Ergebnisse wurden mit quantitativer real-time PCR bestätigt. **Schlussfolgerung I:** die reduzierte transkriptionale Aktivität in der gesunden, contralateralen Hemisphäre in Verbindung mit der frühen Hochregulation von zum DNA-Verlust führender Gene und pro-apoptotischen Genen sowie der Herunterregulation von Axono- und Neurogenese in der Periinfarktregion tragen wahrscheinlich zur reduzierten Neurorehabilitation nach Schlaganfall bei alten Ratten bei. Eine effiziente Neuroprotektion nach Schlaganfall erfordert eine langfristige kontrollierte Senkung der Körpertemperatur. Die Behandlung alter Ratten nach Schlaganfall mit Hydrogensulfid führte zu einer andauernden, tiefen Hypothermie (30,8 ± 0,7º), was eine Reduktion der Infarktgröße um 50% mit gleichzeitiger Reduktion der Phagozytenzahl zur Folge hatte. Auf Transkriptionsebene fanden wir eine Gesamtzunahme der transkriptionalen Aktivität bezogen auf Inflammation und Apoptose. Bezüglich der Verhaltenstestung zeigten die mit Hypothermie behandelten alten Ratten bessere Ergebnisse in Tests, die sensomotorische Fähigkeiten erfordern, bei Ausbleiben evidenter neurologischer Defizite oder oder physiologischer Nebenwirkungen durch diese Therapie. **Schlußfolgerung II:** Prolongierte Hypothermie ist eine einfache und effiziente Methode das durch Schlaganfall verschuldete Defizit bei alten Ratten zu limitieren.

Summary

Aged rats recover poorly after unilateral stroke, whereas young rats recover readily possibly with the help from the contralateral, healthy hemisphere. We asked whether anomalous, age-related changes in the transcriptional activity in the brains of aged rats could be one underlying factor contributing to reduced functional recovery. We analysed gene expression in the periinfarct and contralateral areas of 3-month- and 18-month-old Sprague Dawley rats. Our experimental end-points were cDNA arrays containing genes related to hypoxia signalling, DNA damage and apoptosis, cellular response to injury, axonal damage and regrowth, cell lineage differentiation, dendritogenesis and neurogenesis. The major transcriptional events observed were: (i) Early up-regulation of DNA damage and down-regulation of anti-apoptosis-related genes in the periinfarct region of aged rats after stroke; (ii) Impaired neurogenesis in the periinfarct area, especially in aged rats; (iii) Impaired neurogenesis in the contralateral (unlesioned) hemisphere of both young and aged rats at all times after stroke and (iv) Marked up-regulation, in aged rats, of genes associated with inflammation and scar formation. These results were confirmed with quantitative real-time PCR. **Conclusion I**: reduced transcriptional activity in the healthy, contralateral hemisphere of aged rats in conjunction with an early up-regulation of DNA damage-related genes and pro-apoptotic genes and down-regulation of axono- and neurogenesis in the periinfarct area are likely to account for poor neurorehabilitation after stroke in old rats.

Efficient neuroprotection after stroke requires long-term, regulated lowering of whole body temperature. After stroke, exposure of aged rats to hydrogen sulfide resulted in sustained, deep hypothermia (30.8 ± 0.7°C) that led to a 50% reduction in infarct size with a concomitant reduction in the number of phagocytic cells. At the transcription level, we found an overall decrease in the transcriptional activity related to inflammation and apoptosis. Behaviorally, hypothermia was associated with better performance on tests that require complex sensorimotor skills, in the absence of obvious neurological deficits or physiological side-effects, in aged rats. **Conclusion II**: Prolonged hypothermia is a simple and efficacious method to limit damage inflicted by stroke in aged rats.

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The genomic response of the ipsilateral and contralateral cortex to stroke in aged rats

A.-M. Buga [a, b], M. Sascau [a], C. Pisoschi [b], J. G. Herndon [c], C. Kessler [a], A. Popa-Wagner [a, b, *]

[a] Molecular Neurobiology Laboratory, Clinic of Neurology, University of Greifswald, Germany
[b] University of Medicine and Pharmacy, Craiova, Romania
[c] Yerkes National Primate Research Center, Neuroscience Division, Emory University, Atlanta, GA, USA

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Abstract

Aged rats recover poorly after unilateral stroke, whereas young rats recover readily possibly with the help from the contralateral, healthy hemisphere. In this study we asked whether anomalous, age-related changes in the transcriptional activity in the brains of aged rats could be one underlying factor contributing to reduced functional recovery. We analysed gene expression in the periinfarct and contralateral areas of 3-month- and 18-month-old Sprague Dawley rats. Our experimental end-points were cDNA arrays containing genes related to hypoxia signalling, DNA damage and apoptosis, cellular response to injury, axonal damage and regrowth, cell lineage differentiation, dendritogenesis and neurogenesis. The major transcriptional events observed were: (*i*) Early up-regulation of DNA damage and down-regulation of anti-apoptosis-related genes in the periinfarct region of aged rats after stroke; (*ii*) Impaired neurogenesis in the periinfarct area, especially in aged rats; (*iii*) Impaired neurogenesis in the contralateral (unlesioned) hemisphere of both young and aged rats at all times after stroke and (*iv*) Marked up-regulation, in aged rats, of genes associated with inflammation and scar formation. These results were confirmed with quantitative real-time PCR. We conclude that reduced transcriptional activity in the healthy, contralateral hemisphere of aged rats in conjunction with an early up-regulation of DNA damage-related genes and pro-apoptotic genes and down-regulation of axono- and neurogenesis in the periinfarct area are likely to account for poor neurorehabilitation after stroke in old rats.

Keywords: rat • aging • stroke • gene expression

Introduction

Studies of stroke in experimental animals have identified a variety of interventions with marked neuroprotective effects, but most of these approaches have failed to show benefit in aged human stroke victims. One possible explanation for this discrepancy between laboratory and clinical investigations is the role that age plays in the recovery of the brain from insult.

Although it is well known that aging is a major risk factor for stroke [1] the majority of experimental studies of stroke have been performed on young animals, and therefore may not fully replicate the effects of ischaemia on aged neural tissue. Therefore, studies of experimental stroke in the aged animal are likely to

Q1 *Correspondence to: Aurel POPA-WAGNER, Ph.D.,
Department of Neurology
University of Greifswald
Ellernholzstr. 1-2
17487 Greifswald, Germany.
Fax: +49-38 34-86 68 43
E-mail: wagnerap@uni-greifswald.de

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have more clinical relevance, both for understanding cellular responses to stroke and for identification of beneficial interventions [2–5].

Young rats recover much better than aged rats within 2 weeks from stroke [4] and it has been proposed that an increased tissue repair capacity of young rats may underlie the rapid recovery from ischaemic damage. However, axonal growth does not occur until the second week after stroke [5, 6], calling into question the role of brain plasticity mechanisms in supporting tissue repair and functional recovery in the first few days after stroke. An alternative explanation is that more robust activation of the contralateral hemisphere explains the age-related difference in recovery [7–10].

DNA array technology may provide insight into the mechanisms underlying differences between old and young animals in rate and extent of brain repair and regeneration after stroke. Studies of focal cerebral ischaemia have identified a series of key molecular events and a number of changes in gene regulation following infarct [11–16]. These studies revealed changes in transcriptional activity of a variety of genes related to stress response, inflammation, acute- and delayed cell death. However, these studies have not examined changes in gene expression both in the ipsilateral and contralateral hemisphere to the infarct, and thus do not address the possibility that age related differences in recovery are related to contralateral genetic mechanisms.

In the present study we addressed this question by using defined cDNA arrays. These arrays included genes related to cell injury and survival, cell growth, the hypoxia signalling pathway (including DNA damage), inflammatory-related processes and apoptosis. By comparing gene expression changes in young *versus* aged rats, our goal was to identify post-infarct changes in gene expression following middle cerebral artery occlusion (MCAO) in aged rats that may give us clues as to transcriptional events underlying reduced functional recovery after stroke in aged rats.

Materials and methods

Animals

Eighteen hours prior to surgery, male 3-month-old (young) and 18-month-old (aged) Sprague-Dawley rats were fasted but allowed free access to water to minimize variability in ischaemic damage that can result from varying plasma glucose levels. MCAO was perfomed as described below. Survival times for the study were: 3 days (n = 8 young; n = 7 aged rats) and 14 days (n = 10 for young; n = 10 for the aged rats).

All experiments were approved by the University Animal Experimentation Ethics Board as meeting the ethical requirements of the German National Act on the Use of Experimental Animals.

Reversible occlusion of the middle cerebral artery

Blood flow through the middle cerebral artery was transiently interrupted in deeply anaesthetized rats as previously described [2, 4]. The right middle cerebral artery was slowly lifted with a tungsten hook attached to a micromanipulator until blood flow through the vessel was completely stopped. Both common carotid arteries were then occluded by tightening pre-positioned thread loops. The sharply decreased blood flow was monitored with a Laser Doppler (Periflux 5000, Perimed, Sweden) by positioning the optic tube on the temporal bone of rat skull. After 90 min., the middle cerebral artery and the common carotid arteries were re-opened, allowing full reperfusion of the brain. It should be noted that occlusion of the carotid arteries alone for 90 min. has no noticeable hypoxic effect on the brain. With this model, the infarct is located mostly in the sensorimotor cortex and only rarely in the striatum. If striatal infarction did occur, these animals were excluded from this study.

Following survival times of 3 and 14 days, the rats were deeply anaesthetized and perfused with buffered saline followed by buffered, 4% freshly depolymerized paraformaldehyde. The brain was removed, post-fixed in 4% buffered paraformaldehyde for 24 hrs, cryoprotected in 20% sucrose prepared in 10 mmol/l phosphate buffered saline, flash-frozen in isopentane and stored at (70°C until sectioning.

For real time PCR, seven additional brains per age group and time-point were perfused with buffered saline, cut into 2 mm slices that were dipped in TTC to allow visualization of the infarct core. This procedure allowed us to microdissect the periinfarcted area of cortex and the corresponding cortex area of contralateral healthy hemisphere that were then stored at –70°C until use.

RNA isolation

Total RNA was isolated from the microdissected tissue using TRIzol reagent (Invitrogen life technologies,

Germany) as described by the manufacturer, followed by DNase 1 (Ambion) digestion and further purified using RNeasy Mini extraction kit (Qiagen, Hilden, Germany). To avoid RNA degradation because of secondary hypoxia due to the microdissection procedure, we flash-froze the tissue immediately after microdissection. Purified total RNA was used for cDNA array assay and real time PCR quantification.

cDNA array assay

To analyse gene regulation, we employed commercially available defined oligo microarrays containing known rat genes arranged in three categories: stem cells, hypoxia signalling pathway and apoptosis cDNA microarrays. These arrays contain 258, 96 and 96 known genes respectively (SuperArray, Bethesda, MD) and were processed according to the manufacturer's instructions. In addition, each individual array contains four housekeeping genes and one negative control. Housekeeping genes were included to confirm the integrity of RNA and correct loading of different samples. The list of genes can be found at http://www.superarray.com/ArrayList.php

Briefly, 3 μg of total RNA was annealed with a random primer at 70°C for 3 min., reverse transcribed at 37°C for 25 min. and amplified by linear polymerase reaction (LPR kit, SuperArray) with gene-specific primers in the presence of Biotin-UTP to produce labelled cRNA. The PCR program was 85°C for 5 min.; 30 cycles (85°C, 1 min.; 50°C, 1 min.; 72°C, 1 min.); 72°C for 5 min. After 1 hr pre-hybridization, membranes were hybridized with denatured biotin-labelled cRNA overnight, washed, incubated with streptavidin-alkaline phosphatase conjugate and exposed to X-ray film. The scanned images were processed using web-based GEArray Expression Analysis Suite software (Super Array), data were extracted and gene expression profiles were analysed.

After normalization to housekeeping genes, two sets of data for each age group, given as fold change were generated: that is we compared ipsilateral (periinfarct, pi) *versus* ipsilateral sham controls (ctrl) and contralateral (cl) *versus* contralateral sham controls. Finally, the results are given as the mean of two experiments. Only those genes whose expression is equal to or more than 1.5-fold change were considered as differentially regulated. For down-regulated genes, the threshold was set to 0.5.

Real time quantitative PCR

For real-time PCR, 2 μg of total RNA was reverse-transcribed using random hexamers and the reverse transcription reagents (Superarray, Bethesda, MD). PCR reaction was set up by mixing 10 ng of cDNA, rat primers (Superarray), Master mix (Superarray) and SYBR Green I (Molecular Probes). Real-time PCR amplification was performed as follows: one cycle of 15 min. at 95°C and 45 cycles in three steps each (95°C for 30 sec., 55°C for 30 sec. and 72°C for 30 sec.) using a real-time PCR cycler (SDS 7700, Applied Biosystems). At the end of amplification cycles, primer specificity was checked by appearance of single bands of PCR products in a 1% agarose gel. A standard curve was generated by plotting the log10 [target dilution] of template on the X-axis against the Ct value from serial dilutions of target DNA on the Y-axis.

The relative expression level of genes of interest and housekeeping gene was determined based on the standard curve equation generated for each individual gene. To normalize, the expression level of gene of interest was divided by the expression level of the housekeeping gene from the same sample. Finally, the fold change of an individual gene was calculated by dividing the normalized gene expression level of this gene from experimental sample by the normalized gene expression level of this gene from sham control both for the ipsilateral and the contralateral sides. That is, fold change= ([gene of interest/housekeeping gene]$_{experiment}$]/ [gene of interest / housekeeping gene]$_{sham}$). In addition, we compared baseline gene expression by calculating the ratio of contralateral sham young *versus* contralateral old rats. The sequences of primers used for RT-PCR are given in Table 1s (supplemental data).

Results

Basal transcriptional activities in the cortex of sham-operated young and aged animals

Since baseline differences between gene expression in young and old control rats might affect levels found after infarction, we first summarize the principal findings in control animals.

We studied a total of 442 genes representing growth factors, growth and differentiation-related transcription factors (258 genes), hypoxia signalling pathway (96 genes) and apoptosis arrays (96 genes).

In control animals, the levels of the apoptotic gene Casp7 are increased in the sensorimotor cortex of aged rats (Table 1). Since Casp7 is implicated in the terminal stages of apoptosis, this result suggests that apoptosis is increased in the brains of aged rats. A similar observation has been made in the cortices of aged Fischer 344 rats [17, 18].

Table 1 Ratio (± SD) of baseline gene expression levels in old *versus* young sham control rats

Gene	Category	Ratio (old *versus* young)
Casp 7	Terminal phase of apoptosis	2.08 ± 0.13
Cat	Antioxidant, ROS scavenging	0.42 ± 0.01
Sod2	Antioxidant, ROS scavenging	0.51 ± 0.02
Fabp7	Fatty acid-binding protein 7; lipid metabolism.	2.09 ± 0.28
		0.43 ± 0.05
Igf1r	Insulin-like growth factor receptor	0.27 ± 0.01
Cdh5	Vascular endothelial cadherin	1.93 ± 0.08
Icam5	Intercellular adhesion molecule 5	1.82 ± 0.07
Gjb1		0.36 ± 0.04
Nkx2.2	Transcription factor; oligodendrocytes	0.39 ± 0.04

The calculated ratio is based on RT-PCR results and is given as mean ± SD ($n = 7$).
Statistical significance level was set at $P < 0.05$.

Changes in the mRNAs levels for two major enzymes responsible for reactive oxygen species (ROS) scavenging, catalase (CAT) and superoxide dismutase (SOD) were significantly decreased in the brains of aged rats (Table 1), which is indicative of a reduced capacity to remove radicals from the aging brain.

Fatty acid-binding protein 7 mRNA, which is up-regulated following injury to the axons, [19] was also increased in the aged rat brain (Table 1) suggesting damaged myelin sheets in aged rats [20].

Differentially regulated genes in the post-ischaemic rat brain

We found sixty-one genes (13.8%) that were differentially regulated in the post-ischaemic rat brain (Fig. 1). Of these, 28 genes (6.1% of the total number of genes) were up-regulated. Within the up-regulated genes, 11 genes were increased in both age groups, while nine were up-regulated only in the young rats and eight only in the old. Twenty genes representing 4.5% of the regulated genes were down-regulated. Thirteen genes showed both up- and down-regulation in the two age groups (Fig. 1).

The cumulative number of genes that were up- or down-regulated 1.5-fold for each time-point is shown for the ipsilateral (periinfarct, pi) sensorimotor cortex in Fig. 2A and the contralateral (cl) sensorimotor cortex in Fig. 2B. From these data, it can be inferred that at day 3 after stroke the major age-specific transcriptional effects in the periinfarcted area were (*i*) differential regulation (both up- and down-regulation) of apoptotic genes and (*ii*) a 50% decrease in the number of regulated stem cell-related genes (Fig. 2A). At day 3 after ischaemia, the contralateral, healthy hemisphere of young rats is much more active, at transcriptional level, than that of the aged rats, especially at the level of stem cell-coding genes. At this time-point, age-specific transcriptional events were (*i*) absence of regulation of hypoxia signalling-related genes and (*ii*) down-regulation of apoptosis-related genes (Fig. 2B). At day 14 after stroke we noted a persistent down-regulation of stem cell-related genes in the contralateral (healthy) sensorimotor cortex of aged rats (Fig. 2B).

Up-regulated genes in the ipsilateral sensorimotor cortex of young rats

Temporary occlusion of the middle cerebral artery caused in the first week after stroke the up-regulation of several hypoxia-related genes in the perilesional cortex of young rats (Table 2). Among them we noted vigorous increases in glutathione peroxidase (Gpx1), a gene necessary for antioxidative defence [21]. Genes that were moderately up-regulated in response to energy deprivation included the

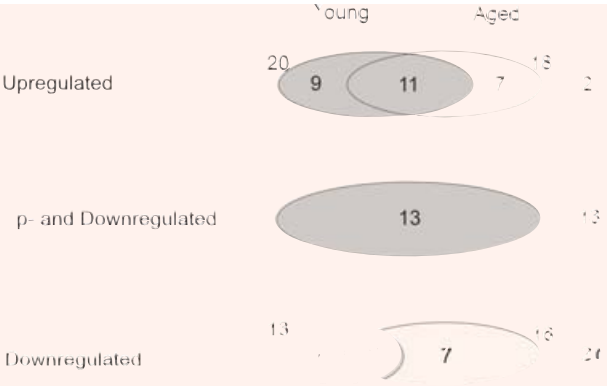

Fig. 1 Diagram shows the number of up- and down-regulated genes in young and aged rat brain. The genes showing both up- and down-regulation during their time course are included in a separate circle.

uncoupling protein 2 (Ucp2), a gene with a key role in antioxidative defence by regulating mitochondrial gluthatione availability [22]. The extent of neuroprotection was also moderately helped by the up-regulation of cystatin B (cstb), a non-caspase cysteine protease inhibitor shown to protect neurons against apoptotic death [23]. In the second week after stroke, the expression of Gpx1 and Ucp2 genes remained at high levels. In addition, the antioxidative capacity was further increased by up-regulation of gluthathione peroxidase 1, CAT and Sssca1 (a gene coding for Sjogren's syndrome/scleroderma autoantigen) [24].

Another gene change that may serve to promote tissue recovery is the up-regulation of transforming growth factor β_1 (TGF-β_1) in the perilesional area of young rats [25]. Tgfb1 is a pleiotropic cytokine with potent neurotrophic and immunosuppressive properties that is up-regulated after injury [26].

Genes related to apoptosis were not up-regulated at day 3 (Table 3). By day 14, however, the number of genes involved in apoptosis like ataxia telangiectasia-mutated homologue, Atm [27] and growth arrest and DNA damage-inducible 45α, (Gadd45a) [28] had increased in young rats.

In the first week after stroke, major transcriptional events also included up-regulation of genes related to neuronal and oligodendrocyte survival, neuronal injury, axonal myelination and neurogenesis. Those changes related to survival include up-regulation of fibroblast growth factor 22 (Fgf22) [29], nerve growth factor β (Ngfb) [30] and insulin-like growth factor receptor (Igfr1) [31]. Related to neuronal injury is also fatty acid-binding protein 7 (Fabp7) [19]. Changes related to myelination include oligodendrocyte transcription factor 1 (Oligo1)[31], NK2 transcription factor related locus 2 Drosophila (Nkx2-2_predicted) [32] and gap junction membrane channel protein β 1(Gjb1). Two up-regulated factors related to neurogenesis are Frizzled homologue 8 (Fzd8) [33] and Gata-binding protein (Gata2) [34] (Table 4).

We also noted high levels of mRNA coding for procollagen type I, α (Col1a1), a gene associated with vascular remodelling [35] by providing a substrate for neurite outgrowth, respectively [36]. Likewise, the matrix metalloproteinase 14 gene (Mmp14), which is critical to creating a permissive growth environment for neurites [36], was moderately increased in the infarcted area (Table 2).

In the second week following stroke, several genes returned to control values (Fgf22, Fzd8, Gata2 and Igfr1) while several other new genes were up-regulated. These include cystatin C (Cst3), a gene that has been linked to glial development [37] and glial cells missing homologue (Gcm2), a transcription factor that is involved in specification and differentiation of certain neuronal and glial lineages [38, 39]. Also up-regulated in the second week were the neuronal survival factors like insulin-like growth factor 2 (Igf2) [40] and inhibin β B (Inhbb) [41] (Table 4).

Finally, the gene coding for cadherin 5 (Cdh5), was increased between day 3 and day 14 after stroke. Since Cdh5 has cell-adhesion activity and is specifically expressed in vascular endothelial cells, this finding suggests that the blood-brain barrier of aged rats is still compromised (Table 4).

Dow-regulated genes in the ipsilateral sensorimotor cortex of young rats

In the first week after stroke, major transcriptional events included down-regulation of genes involved in neurogenesis, axonal growth and maturation, dendritic injury, axonal migration and growth, and in cell adhesion. For example, neural cell precursor proliferation sonic hedgehog homologue (Shh) [42–44] was down-regulated. Genes involved in axonal growth and maturation that were also down-regulated include neurofilament light chain (Nefl) [45, 46], chromogranin A [47] and growth-associated protein GAP-43 [48]. Bone morphogenetic protein receptor

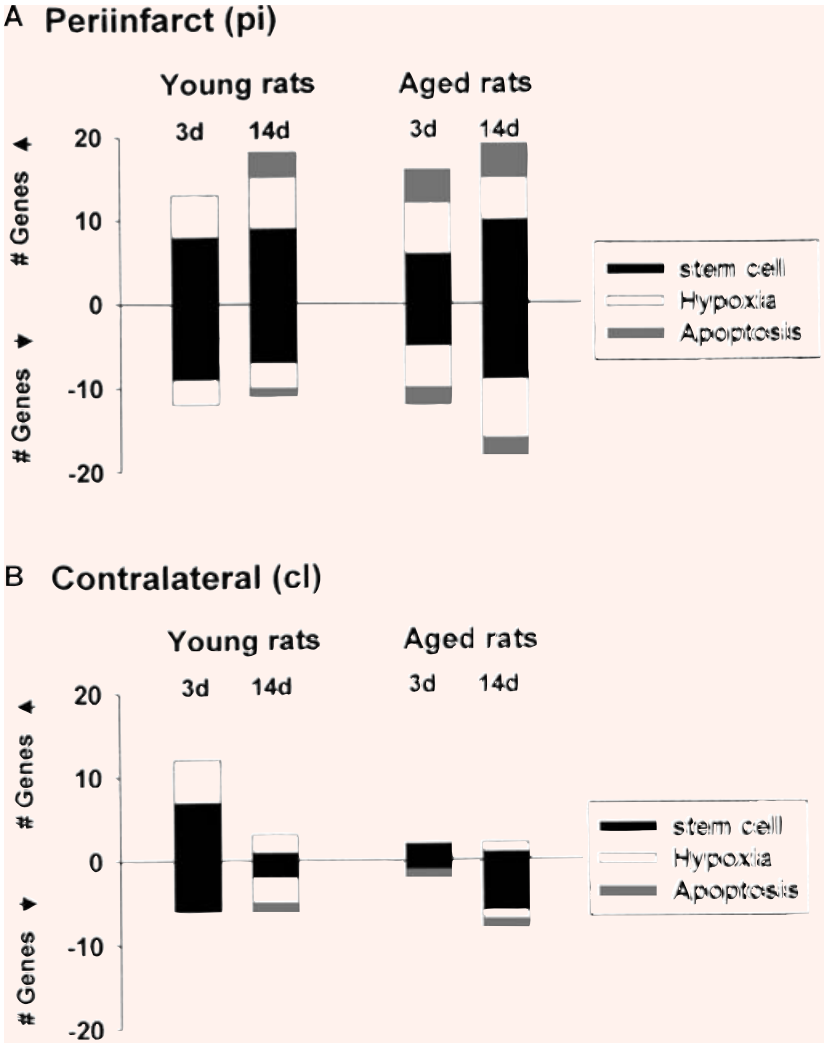

Fig. 2 Time course of changes in the numbers of up- and down-regulated genes in post ischaemic brain. The numbers of genes up- and down-regulated more than 1.5-fold in the periinfarct (**A**) or contralateral area (**B**) at two time-points of young or aged rats were presented in the form of bar graphs. Stem cell, hypoxia signalling pathway and apoptosis-related genes are presented as black, empty and grey boxes, respectively.

type 2 (Bmpr2) [49], which is involved in dendritic injury and dendritogenesis also showed decreased activity. Also down-regulated were α and δ catenins (Ctnna2 and Ctnnd2)[50], necessary for axonal migration, intercellular adhesion molecule 5 (telencephalin) [51] and integrin β 5, a vitronectin receptor [52] (Table 4).

In the second week after stroke, down-regulated genes included fibroblasts growth factor receptor 1 (Fgfr1), a gene implicated in axonal growth [53], and basic helix-loop-helix domain containing class B2 (Bhlhb2), a gene that may play a role in regulating neuronal differentiation during development and adaptive neuronal plasticity and neurite outgrowth in the adult [54] (Table 4). Glucose phosphate isomerase (Gpi) was also decreased. Gpi is a housekeeping gene encoding for phosphoglucose isomerase that has been found to be identical to neuroleukin, which has neurotrophic and lymphokine properties [55]. Neuropeptide Y (Npy) was also decreased at this time-point. Npy is a gene involved in regulation of energy balance (Table 2), and which forms part of the 'lipostat' system along with leptin and corticotropin-releasing hormone.

In addition, genes implicated in axonal growth/migration like Shh [43] and catenin α 2 and catenin δ 2 remained at low levels at all time-points studied. Finally, genes important for synaptic transmission such as myosin heavy polypeptide 6 (Myh6) [56] was down-regulated at all time-points studied (Table 4).

Table 2 Hypoxia signalling pathway-related gene expression profiles

Gene name	Genbank Accession no.	Description	Fold change							
			3-months-old rat				18-months-old rat			
			Day 3		Day 14		Day 3		Day 14	
			pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl
Hypoxia related gene										
Col1a1	NM_007742		3.69		7.02		4.44		17.18	
cstb	NM_007793	Cystatin .B	1.52	1.52			1.56			
Gpx1*	NM_008160	Glutathione peroxidase 1	5.38		5.18		3.07		3.01	
Mmp14	NM_008608	Matrix metallopeptidase 14 (membrane-inserted)	1.55							
Ucp2*	NM_011671	Uncoupling protein 2 (mitochondrial, proton carrier)	2.20	2.20	3.86		3.86		4.39	
Rps2	NM_008503	Ribosomal protein S2		2.43		0.65	2.47		1.93	
Sod2*	NM_013671	Superoxide dismutase 2, mitochondrial		1.63			0.66		0.38	0.63
Cat*	NM_009804	Catalase			2.39					
Sssca1	NM_020491	Sjogren's syndrome/scleroderma Autoantigen 1 homologue (human)			2.12					
Tgfb1	NM_011577	Transforming growth factor, β 1			2.33	2.33				
Pea15	NM_011063	Phosphoprotein enriched in astrocytes 15				1.57				
IL6	NM_031168	Interleukin 6					2.13			
Prpf40a	NM_018785	PRP40 pre-mRNA processing factor 40 homologue A (yeast)							2.25	2.65
Chga*	NM_007693	Chromogranin A	0.44				0.39		0.33	
Gap43*	NM_008083	Growth associated protein 43	0.65						0.61	
Vegfa*	NM_009505	Vascular endothelial growth factor A	0.64	1.50			0.56			
Bhlhb2	NM_011498	Basic helix-loop-helix domain containing, class B2			0.23					
Gpi1	NM_008155	Glucose phosphate isomerase 1			0.57	0.61	0.54		0.42	

Continued

Table 2 Continued

Gene name	Genbank Accession no.	Description	Fold change							
			3-months-old rat				18-months-old rat			
			Day 3		Day 14		Day 3		Day 14	
			pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl
Npy	NM_023456				0,30		0.50		0.39	
Camk2g	NM_178597	Calcium/calmodulin-dependent protein kinase II gamma				0.41				
Plod3	NM_011962	Pro-collagen-lysine, 2-oxoglutarate 5-dioxygenase 3							0.41	
Tuba1*	NM_011653								0.59	

Fold change is calculated as the ratio of experiment to sham control after they were normalized to housekeeping genes obtained from two independent experiments. Genes whose average fold induction was equal to or higher than 1.5 and equal or lower than 0.66 were selected. Genes were listed according to up or down-regulation of genes and alphabetic order. *Represents genes were not previously reported in the after stroke rat brain. Genes which have been validated by RT-PCR were marked by colour.
pi, periinfarct, lesional hemisphere; cl, contralateral, healthy hemisphere; ctrl, sham control.

Young rats, contralateral sensorimotor (undamaged) cortex

In young rats 3 days after stroke, 12 genes (2.7% of the total) were up-regulated and six (1.3%) were down-regulated within the contralateral sensorimotor cortex. By day 14, the number of up-regulated genes decreased from 12 to three (0.7%), while the number of down-regulated genes increased (Fig. 2B).

Among the genes up-regulated at 3 days were several related to oligodendrocytes regeneration, remyelination and communication, including Oligo1, NK2 transcription factor related locus 2 Drosophila (Nkx2-2_predicted) and gap junction membrane channel protein β 1(Gjb1; see Table 4). In the second week, a gene coding for a phosphoprotein enriched in astrocytes (PEA-15) was up-regulated. PEA-15 is a 15 kD acidic serine-phosphorylated protein which is expressed in different cell types, especially in the central nervous system (Table 2). Recent data indicates that PEA-15 has an anti-apoptotic role, presumably within astrocytes [57].

Genes down-regulated in the first week in the contralateral hemisphere of the young rats included intercellular adhesion molecule5 (telencephalin) (Icam5) and Nefl. The first of these is involved in neuritic outgrowth [58] while the second is required for axonal maturation [59]. Myh6 [56], a gene important for synaptic transmission, was also decreased in this region in the first week (Table 4).

In the second week after stroke, calcium- / calmodulin-dependent protein kinase II χ (Camk2g) and Gpi were both down-regulated. Camk2g is important for dendritogenesis [60], and the Gpi-coding product has neurotrophic properties [55] (Table 2).

Up-regulated genes in the ipsilateral sensorimotor cortex of aged rats

Temporary occlusion of the middle cerebral artery caused the up-regulation of several hypoxia-related genes in the perilesional cortex of aged rats (Table 2). Unique to aged rats was the up-regulation of ribosomal protein 2 (Rps2), which under normal cellular conditions is a substrate for PRMT3 (protein arginine methyltransferase 3) that catalyse the formation of asymmetric dimethylarginine [61]. A likely mechanism for this is that Rps2 is synthesized by reactive astrocytes that are present in greater number in the perilesional area of aged rats [4].

Gpx1, a gene necessary for antioxidative defence, was up-regulated in old rats, but at a lower level than observed in young rats. Further, tissue protection was achieved by a strong up-regulation of the Ucp2, a gene with a key role in energy availability and

Table 3 Apoptosis-related gene expression profiles

Gene name	Genbank Accession no.	Description	Fold change							
			3-month-old rat				18-month-old rat			
			Day 3		Day 14		Day 3		Day 14	
			pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl
Apoptosis-related gene										
Atm	NM_007499	Ataxia telangiectasia mutated homologue (human)			2.64				1.97	
Gadd45 a*	NM_007836	growth arrest and DNA-damage-inducible 45 α			1.81		3.56		3.84	
Hus1	NM_008316	Hus1 homologue (S. pombe)			1.52		2.78		1.64	
Mdm2	NM_010786	transformed mouse 3T3 cell double minute 2					2.67			
Tnfrsf7	NM_001033126	Tumour necrosis factor receptor superfamily, member 7					4.60			
Casp7*	NM_007611	Caspase 7			0.49	0,22			3.34	
Traf1	NM_009421	TNF receptor-associated factor 1					0.29	0,39	0.49	0.43
Traf4	NM_009423	TNF receptor-associated factor 4							0.50	
Trp53	NM_011640	Transformation-related protein 53					0.62			

Fold change is calculated as the ratio of experiment to sham control after they were normalized to housekeeping genes obtained from two independent experiments. Genes whose average fold induction was equal to or higher than 1.5 and equal or lower than 0.66 were selected. Genes were listed according to up- or down-regulation of genes and alphabetic order.
*Represents genes were not previously reported in the after stroke rat brain. Genes that have been validated by RT-PCR were marked by colour.
pi, periinfarct, lesional hemisphere; cl, contralateral, healthy hemisphere; ctrl, sham control.

antioxidative defence by regulating mitochondrial gluthatione availability. Cystatin B (cstb) was also up-regulated, although less strongly than Ucp2. Cstb is a non-caspase cysteine protease inhibitor shown to protect neurons against apoptotic death.

In the second week after stroke, the expression of Gpx1 and Ucp2 genes remained at high levels in the contralateral hemisphere of old rats. However, in contrast to the young rats, the antioxidative capacity was not supplemented by increases in CAT and SOD gene expression. Furthermore, tissue recovery was not supported by increases in Tgfb1, a cytokine whose expression was associated with increased neuroprotection by suppressing inflammation, promotes neurogenesis and prevents apoptosis (Table 2).

In contrast to the young rats at day 3, aged rats exhibited strong up-regulation of genes involved in DNA damage, cell cycle arrest and apoptosis (Table 3). In particular, aged rats rapidly up-regulated genes such as growth arrest and DNA-damaged inducible 45 α (Gadd45α), telangiectasis mutated homologue (human) (Atm_mapped), Hus1 homologue (S. pombe) (Hus1_predicted), transformed mouse 3T3 cell double minute 2 (Mdm2), caspase 7 and tumour necrosis factor receptor superfamily member 7 (Tnfrsf7, also called CD27). These genes are required for DNA damage-induced apoptosis. These changes are probably related to the simultaneous decrease of anti-apoptotic genes expression such as Traf1, Traf 4 and Trp53 [62, 63] in the same

Table 4 Stem cell-related gene expression profiles

Gene name	Genbank Accession no.	Description	Fold change							
			3-month-old rat				18-month-old rat			
			Day 3		Day 14		Day 3		Day 14	
			pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl
Stem cell related genes										
Fabp7*	NM_021272	fatty acid-binding protein 7, brain	3.40		4.17		7.48		8.07	
Fgf22	NM_023304	Fibroblast growth factor 22	2.28	2.15						
Fzd8	NM_008058	frizzled homologue 8 (Drosophila)	4,61	2.37						0.61
Gata2	NM_008090	Gata-binding protein 2	2.17			0.36			0.44	0.40
Igf1r*	NM_010513	Insulin-like growth factor 1 receptor	2.70		0.56		0.62			
Ngfb	NM_013609		2.09	2.03	2.00					
Nkx2-2*	NM_010919	NK2 transcription factor related, locus 2 (Drosophila)	1.59	2.98	2.82					
Oligo1	NM_016968	oligodendrocyte transcription factor 1	2.38	1.65	1.81		1.86		1.51	
Gjb1*	NM_008124	Gap junction membrane channel protein β 1		2.35	3.12				0.38	
Ptch1	NM_008957	Patched homologue 1		1.81	0.36					
Cst3	NM_009976	cystatin C			3.11				5.27	1.67
Gcm2	NM_008104	Glial cells missing homologue 2 (Drosophila)			2.04	2.21				
Igf2	NM_010514	insulin-like growth factor 2			7.38				14.61	
Cdh5*	NM_009868	Cadherin 5					1.59		2.30	
Ptprc	NM_011210	protein tyrosine phosphatase, receptor type, C					3.67		5.73	
Ptges3	NM_019766	prostaglandin E synthase 3 (cytosolic)					2.60	1.56	2.09	0.66
Tgfbr1	NM_009370	transforming growth factor, β receptor I					7.41		6.78	
Cdkn1b	NM_009875	Cyclin-dependent kinase inhibitor 1B						3.97		
Bmpr2*	NM_007561	Bone morphogenetic protein receptor, type 2	0.51						0.60	

Continued

Table 4 Continued

Gene name	Genbank Accession no.	Description	Fold change							
			3-month-old rat				18-month-old rat			
			Day 3		Day 14		Day 3		Day 14	
			pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl	pi/ctrl	cl/ctrl
Ctnna2	NM_009819		0.41	0.1	0.17				0.35	
Ctnnd2	NM_008729	Catenin,delta 2	0.50	0.33	0.27		0.26	0.57	0.17	
Ctnnd2	NM_008729	Catenin,delta 2	0.50	0.33	0.27		0.26	0.57	0.17	
Fgfr1*	NM_010206	Fibroblast growth factor receptor 1			0.34		0.57		1.77	
Icam5*	NM_008319	Intercellular adhesion molecular 5, telecephalin	0.43	0.66					1.55	
Inhbb*	NM_008381	Inhibin β-B	0.58		2.30		0.65		0.60	0.53
Itgb5*	NM_010580	Integrin β 5	0.40							
Myh6	NM_010856	Myosin, heavy polypeptide 6, cardiac muscle, α	0.66	0.29	0.30					
Nefl	NM_010910	neurofilament, light polypeptide	0.30	0.54			0.66		0.41	
Shh	NM_009170	Sonic hedgehog	0.22	0.22	0.22	0.22				0.51
Foxg1	NM_008241	Forkhead box G1							0.49	0.62
Mtap2	XM_894407	Microtubule-associated protein 2							0.64	

Fold change is calculated as the ratio of experiment to sham control after they were normalized to housekeeping genes obtained from two independent experiments. Genes whose average fold induction was equal to or higher than 1.5 and equal or lower than 0.66 were selected. Genes were listed according to up- or down-regulation of genes and alphabetic order.

*Represents genes were not previously reported in the after stroke rat brain. Genes that have been validated by RT-PCR were marked by colour.

pi, periinfarct, lesional hemisphere; cl, contralateral, healthy hemisphere; ctrl, sham control.

region. Caspase 7, an additional pro-apoptotic gene, was also strongly activated in the second week after stroke.

In addition, inflammatory- and stroke-related genes, prostaglandin E synthase 3 (Ptges3) [64], protein tyrosine phosphatase, receptor type C (Ptprc) also known as leukocyte-common antigen (LCA) or CD45 [65] (Table 4) and interleukin 6 (Il6) [66] were increased in the aged, but not the young, perilesional cortex (Tables 2 and 4). In particular, increased expression of Ptprc and Ptges3 genes persisted in the second week after stroke (Table 4). Closely related to increased inflammation, there is a robust overexpression of the gene coding for transforming growth factor receptor type I (TGFR1) thought to be implicated in fibrosis and therefore, scar formation [67] (Table 4).

Aged rats still have the capability to mount a robust cytoproliferative response to cerebral ischaemia [68]. Similar to the young rats, the aged rats displayed vigorous increases in pro-collagen type I, α I (Col1a1), a gene associated with hypoxia-induced vascular remodelling and neurite outgrowth. Interestingly, the levels of Col1a1 mRNA vastly exceeded those of young rats in the second week after stroke (Table 2).

Down-regulated genes in the ipsilateral sensorimotor cortex of aged rats

In the first week after stroke, several genes with a role in axonal outgrowth and neurogenesis were down-regulated in the damaged hemisphere of old rats. These included Shh, an axonal chemoattractant [42–44] and Fgfr1, an axonal growth factor [53]. In contrast, Fgfr1 was not down-regulated at 3 days after stroke (Table 4). Also, down-regulated was the gene encoding chromogranin A (Chga) which is required for neurogenesis [47] (Table 2). RNA-splicing factor Prp40, a negative regulator of Notch signalling, was up-regulated in old, but not young rats [69]. Because the Notch pathway is a versatile regulator of axonal growth [70], this finding suggests that axonal and dendritic growth in aged rats is severely restricted. Other genes that were down-regulated include growth-associated protein 43, tubulin α1 (Tub1a), Bmpr2 and microtubule-associated protein 2 (Mtap2). GAP43 is required for neurogenesis, axonal growth and maturation [71]. Bmpr2 is involved in dendritic injury and dendritogenesis [49]. Mtap2 and Nefl genes are required for axonal maturation (Table 4). Genes coding for neuronal survival factors, such as Inhbb, were also down-regulated in the second week after stroke, a decisive time-point for successful regeneration [71] (Table 4). Along this line, pro-collagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (Plod3; synonym, LH3) that is critical for growth cone migration [72], and Gpi were down-regulated at all time-points studied [41]. Neurogenesis was further impaired by down-regulation of Tuba1 that in necessary for proper neuronal migration [73] and down-regulation of genes necessary for axonal migration, such as α and δ catenins [50] and cell adhesion like intercellular adhesion molecule 5 (Icam5, also called telencephalin) [58] (Table 4).

In the second week after stroke, down-regulated genes included Fgfr1 that was implicated in axonal growth [53]. In addition, genes implicated in neurogensis and axonal growth/migration like Shh [43] and catenin α 2 and catenin β 2 (Table 4) and Npy(Table 2), a gene involved in regulation of energy balance, were kept at low levels at all time-points studied. Surprisingly, in contrast to the young rats, the gene encoding Myh6, and which is important for synaptic transmission [56], was not down-regulated after stroke in the brains of aged rats.

Regulated genes in the contralateral (undamaged) sensorimotor cortex of aged rats

Generally, the healthy contralateral sensorimotor cortex of the aged rats was transcriptionally less active than that of the young rats, especially in the first week after stroke. Aged rats had six-fold fewer up-regulated genes than young rats at all time-points studied. However, the number of down-regulated genes in aged rats in the second week after stroke was 6-fold greater than the number down-regulated in first week (Fig. 2B). The most prominent change in aged rats was a robust increase in the expression of the anti-proliferation gene, cyclin-dependent kinase inhibitor 1B (Cdkn1b; synonyms: p27, p27Kip1) (Table 4), which inhibits the activity of cyclin-CDK complexes and plays a central role in neuronal migration, that is Cdkn1b up-regulation would impair neurogenesis [74]. RNA-splicing factor Prp40, a negative regulator of notch signalling [69], was also up-regulated. The Notch pathway is a versatile regulator of cell fate specification, growth, differentiation and patterning processes in metazoan organisms. In particular, in the developing nervous system the Notch signalling pathway specify axonal growth cones [70]. Several changes in gene regulation were observed that could enhance the Notch pathway, including down-regulation of genes required for neurogenesis and axonal growth/migration (Ctnnd2, Foxg1, Ptges3, Gata2, Fzd8, Sod2, Traf1, Shh) and of one gene required for neuronal survival factors (Inhbb).

Verification of microarray data by real-time quantitative PCR

To confirm the microarray data, real-time quantitative PCR was performed. We randomly selected Bmpr2, brain fatty acid-binding protein, Cdh5, fibroblast growth factor 1 receptor, inhibin β, insulin-like growth factor 1 receptor, integrin β 5 and intercellular adhesion molecular 5 from the stem cell-related cDNA array (Fig. 3). We also selected Chga, Ucp2, Gpx1, SOD2, CAT, growth-associated protein 43, tubulin (1, vascular endothelial growth factor A from hypoxia signalling pathway (Fig. 4). In addition, we chose caspase 7 and Gadd45a from the apoptosis array (Fig. 5). The expression pattern of these genes from

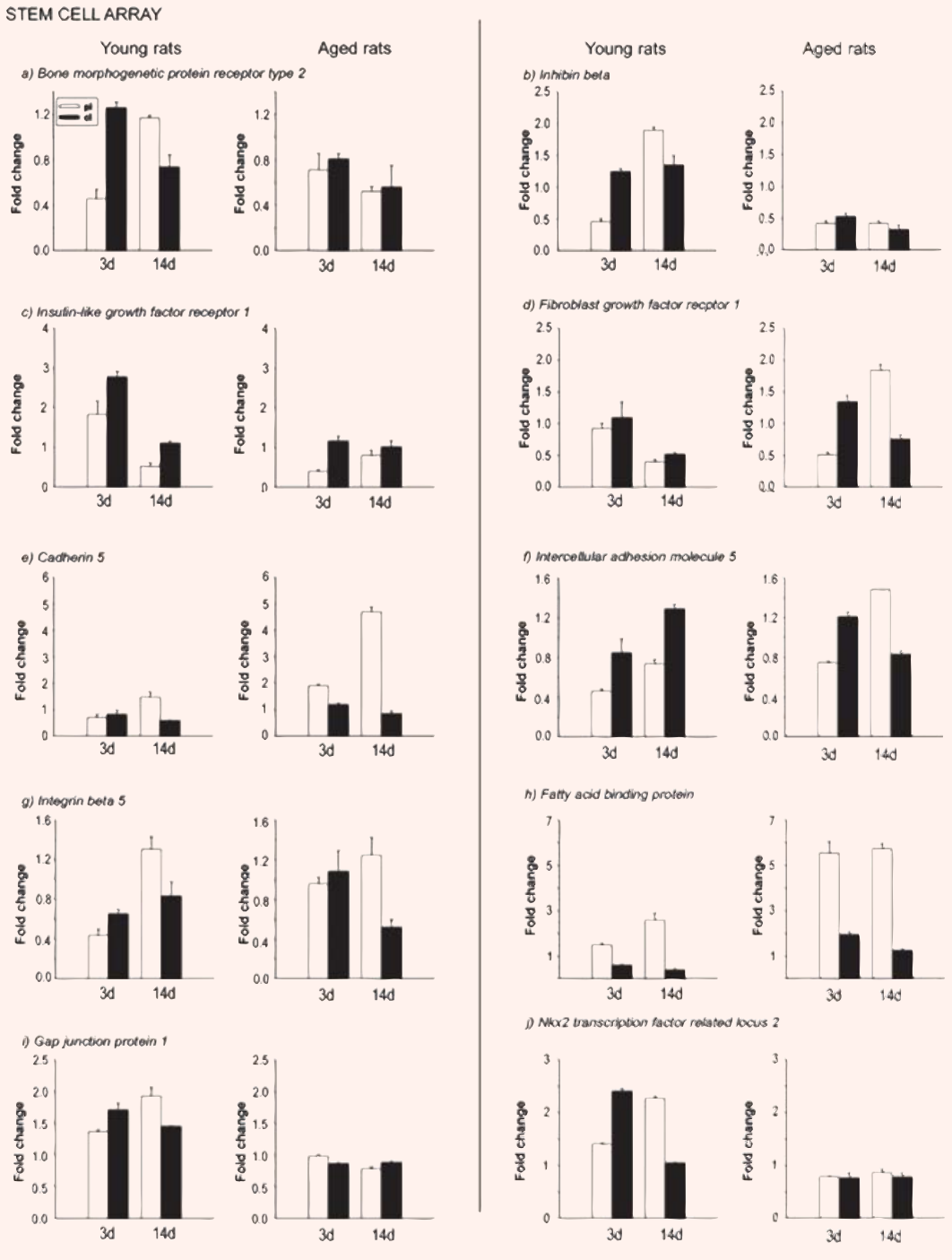

Fig. 3 Validation of altered stem cell-related gene expression by real-time quantitative PCR. Selected genes identified from the macroarray analysis were further confirmed by real-time PCR. The expression profiles are presented in the form of column graphs. The numbers on the vertical axis represent the average fold change. The horizontal axis denotes the post stroke time-points. pi, periinfart; cl, contralateral; 3d, after stroke day 3; 14d, after stroke day 14.

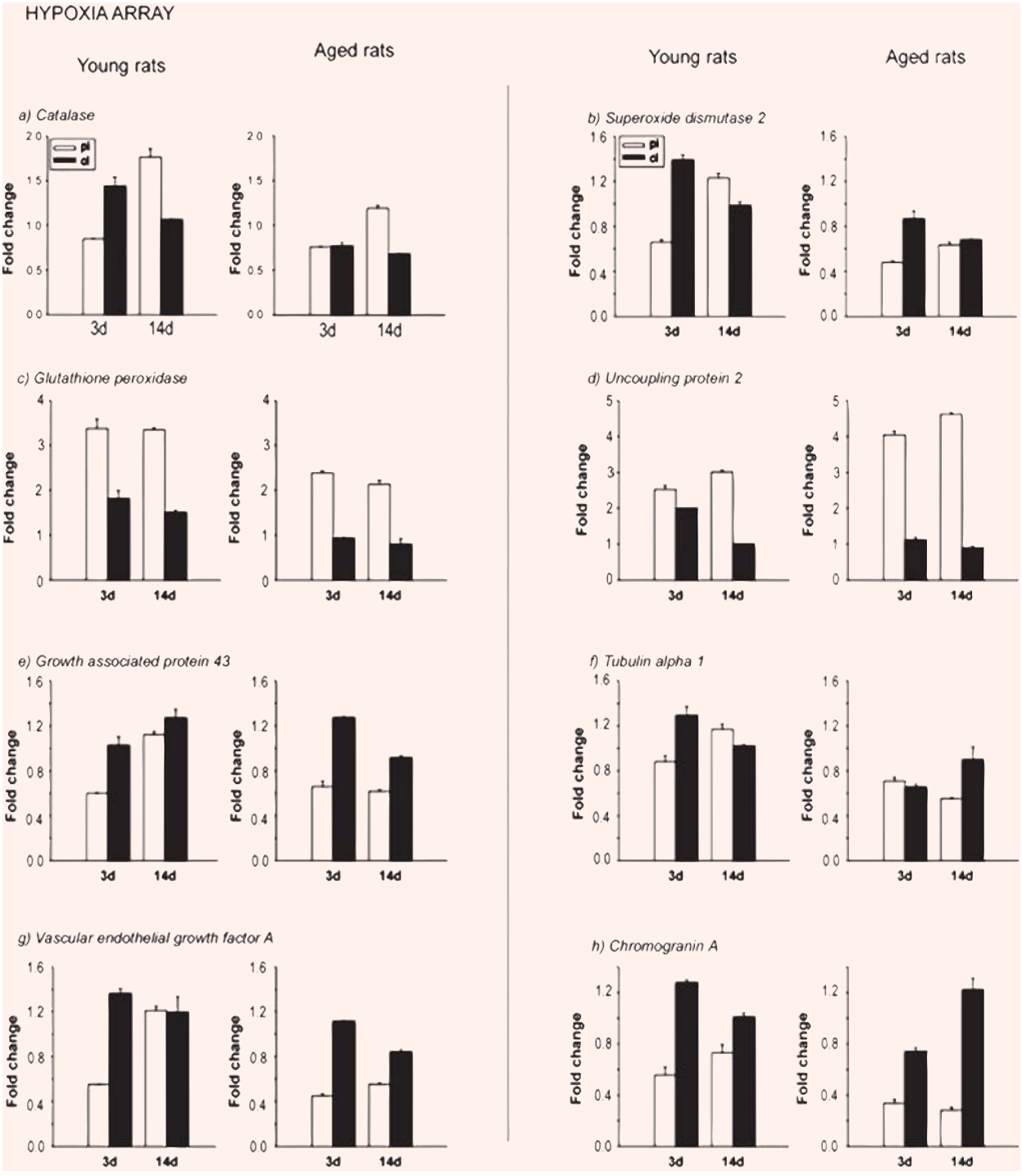

Fig. 4 Validation of altered hypoxia signalling pathway-related gene expression by real-time quantitative PCR. Selected genes identified from the macroarray analysis were further confirmed by real-time PCR. The expression profiles are presented in the form of column graphs. The numbers on the vertical axis represent the average fold change. The horizontal axis denotes the post stroke time-points. pi, periinfart; cl, contralateral; 3d, after stroke day 3; 14d, after stroke day 14.

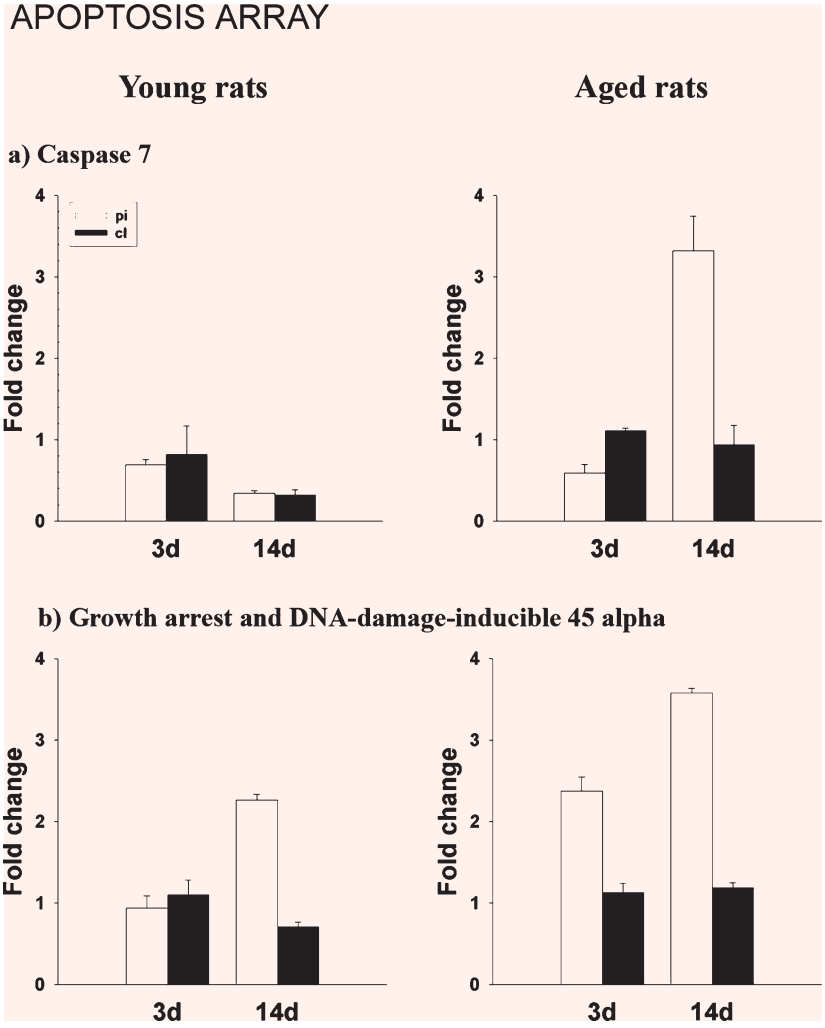

Fig. 5 Validation of altered apoptosis-related gene expression by real-time quantitative PCR. The selected genes identified from the microarray analysis were further confirmed by real-time PCR. The expression profiles are presented in the form of column graphs. The numbers on the vertical axis represent the average fold change. The horizontal axis denotes the after stroke time-points. pi, periinfart; cl, contralateral; 3d, after stroke day 3; 14d, after stroke day 14.

real-time PCR matched well with the expression profile obtained by microarray analysis.

Discussion

It has been hypothesized that the ipsilateral and the contralateral hemispheres work in synergy to promote functional recovery after stroke in young rats. Using categorized DNA arrays we found inappropriate gene regulation in response to stroke both in the ipsilateral and the contralateral hemisphere of aged rats. The gene expression profile in the brains of post-stroke aged rats is indicative of increased cell death due to DNA damage and apoptosis, especially in the first week after stroke. Similarly, we report persistent down-regulation of genes that are required for neurogenesis after stroke in aged rats.

Up-regulation for DNA damage and down-regulation of anti-apoptosis-related genes in the periinfarcted region of aged rats after stroke

We [4], and others [75–77] found that aged rats were more severely impaired by stroke and showed diminished functional recovery as compared with young rats. Therefore, in the following discussion we will assume that the time course of gene changes occurring in the brains of young rats represents the optimal response to stroke.

Early up-regulation of genes related to DNA damage, cell cycle arrest and apoptosis after cerebral ischaemia in aged rats

The first week after stroke is crucial for successful recovery in human stroke victims and in animal models of stroke. This period is associated with both immediate and late neuronal death that will determine the extent of overall cell loss in the hypoperfusion area (formerly designated the 'penumbra').

Our results indicate that one major genetic event that may lead to increased cellular death in the hypoperfusion area in aged rats is the rapid up-regulation of a group of genes related to DNA damage, cell cycle arrest and apoptosis. In the first 3 days after stroke there was a massive up-regulation of DNA damage-related genes like Gadd45a, Hus1, Mdm2 and Tnfrsf7 in the perilesional cortex of aged rats as compared to young at the same time-point. Cell death in aged rat brains was accelerated due to down-regulation of anti-apopotic genes, such as Traf1, Traf4 and Trp53. In contrast, young rats did not express DNA damage in the first 3 days after stroke. By day 14, the number of DNA damage-related genes in the perilesional cortex of young rats too increased to equal those of aged rats that were kept at high levels at all time-points (summarized in supplemental Fig. 1A).

It has been proposed that Mdm2 is an indicator of DNA damage in the brain early after an ischaemic insult in a similar way to Gadd45_ [78]. The role of Hus1 and Atm in the post stroke rat brain are not known. The DNA damage-induced chromatin binding has been shown to depend on the activation of the checkpoint kinase Atm, and is thought to be an early checkpoint signalling event [79].

Another dramatic change we observed was a 4-fold increase in the down-regulation of anti-apoptotic genes Traf 1, Traf4 and Trp53 in the lesioned area of post-ischaemic aged rats brains. In the contralateral hemisphere we noted the early increase in the expression of the anti-apoptotic gene Cstb in the brains of young as compared to aged rats.

Trp53 belongs to the tumour suppressor p53 superfamily. Mutations in this locus affect cell-cycle regulation and apoptosis. After brain trauma, Traf1 and Traf4 were polyubiquitinated in lipid rafts. Subsequently, the signalling complex contained activated caspase-8, thus initiating apoptosis [80].

Tnfrsf7 and caspase 7 (Casp 7) play an important role in mediating CD27-binding protein induced apoptosis [81]. At 14 days after stroke, both of these were strongly up-regulated in the perilesional area of aged but not young rats. Since Casp7 is already increased in control aged rat brains, we infer that ischaemia will exacerbate a death mechanism that is already operational in aged brains.

There was no change in the contralateral hemisphere of either age group in regulation of DNA damage or apoptosis-related genes. There was only one pro-apoptotic gene, Casp7, that was down-regulated in young rats and one down-regulated gene in the aged rats, Traf1 (summarized in supplemental Fig. 1B).

Genes associated with inflammation and scar formation are strongly up-regulated in aged rats

Ischaemia/reperfusion injury is associated with the proliferation and hypertrophy of astrocytes and with inflammatory responses. In aged rats there were in total five up-regulated inflammation-related genes in the peiinfarcted region (Ptprc, Ptges3, Tgfbr1, IL6, Rps2), while in the young rats there was only one up-regulated gene, Tgfb1.

Ptges3 may contribute to production of prostaglandin E2 from arachidonic acid. It was also found to be significantly increased in the rat brain after challenge with lipopolysaccharide (LPS) [64]. In the present study, increased levels of this inflammatory gene Ptges3 persisted into the 2nd week post-stroke. The inflammatory cytokine IL-6 is clearly implicated in the response to central nervous system (CNS) injury, but whether this response is neuroprotective or pathological is uncertain [82, 83].

Aged, but not young, rats exhibited up-regulation of Rps2, which under normal cellular conditions is a substrate for PRMT3 . A likely explanation of the up-regulation we observed is that Rsp2 is synthesized by reactive astrocytes [84] which are present in great number in the perilesional area of aged rats [4].

TGF-β1 was up-regulated only in young rats. Tgfb1 is a pleiotropic cytokine with potent neurotrophic and immunosuppressive properties that is up-regulated after injury. In vitro, Tgfb1 protects neurons against excitotoxicity by inhibiting t-PA-potentiated NMDA-induced neuronal death by

up-regulation of type-1 plasminogen activator inhibitor (PAI-1) in astrocytes 4. In addition, Tgfb1 has been recently characterized as an antiapoptotic factor in a model of staurosporine-induced neuronal death [25]. It has recently been demonstrated that Tgfb1 suppresses inflammation and promotes survival in adult CNS after axonal injury [26] (as summarized in supplemental Fig. 2).

In general, genes influencing inflammatory processes were not significantly modified in the contralateral hemisphere of either young or aged rats.

Neuroprotection, including antioxidative defence and neuronal survival factors is diminished in the perilesional cortex of aged rats

Neuroprotection conferred by antioxidative defence

In the young rats the number of genes conferring neuroprotection in the damaged hemisphere exceeded by a factor of 2 those expressed in the aged rats at all time-points. The degree of neuroprotection in aged rats was further decreased by a 2-fold increase in the number of down-regulated genes coding for antioxidative defence and neuronal survival factors so that, in total, aged rats had a 4-fold decrease in the number of genes conferring neuroprotection (summarized in supplemental Fig. 3A).

In the contralateral hemisphere the effect on neuroprotective genes expression was even more damaging in aged rats, which did not express any protective gene, while young rats up-regulated seven such genes. In the second week after stroke there was no difference in the number of expressed gene numbers in the two age groups (summarized in supplemental Fig. 3B).

Oxidative damage may be caused by ROS, including superoxide [85, 86]. Counteracting oxidative stress through up-regulation of mitochondrial antioxidants is one of the cell survival mechanisms operating shortly after cerebral ischaemia. Failure to increase the expression of antioxidant systems may increase the sensitivity to oxidative stress [14, 87] and contribute to poor recovery after cerebral ischaemia.

In the first week after stroke, the antioxidative capacity in young rats was supported by up-regulation of Ucp2 (mitochondrial, proton carrier)(Ucp2) and Gpx1. For example, mice lacking Gpx1 ($Gpx1^{-/-}$ mice) which is indicative of reduced protection, have increased infarct size after MCAO [88]. In the second week after stroke, the antioxidative capacity of young rats was further improved by up-regulation of CAT, which has been intensively studied as an antioxidant in Sjogren's syndrome/scleroderma pathology. The expression of Sssca1 gene may reflect the generation of faulty proteins by oxidation [24].

The antioxidative capacity of aged rats was reduced as compared to young rats. SOD2, mitochondrial (Sod2), another component of the antioxidant system, was down-regulated in the perinfarcted area of aged rats while Cat and Sssca1 were not up-regulated at all in aged animals.

Our finding of decreased levels of both CAT and SOD2 implies both a decline in the antioxidative capacity and synaptic plasticity with increasing age [89]. Taken together, these data suggest that the antioxidative system is not fully operational in aged rats (supplemental Fig. 3A and B, grey bars).

Neuroprotection due to increased expression of neuronal survival factors

Another age difference we detected was the more robust activation in young rats of genes coding for neuronal survival factors. Thus, in the first week after stroke, genes such as Fgf22, Ngfb, Igf1r1, Olig1 and Gjb1 were up-regulated in young, but not aged rats (as summarized in supplemental Fig. 3A, black bars).

Injury-related regulated genes have been implicated in numerous cellular processes, including proliferation, migration, differentiation and survival [90]. In particular, Fgf22 plays a significant role as a neuronal and oligodendrocyte survival factor [29]. Moreover, using clustering of synaptic vesicles in cultured neurons from mouse brain as an assay, FGF22 was identified as a major active organizing species [91]. In line with our results, Fgf22 was up-regulated in the skin of young but not aged mice in a model of skin injury [29].

Insulin-like growth factor-I (IGF-I) has been shown to be a potent agent in promoting the growth and

differentiation of oligodendrocyte precursors, and in stimulating myelination during development and following injury [31]. Recent evidence shows that endogenous IGF-1 also plays a significant role in recovery from insults such as hypoxia-ischaemia and that giving additional exogenous IGF-1 can ameliorate damage.

During the second week, when recovery at tissue level is evident, young rats exhibited up-regulation of several neuronal survival factors including Ngfb, Igf2, inhibin β (Inhbb) (also called activin), Oligo1, Cyt3, Pea-15 and TGF-β.

There is evidence that each of these factors is related to neuronal survival or growth. For example, Ngfb is a neurotrophic factor that is critically involved in the development and maintenance of both the peripheral and central nervous system [92] and it is up-regulated in response to cerebral ischaemia [93].

Activin is a neuronal survival factor that is rapidly increased after transient cerebral ischaemia and hypoxia in mice [94]. Activin is a member of the TGFB family that is involved in cell differentiation, hormone secretion and regulation of neuron survival [95]. These findings suggest that endogenously activated autocrine loops of activin-Nodal signalling promote ES cell self-renewal [41]. Igf2, also called somatomedin A, is a neuronal survival factor that acts neuroprotectively if administered intracerebroventricularly after cerebral ischaemia [96].

CysC is an endogenous cysteine proteases inhibitor produced by mature astrocytes in the adult brain and is vigourously up-regulated in the ipsilateral hemisphere of both and young rats after stroke. Cystatin-B is expressed by neural stem cells and by differentiated neurons and astrocytes. Mice with a gene deletion of CSTB exhibit increased apoptosis of specific neurons [37, 97].

PEA-15 is a small protein (15 kD) that was first identified as an abundant phosphoprotein in brain astrocytes and subsequently shown to be widely expressed in different tissues and highly conserved among mammals [98].

Neuroprotection is completely absent in the contralateral hemisphere of aged rats after stroke

A major difference between young and aged rats was seen in the number of neuroprotective genes that were up-regulated in the contralateral hemisphere of young *versus* aged rats. There were in total seven up-regulated such genes in the contralateral hemisphere of young rats, two coding for antioxidative defence (Ucp2 and Sod2) and five coding for growth and survival factors (Fgf22, Ngfb, Oligo1, Gjb1 and Vegfa) (summarized in supplemental Fig. 3B).

Neurogenesis is not fully supported in the periinfarcted area, especially in aged

We found that neurogenesis is not fully supported in the periinfarcted area of both age groups and especially in the aged rats (supplemental Fig. 4A and B).

Both age groups expressed high levels of Fabp7 mRNA in the perilesional area during the 2-week study period. We found that the extent of axonal damage, as indicated by higher levels of expression for Fabp7 mRNA in the perilesional area, is greater in aged rats than in young rats. Since the basal levels of Fabp7 mRNA were also increased in the control aging brain, we hypothesize that the increased levels in Fabp7 transcript in the aged rats brain after injury are in part due to age-associated increases in Fabp7 mRNA levels. In the normal aging brain, myelin sheaths show signs of breakdown [99] and the turnover rates of cerebroside and GM1 increased in senescence. The latter phenomenon may indicate an enhanced myelin turnover in senescence [100]. Previous work also indicated age-related increases in Fabps [101] and implicated Fabp7 as a potential marker of axonal injury after cerebral infarction and, generally, of brain damage [101, 102, 19].

Specific to the aged rats was the up-regulation of myelin-injury specific gene, NK2 transcription factor related, locus 2 (Drosophila)(Nkx2-2). Up-regulation of this gene has been demonstrated in NG2-expressing oligodendrocyte precursor cells surrounding the hypoperfusion area after MCAO [32].

Myosin VI (Myo6) is a minus end-directed actin-based motor component found in neurons that express Trk receptors. It has been reported that Myo6 is necessary for brain-derived neurotrophic factor (BDNF)-TrkB-mediated facilitation of long-term potentiation and synaptic plasticity in postnatal day 12–13 hippocampus [56].

Cerebral ischaemia causes cellular death and dissolution of neuronal networks with subsequent activation of genes involved in cellular adhesion. Specific

to the aged rats was the up-regulation genes with cell adhesion activity (Cdh5, Icam5) though the significance of down-regulation for tissue recovery is not presently understood.

After the infarct area is stabilized, repair mechanisms involving stem cells may become active, especially in the second week after stroke. In young rats, there were seven up-regulated genes with important roles in neurogenesis (Fzd8, Gata2, Gcm2, Oligo1, Gjb1, Colla1, Mmp14).

Wnt signalling plays critical biological roles during normal embryonic development and homeostasis in adults. In the canonical pathway, binding of Wnt ligands to the Fzd receptor and the low-density lipoprotein-related receptor (LRP) 5 or LRP6 co-receptor initiates downstream signalling events leading to gene activation by β-catenin and the T cell factor (TCF)-lymphoid enhancer factor (LEF) family transcription factor complex [33].

An essential role of Gata transcription factors in sympathetic neuron development has also been recently described [34].

Gcm2 is a transcription factor whose expression is restricted to post-embryonic stages and that is required for specification and differentiation of certain neuronal and glial lineages [38, 39].

We found a number of genes implicated in re-myelinization, such as Nkx2-2 and Olig1 that were up-regulated in the contralateral hemisphere of young rats at 3 days after ischaemia, but not in aged rats. Both Nkx2-2 and Olig1 are transcription factors that play an important role in the differentiation of oligodendrocyte progenitor cell (OPC) into re-myelinating oligodendrocytes, myelinogenesis and in axonal recognition [103, 104]. In this light, the aged rats are at disadvantage in myelin repair because, as we found, Nkx2-2 gene activity was substantially decreased even in intact aged rats as compared to their younger counterparts [106].

Pro-collagen, type I, α 1 (Col1a1) is a gene associated with hypoxia-induced vascular remodelling [35]. Very likely, neovasculogenesis and indirectly pro-collagen type I α are required for neurite outgrowth, respectively [36]. Surprisingly, in aged rats the levels of pro-collagen, type I, α 1 mRNA vastly exceeded those of young rats in the second week after stroke suggesting that neovasculogenesis is not impaired after stroke in aged rats. Since there is a reported down-regulation of several collagen genes (*e.g.* Col1a1 and Col3a1) in the aging lung from C57BL/6 mice [107], we infer that the middle-aged rat brain is still capable of robust up-regulation of genes expression upon injury.

The Mmp14 gene, which is critical to creating a permissive growth environment for neurites [36], was up-regulated to modest levels in the periinfarcted area of young rats only.

Down-regulation of axonal growth- and dendritogenesis-related gene expression in both age groups

Axonal re-growth after injuries to the CNS is central to successful functionality of the damaged area and is generally known to be impaired in higher vertebrates. Indeed, in the first week after stroke, no genes important for axonal growth were up-regulated in either age group (supplemental Fig. 4A and B). On the other hand, genes with axonal growth activity like Shh, patched homologue 1 (Ptch1), Fgfr1, catenin α 2 (Ctnna2), catenin δ 2 (Ctnnd2), growth-associated protein 43 (Gap43), α-tubulin (Tuba), forkhead box G1 (Foxg1) and Plod3, were down-regulated in the lesioned cortex of both young and aged rats.

The gene coding for hedgehog homologue (Shh) is a signalling pathway component that plays a role in neurite outgrowth and neurogenesis by regulating proliferation of adult neural stem cells [44]. Shh belongs to a family of signalling glycoproteins implicated in embryonic development. Shh displays inductive, proliferative, neurotrophic and neuroprotective activities on various neural cells and signals through a receptor complex associating Patched (Ptch1) and Smoothened (Smo) [94].

Specific to the aged rats was the down-regulation of several axonal growth and neurogenesis-specific genes including Gata2, Gjb1, Foxg1, Plod3, Mtap2 and Tuba1. Gata2 and Gata3 have been demonstrated, in the chick and mouse respectively, to be essential members of the transcription factor network controlling sympathetic neuron development [34].

Gjb1, a component of gap junctions, was strongly up-regulated at 3 days after ischaemia in young, but down-regulated in aged rats, suggesting poor cell–cell communication in the perilesional area in the brains of aged rats. Previous work showed that Gjb1, also known as Cx22, is expressed in oligodendrocytes and facilitated cell–cell communication [107].

Foxg1 is an evolutionarily conserved, winged-helix transcriptional factor that controls telencephalic neurogenesis, suggesting that Foxg1 controls precursor proliferation *via* regulation of Fgf signalling and differentiation *via* regulation of Bmp signalling.

Axonal growth cone migration is further impaired by down-regulation of genes that are important to establish appropriate migration cues such as the gene coding for Plod3. Plod3 has a role in matrix remodelling and modifies collagen IV residues to render them growth-permissive [72]. The down-regulation of these genes suggests that failure to grow axons may have many causes, each acting at different levels.

Other genes involved in neuritic outgrowth and differentiation were also down-regulated. These included basic helix-loop-helix domain containing, class B2 (Bhlhb2), Chga, Icam5 (telencephalin), synaptic transmission required gene, myosin and heavy polypeptide 6 (Myh6). Bhlhb2 is up-regulated in the hippocampus after transient forebrain ischaemia and may play a role in regulating neuronal differentiation during development and adaptive neuronal plasticity and neurite outgrowth in the adult [54, 108].

The Chga gene was down-regulated in the first week after stroke in young rats and at all time-points in the aged. Activation of Chga promoter in hippocampal progenitor cells led to neurite outgrowth, indicative of a role for Chga in neuronal differentiation [47]. Icam5 (telencephalin) is a dendrite-expressed membrane glycoprotein of telencephalic neurons in the mammalian brain. Additionally, the Icam5/actinin interaction is involved in neuritic outgrowth [58].

The growth and morphological differentiation of dendrites are critical events in the establishment of proper neuronal connectivity and neural function. The expression of Bmpr2 is required for BMP-dependent induction of the dendritic arbour in cortical neurons (Bmpr2) [49].

One possible cause of the absence of axonal growth at the lesion site is the opposite expression of genes which otherwise should be co-ordinated. For example, the Fzd8, which is robustly up-regulated in the ipsilateral hemisphere of young rats, does not co-ordinate with the gene expression for α N-catenin, a gene that is down-regulated at the same time-points.

Neurogenesis is impaired in the contralateral (unlesioned) hemisphere of both young and aged rats at all times after stroke

The contralateral hemisphere of young rats seems to support some neurogenesis by up-regulating several genes related to axonal injury (Nkx2-2), glial differentiation (Gcm2), embryonic development (Ptch1) and neurogenesis (Fzd8). Additionally, the synaptic transmission-relevant gene, Myh6 was down-regulated. Aged rats, in contrast, up-regulated genes suppressing cell division (Cdkn1b) and axonal growth (Prp40a), both of which are required for neurogenesis (summarized in supplemental Fig. 4B).

In the second week, a period decisive for axonal growth, the only up-regulated gene in aged rats was PRP40 pre-mRNA processing factor 40 homologue A (Prpf40a).

The neurogenesis-required genes Fzd8 and Foxg1 were down-regulated in old rats only. Aged rats also failed to up-regulate the axonal and myelin injury-related gene (Nkx2-2) and the Gmc2 gene, a gene is important for differentiation of certain glial and neuronal lineages. They also failed to up-regulate Ptch1 that is required for reactivation of developmental mechanisms after injury. Both age groups had many down-regulated neurogenesis-related genes (Gata2, Ctnnd2, Shh).

An overview of major genetic events in the ipsilateral and contralateral hemisphere tentatively associated with functional recovery of the after stroke in young and aged rats is given is Fig. 6 and 7.

The substrates that mediate recovery of motor function after stroke are incompletely understood. Although the effect of age on cerebral ischaemia and recovery after stroke has been the focus of several recent reports [109, 110], the contribution of the contralateral hemisphere to neurorestoration has not been addressed at gene expression level. Our study shows that the contralateral, healthy hemisphere in young rats is much more active at transcriptional level than that of the aged rats at day 3 after ischaemia, especially at the level of stem cell and hypoxia signalling coding genes. A possible explanation for this is the temporary decrease in transcallossal disinhibition allowing misbalanced gene expression in the contralateral hemisphere [111–113]. However, at 3 days after stroke, tissue in the

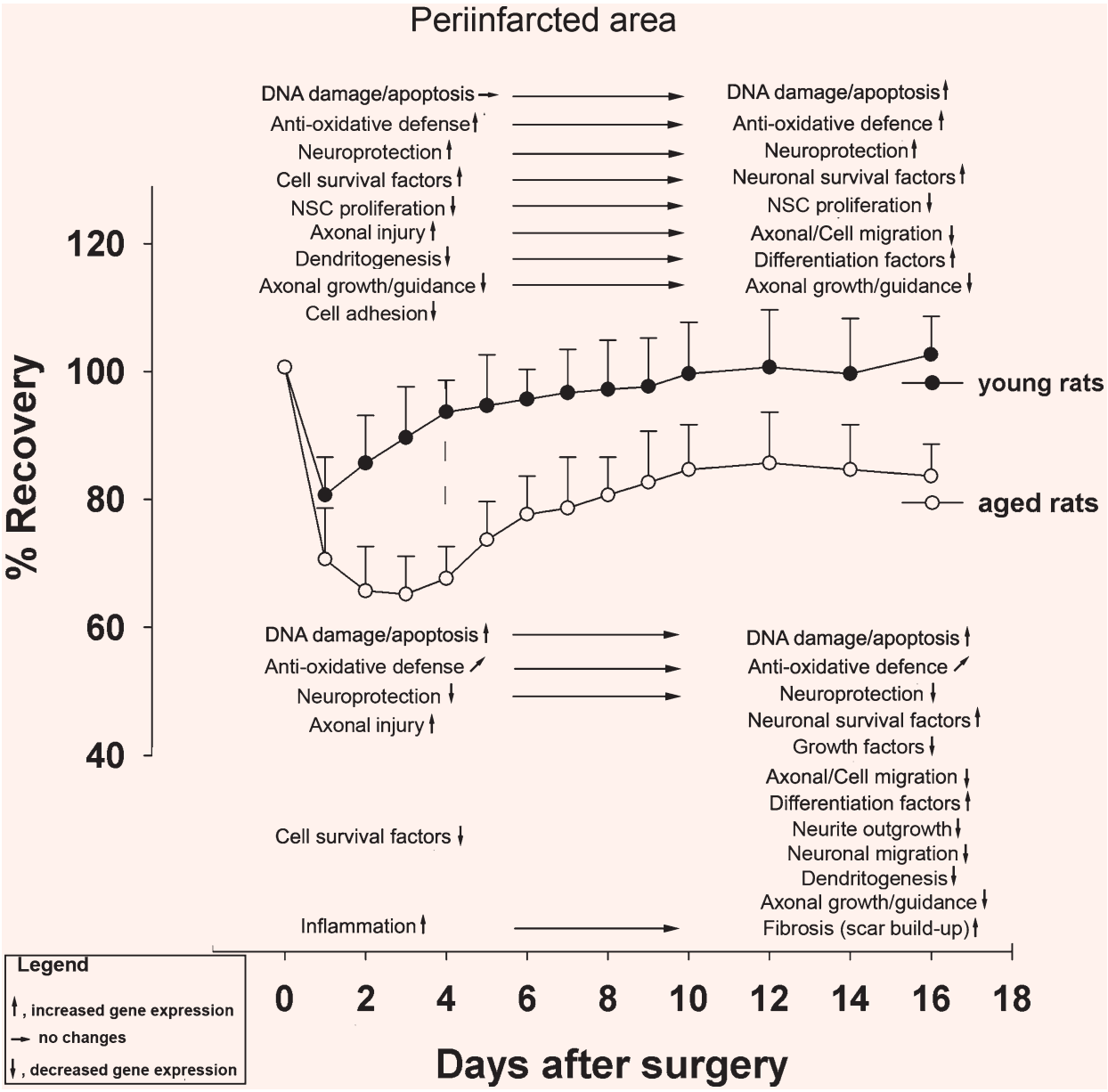

Fig. 6 Overview of major genetic events in the ipsilateral hemisphere tentatively associated with functional recovery of the after stroke in young and aged rats.

hypoperfusion region remains highly vulnerable to cell death, making it unlikely that the infarcted area could support tissue regeneration and recovery of function. Instead, we hypothesize that the contralateral sensorimotor cortex may contribute to functional recovery by taking over some function of the damaged hemisphere *via* uncrossed corticospinal tract fibres [7]. Later in the recovery process, regions adjacent to the lesioned area become activated and take over areas previously innervated by the lost neurons [114]. More complete recovery after stroke may ultimately require both the local reorganization of the ipsilateral perilesional cortex and inputs from the contralateral hemisphere [8, 9].

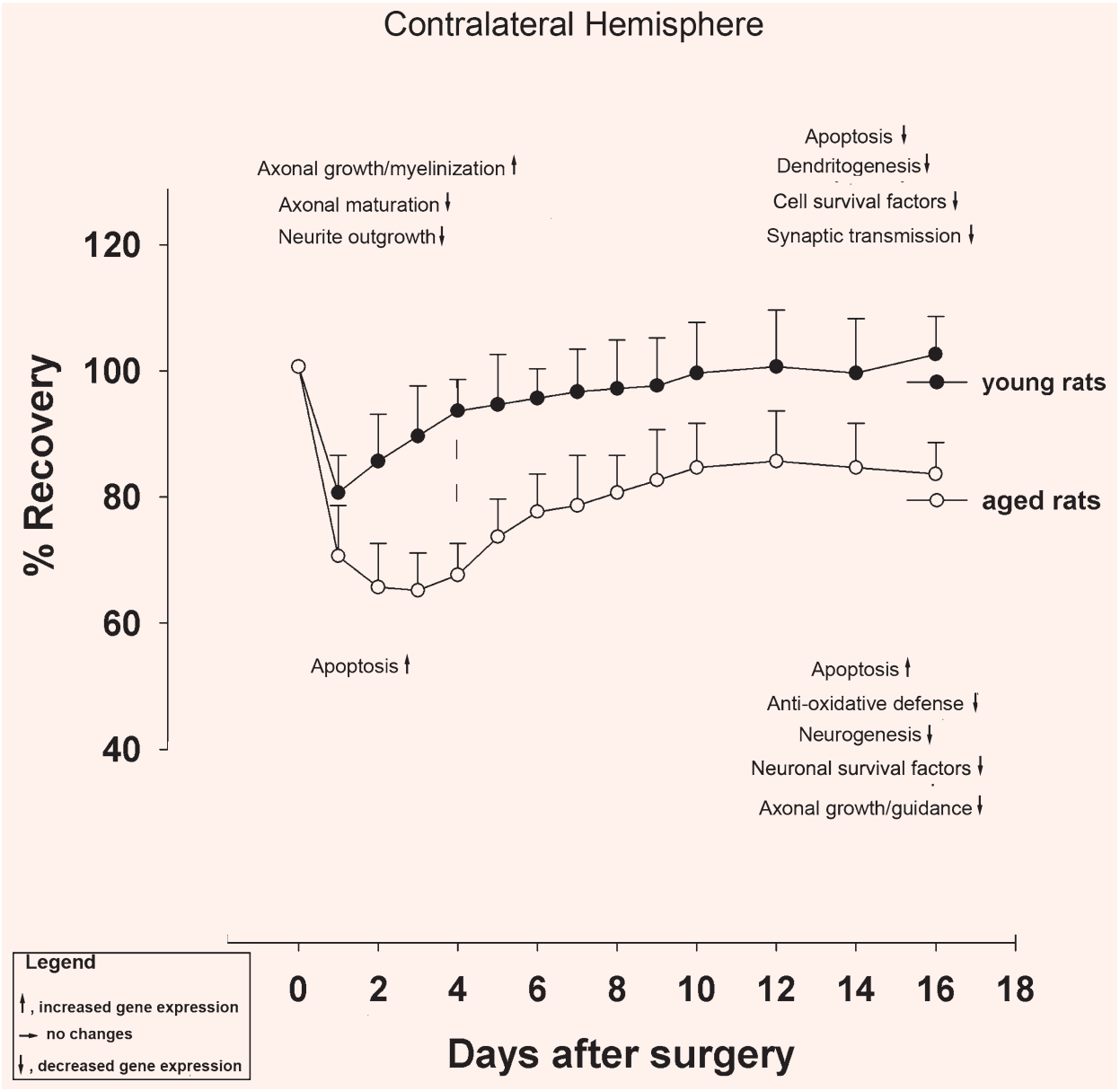

Fig. 7 Overview of major genetic events in the contralateral hemisphere tentatively associated with functional recovery of the after stroke in young and aged rats.

Profiles of gene expression after stroke are likely to be influenced by basal levels, that is by changes gene expression associated with the aging process itself.

Following insult to the brain, old rats are capable of up-regulating gene expression, but the response is often blunted and temporally uncoordinated, in comparison to the response in young rats [115–122].

Many aspects of age-related changes in gene expression in the normal aging brain have been summarized recently [123]. Recently, gene profiling in the hippocampus of young and aged rats revealed that

normal aging is associated with down-regulation of axonal growth, cytoskeletal assembly/transport, signalling and lipogenic/uptake pathways, concomitant with up-regulation in immune/inflammatory, lysosomal, lipid/protein degradation, cholesterol transport, TGF and cAMP signalling pathways in the hippocampus [124].

Conclusions

Reduced transcriptional activity in the healthy, contralateral hemisphere of aged rats, in conjunction with an early up-regulation of DNA damage-related genes and pro-apoptotic genes and down-regulation of axono and neurogenesis in the periinfarct area, is likely to account for poor neurorehabilitation after stroke in old rats. Our results suggest several ways to improve functional recovery after stroke in the aged such as transcranial stimulation of the contralateral hemisphere or application of pharmacologic agents aimed at reducing the DNA damage and apoptosis in the periinfarcted area in the first week after stroke.

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Long-term hypothermia reduces infarct volume in aged rats after focal ischemia

Baltromejus Florian[a], Raluca Vintilescu[a,c], Adrian Tudor Balseanu[a,c], Ana-Maria Buga[a,c], Olaf Grisk[d], Lary C. Walker[b], Christof Kessler[a], Aurel Popa-Wagner[a,*]

[a] Department of Neurology, University of Greifswald, Ellernholzstr. 1-2, 17487 Greifswald, Germany
[b] Yerkes National Primate Research Center and Department of Neurology, Emory University, Atlanta, GA, USA
[c] University of Medicine and Pharmacy, Craiova, Romania
[d] Department of Physiology, University of Greifswald, Germany

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ABSTRACT

In aged humans, stroke is a major cause of disability for which no neuroprotective measures are available. A viable alternative to conventional drug-based neuroprotective therapies is brain/body cooling, or hypothermia. In animal studies of focal ischemia, short-term hypothermia consistently reduces infarct size. Nevertheless, efficient neuroprotection requires long-term, regulated lowering of whole body temperature. Focal cerebral ischemia was produced by reversible occlusion of the right middle cerebral artery in 17-month-old male Sprague–Dawley rats. After stroke, the aged rats were exposed for 2 days to a mixture of air and a mild inhibitor of oxidative phosphorylation, hydrogen sulfide (H_2S), which resulted in sustained, deep hypothermia ($30.8 \pm 0.7\,^{\circ}C$). Long-term hypothermia led to a 50% reduction in infarct size with a concomitant reduction in the number of phagocytic cells. At the transcription level, hypothermia caused a reduction in the mRNA coding for caspase 12, NF-kappa B and grp78 in the peri-infarcted region, suggesting an overall decrease in the transcriptional activity related to inflammation and apoptosis. Behaviorally, hypothermia was associated with better performance on tests that require complex sensorimotor skills, in the absence of obvious neurological deficits or physiological side effects, in aged rats. *Conclusions*: Prolonged, H_2S-induced hypothermia is a simple and efficacious method to limit the damage inflicted by stroke in aged rats.

Despite promising results obtained in experiments with young animals, human stroke trials of neuroprotectants that should act after thrombolysis to limit infarct expansion and to promote tissue recovery have not yielded satisfactory results [6,14,25]. One potential explanation for this incongruity between laboratory and clinical findings is the role that age plays in the recovery of the brain from insult. Although aging is a well-known risk factor for stroke [3], the majority of experimental studies of stroke have been performed on young animals, and therefore may not fully mimic the effects of ischemia on neural tissue in aged subjects [9]. In this regard, the aged post-acute animal model is clinically most relevant to stroke rehabilitation and cellular studies [2,16,19].

A viable alternative to conventional drug-based therapies is hypothermia, either via cooling of the brain or the entire body. Studies have demonstrated that animals subjected to mild hypothermia (30–35 °C) for up to 4 h after an ischemic event have reduced mortality and neuronal injury, and improved neurological outcome [13,18,21]. However, taking into account the time-range of ischemic penumbra development, short-term hypothermia might not be sufficient to limit tissue damage. Additionally, such studies have been hampered by the lack of a simple method to pharmacologically induce long-term, regulated lowering of whole body temperature. Recently, a suspended animation-like state was induced in mice by hydrogen sulfide (H_2S), a weak, reversible inhibitor of oxidative phosphorylation that can reduce whole body temperature to as low as 15 °C [4].

Previously we have shown that the phagocytic activity of brain macrophages in the first 3 days post-stroke may contribute to the early, rapid development of the infarct in aged animals [2]. We reasoned that long-term lowering of body temperature during the first 2 days after stroke may diminish the inflammatory reaction in the brain, and as such may have a greater beneficial effect than short-term hypothermia lasting up to 4 h. We now report that the post-stroke exposure of aged rats to H_2S-induced hypothermia for 48 h causes a 50% reduction in infarct size without obvious neurological deficits or physiological side effects.

Male Sprague–Dawley rats were kept under standard laboratory conditions. A total of 24 aged (17–18 months) rats were used in this study. Body weights ranged from 520 to 600 g. The rats had free

* Corresponding author. Fax: +49 3834 866843.
E-mail address: wagnerap@uni-greifswald.de (A. Popa-Wagner).

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access to water, and food was available *ad lib* except under certain experimental conditions (see below).

To evaluate changes in neurological function associated with ischemia, locomotor function and learning ability were assessed before and after surgery. All testing was performed blindly between 9 a.m. and 11 a.m. by the same investigator. Results obtained before surgery were used to define 100% functionality for each animal on each test, and functional recovery was expressed as percent recovery relative to the pre-surgery baseline.

The Rotarod beam-walking task assesses fine vestibulomotor function in the middle cerebral artery occlusion (MCAO) model. Each rat was tested for its ability to negotiate a rotating (6 rpm) horizontal rod. The score assessment was as follows: **0**, rat falls immediately; **1**, rat does not walk forward, but stays on the Rotarod; **2**, rat walks, but falls before reaching the goal; **3**, rat traverses the rod successfully, but the limbs are used asymmetrically; **4**, the left hindlimb is used less than 50% of the time taken to traverse the rod; **5**, the rat successfully traverses the rod, but with minor difficulties; **6**, no mistakes, symmetric movements.

Spatial learning was evaluated in the labyrinth food-finding test (maze test) in which food serves as a reward for correctly navigating the maze. We assigned a score of **0** for successfully finding the baited arm. Because the apparatus was made up of 3 T-mazes, the rats could commit 1, 2 or 3 errors in each trial.

We tested the ability of each animal to maintain its position at a given angle on an inclined plane. The relative angle at which a rat could no longer maintain its position was taken as a measure of the degree of functional impairment. This test was conducted once before surgery and daily thereafter.

Blood flow through the middle cerebral artery was transiently interrupted as previously described [23] in 19 aged rats. An additional group of 5 aged rats was used for determination of H_2S concentrations in plasma and brain tissue.

After 90 min, the middle cerebral artery and the common carotid arteries were re-opened, allowing full reperfusion of the brain. One hour after the resumption of blood flow, individual rats were exposed for 48 h to an atmosphere containing 80 ppm hydrogen sulfide (H_2S) and 19.5% O_2 achieved by mixing room-air with 5000 ppm H_2S-balanced nitrogen at a flow rate of 3 l/min. H_2S is a weak, reversible inhibitor of oxidative phosphorylation that has been shown to induce a suspended animation-like state in mice by lowering the whole body temperature to 15 °C during an exposure time of 6 h [4]. Water was available during this period, although no appetitive activity was observed. Carbon dioxide, O_2 and H_2S were measured continuously using appropriate gas detectors (GfG, Dortmund, Germany) placed directly in the cage. Body temperature was measured telemetrically (DSI, Tilburg, The Netherlands). The temperature outside the experimental box was maintained at 21 °C in a well-ventilated room. Control rats were kept at 21 °C under normal atmospheric conditions. All experiments were approved by the local review board according to the standards defined by the European Communities Council Directive (86/609/EEC).

After 48 h of exposure to H_2S, 1 ml of blood was withdrawn from the left femoral artery, centrifuged, and the plasma was aspirated and stored at −65 °C prior to assay for H_2S. In addition, brain tissue collected at sacrifice (below) was homogenized in 100 mM ice-cold potassium phosphate buffer (pH 7.4). Aliquots of plasma and brain homogenate (120 μl) were mixed with distilled water (120 μl), trichloroacetic acid (10%, wt/vol, 120 μl), zinc acetate (1%, wt/vol, 60 μl), *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (20 μM; 40 μl) in 7.2 M HCl and $FeCl_3$ (30 μM; 40 μl) in 1.2 M HCl and absorbance of the resulting solution measured at 670 nm after 15 min [1]. All samples were assayed in duplicate. The H_2S concentration of each sample was calculated against a calibration curve of sodium hydrosulfide (NaHS).

Two weeks after stroke, penumbral tissue from the hypothermic and control groups was used for relative gene expression analysis of the inflammation-related genes caspase 12, NF-kappa B and grp78. Total RNA was isolated, transcribed into cDNA, and used for real-time PCR as detailed previously [12]. Real-time PCR amplification was performed as follows: one cycle of 15 min at 95 °C and 45 cycles in three steps each (95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s) using a real-time PCR cycler (MyIQ, BioRad). Each quantitative PCR was performed in triplicate. Target cDNA was amplified by primer sets of caspase 12, NF-kappa B and grp78 and a Quantitec kit supplied by Qiagen (Hilden, Germany). The rat GAPDH gene served as the normalization gene.

Sections (25 μm) were cut on a freezing microtome and processed for immunohistochemistry as free-floating material as previously described [2]. In short, after blocking in 3% donkey serum, 10 mmol/l PBS, and 0.3% Tween 20, sections were incubated overnight at 4 °C with monoclonal antibodies recognizing a cytoplasmic determinant of brain macrophages (clone ED1, 1:400; Serotec, UK) or the neuronal nuclear marker, mouse anti-NeuN (1:1000, Upstate/Chemicon, CA, USA), diluted in PBS containing 3% normal donkey serum and 0.3% Tween 20. The primary antibody was detected using the ABC system (Vectastain Elite Kit, Vector, Burlingame, CA). Infarct volume was determined in every 20th section by Nissl staining or by immunostaining with NeuN, as previously described [2].

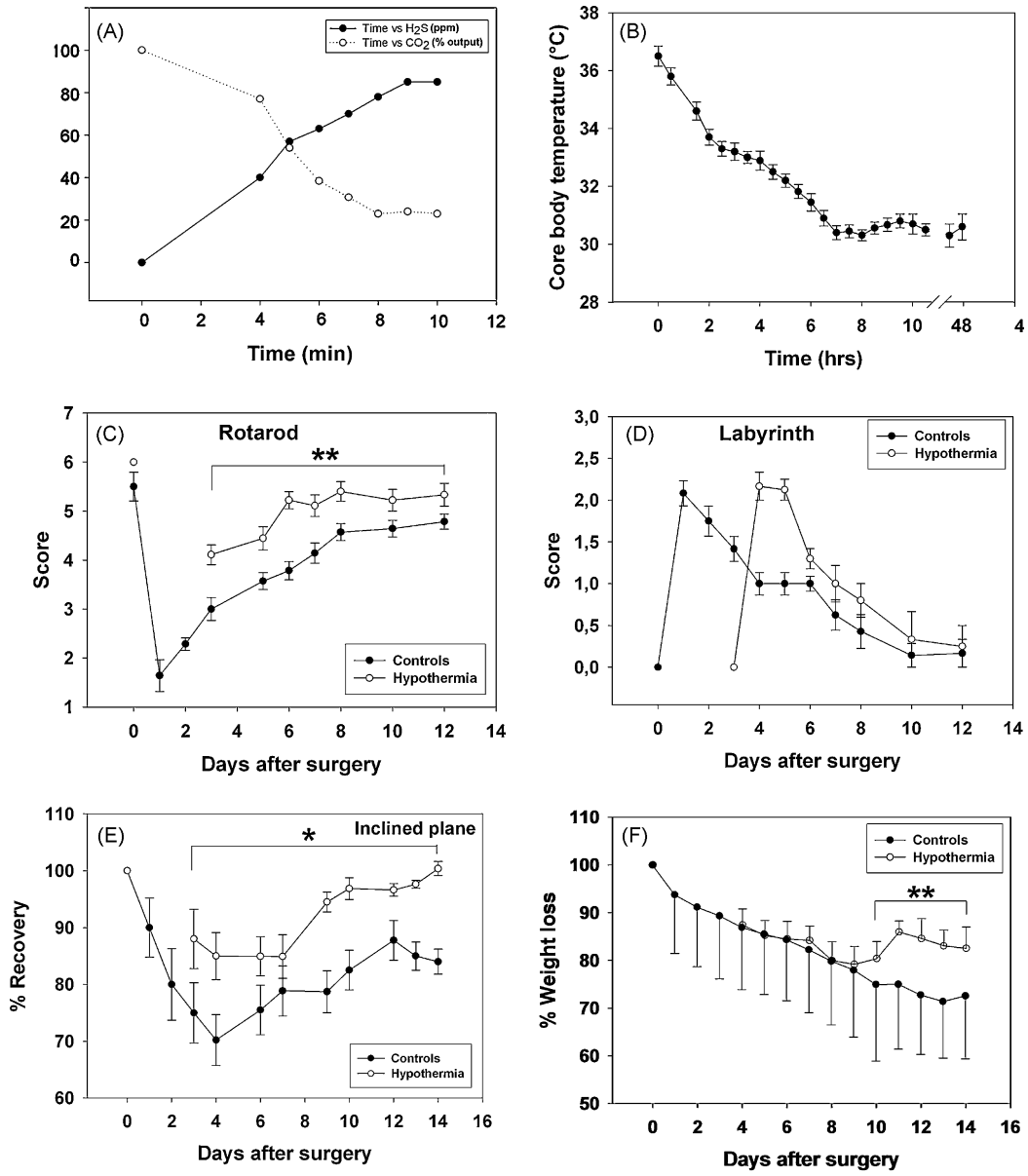
A quantitative estimate of the number of ED1-immunopositive cells of macrophagic or activated phenotype was obtained by counting the cells in 25-μm-thick sections in area units measuring 250 μm × 250 μm, employing a 'random-systematic' protocol (random start point for a systematic series of every 10th section through the infarcted volume) using LUCIA software (NIKON, Duesseldorf, Germany). The area occupied by cells of interest was 30% of the total stained area.

To eliminate false-positive particles, the cross-sectional area of nuclei was set between 70 and 110 μm^2 for activated microglia. Structures with areas lying outside this range were eliminated from the final count. Activated microglial cells and macrophages have distinct morphologies, but the cross-sectional areas of nuclei are nearly equal; therefore, the distinction between activated microglial cells and phagocytic macrophages was undertaken manually by microscopic evaluation. The somata of activated microglia measured approximately 5 μm × 18 μm. Since the tissue sections were 25 μm in thickness, ED1-positive cells were counted by focusing through the entire section. Cells in the uppermost focal plane were ignored to avoid oversampling errors by counting cell caps. An integration of ED1-positive cells was achieved by multiplying the number of counted cells per section times the section interval.

The main effect of treatment (hypothermia) was evaluated by one-way analysis of variance analyses using SPSS software (SPSS Inc., Chicago, IL). The level of significance (two-tailed threshold) was set at $p \leq 0.05$. Data are presented as means ± standard deviations.

In our experimental paradigm, carbon dioxide output declined rapidly in proportion to increasing H_2S concentrations, and reached the lowest levels after 8 min (Fig. 1A). Exposing aged rats to an atmosphere containing H_2S after stroke led to a gradual decrease in whole body temperature until it stabilized at 30.8 ± 0.7 °C after 6 h (Fig. 1B). 48 h after commencing exposure to H_2S, the animals were returned to normal environmental conditions.

After returning to room temperature, the rats recovered within minutes and did not show any signs of neurological or physiological deficits beyond those caused by the stroke. On the contrary, the recovery of complex motor function was significantly better in animals kept under hypothermia (Fig. 1C). Recovery of spatial learning,



(G) Total inhaled dose of H_2S, and plasma- and brain tissue-concentrations of H_2S after 48 hrs of exposure to 80 ppm H_2S.

Group[1]	Total inhaled dose[2]	[H_2S] in plasma[3]	[H_2S] in brain tissue[4]	Heart rate
Controls	ND	17.32 ± 3.3 µM	1.12 ± 4.6 µg/g	123.8 ± 4.6
Hypothermia	16.7 µg/min	27.5 ± 2.3 µM	2.70 ± 5.3 µg/g	64 ±7

[1]Five rats were used for these measurements
[2]Based on a ventilation rate of 150 ml/min.
[3,4]Note that hydrogen sulfide is only moderately water soluble and thus not well-absorbed by the moist tissues of the upper respiratory tract. The given value represents an increase of 19% over control levels
ND, not detectable.

Fig. 1. (A) Exposure of aged rats to an atmosphere containing H_2S after stroke led within minutes to an 80% reduction in CO_2 output. Data are from one animal, but are representative of all animals. (B) H_2S induced a decrease in body temperature in aged rats, which stabilized at 30.8 ± 0.7 °C after 6 h. (C) The recovery of complex motor function (Rotarod) was significantly improved ($p < 0.03$) in animals kept under deep hypothermia. (D) In the labyrinth test, recovery of spatial learning based on positive reinforcement, working and reference memory did not differ significantly between the two groups. (E) On the inclined plane, the beneficial effect (15% increase; $p < 0.05$) of hypothermia extended over the entire study period. (F) Body weight regulation was significantly improved in hypothermic animals post-stroke compared to animals kept at room temperature ($p < 0.025$). (G) Total inhaled dose of H_2S, and plasma- and brain tissue-concentrations of H_2S after 48 h of exposure to 80 ppm H_2S.

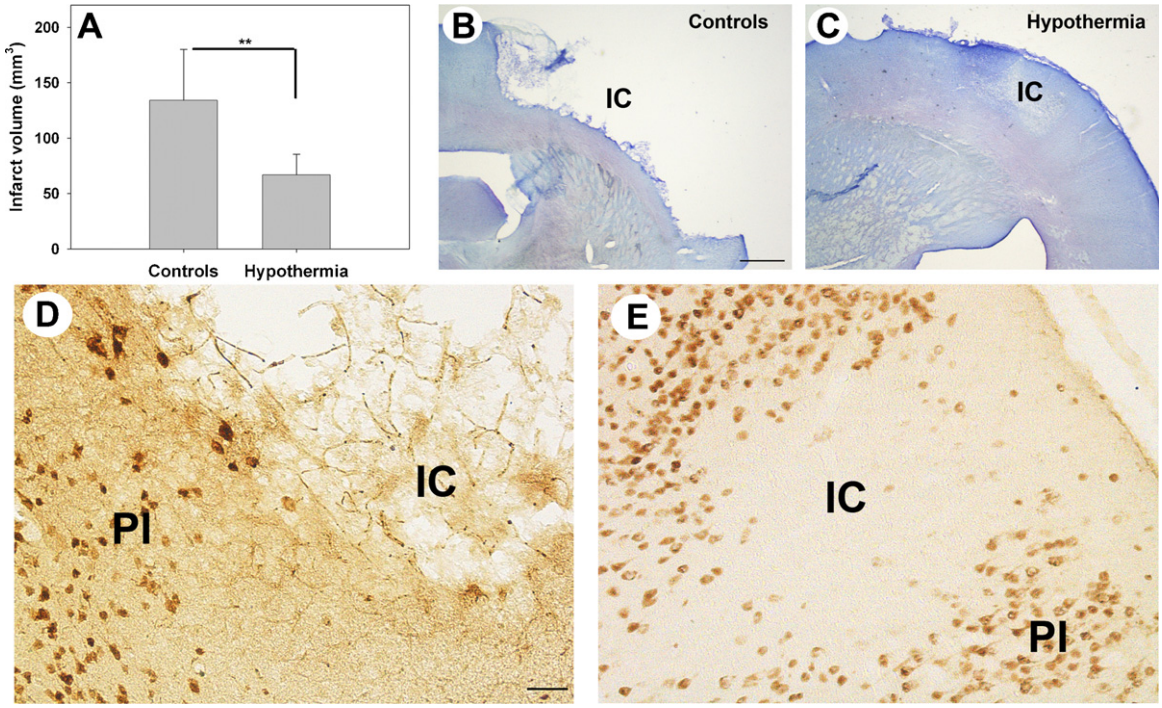


Fig. 2. (A) At two weeks post-stroke, the volume of the infarct was reduced by 50% in hypothermic animals ($p < 0.01$) as measured by Nissl staining (B vs. C) and by immunohistochemical staining with NeuN (D vs. E). Bars: 800 μm (C) and 100 μm (E). Abbreviations: IC, infarct core; PI, peri-infarct.

working memory and reference memory did not differ significantly between the two groups (Fig. 1D). On the inclined plane task, the beneficial effect (15% increase in performance over controls) of hypothermia extended over the entire study period (Fig. 1E).

After stroke, the animals ate less and body weight declined progressively, whether or not they were kept under long-term hypothermia (Fig. 1F). Nevertheless, in contrast to controls, animals kept for 2 days in the H_2S atmosphere began to regain weight by day 10 post-stroke (Fig. 1F). The concentration of sulfide in rat plasma and brain tissue was relatively constant regardless of the exposure duration. Total inhaled dose of H_2S, and plasma- and brain tissue-concentrations of H_2S after 48 h of exposure to 80 ppm H_2S, are shown in Fig. 1G.

Histologically, we found by Nissl staining (Fig. 2B and C) a significant reduction in the infarct volume of animals kept under hypothermia (Fig. 2A). Immunohistochemistry with NeuN, a marker of neuronal nuclei used to reveal fine details of the infarcted area [2], showed that the infarct core appeared to be well-preserved in animals kept under hypothermia (Fig. 2E) as compared to control animals, in which the infarct core appeared to be relatively disintegrated (Fig. 2D).

Using a monoclonal antibody specific for macrophages, we found 2 distinct populations of ED1-positive cells in the infarct core. In the control group, there were approximately equal numbers of cells displaying macrophage-like and activated microglia-like phenotypes (Fig. 3A and C), while in the hypothermia group, the activated microglia-like phenotype predominated (Fig. 3B and C).

Measurement of mRNAs for the inflammatory markers caspase 12, NF-kappa B and grp78 in the peri-infarcted region revealed that, by day 14, the transcript levels of all three inflammatory markers were significantly lower in the hypothermia group than in the control group (Fig. 3D).

We report that exposure of aged rats after stroke to H_2S-induced hypothermia for 48 h causes a 50% reduction in infarct size without causing obvious neurological or physiological side effects. In fact, the hypothermic rats resume normal activities within minutes after returning to normal environmental conditions, and they scored significantly better in tests that require complex sensorimotor skills, such as the Rotarod and inclined plane tasks, suggesting a neuroprotective effect of prolonged hypothermia. Additionally, after the first week post-stroke, rats maintained under hypothermia began to gain weight more rapidly than did control rats. It should be noted that exposure of rats to 80 ppm H_2S for 90 days did not result in toxicologically relevant gross pathology or alterations in hematological indices [10].

The physiological actions of hydrogen sulfide are not yet fully understood. H_2S is a potent vasodilator that is produced endogenously in heart and vascular tissue, and it is increasingly recognized as an important signalling molecule in the cardiovascular and nervous systems. In our aged rat stroke model, one clear effect of hypothermia was the relative preservation of the infarct core, suggesting that the phagocytic activity of microglia was diminished by the hypothermic conditions. Transcriptionally, hypothermia was associated with decreased levels of mRNAs coding for caspase 12, NF-kappa B and grp78. Unlike other caspases that have functions related to apoptosis, caspase 12 may be pro-inflammatory and is implicated in cytokine processing and the regulation of inflammation [20]. We hypothesize that a reduction in the levels of casp12 leads to decreased levels of other inflammatory mediators such as NF-kappa B and grp78, as underscored by our finding that the number of phagocytic cells is reduced in the penumbra of rats subjected to prolonged hypothermia.

Numerous studies also have demonstrated a protective effect of H_2S in myocardial ischemia [29]: (1) intravenous treatment with sodium hydrosulfide, an H_2S donor, significantly reduces myocardial infarct size [27] and contributes to cardioprotection during ischemia-reperfusion injury [28]; (2) H_2S preconditioning protects the heart against ischemia-reperfusion insults at least partly by ameliorating intracellular Ca^{2+} handling [22]; (3) the hydrogen sulfide-releasing derivative of diclofenac, S-diclofenac, had marked anti-ischemic activity in ischemic-reperfused rabbit hearts [12]; and (4) delivery of H_2S at the time of reperfusion limits infarct

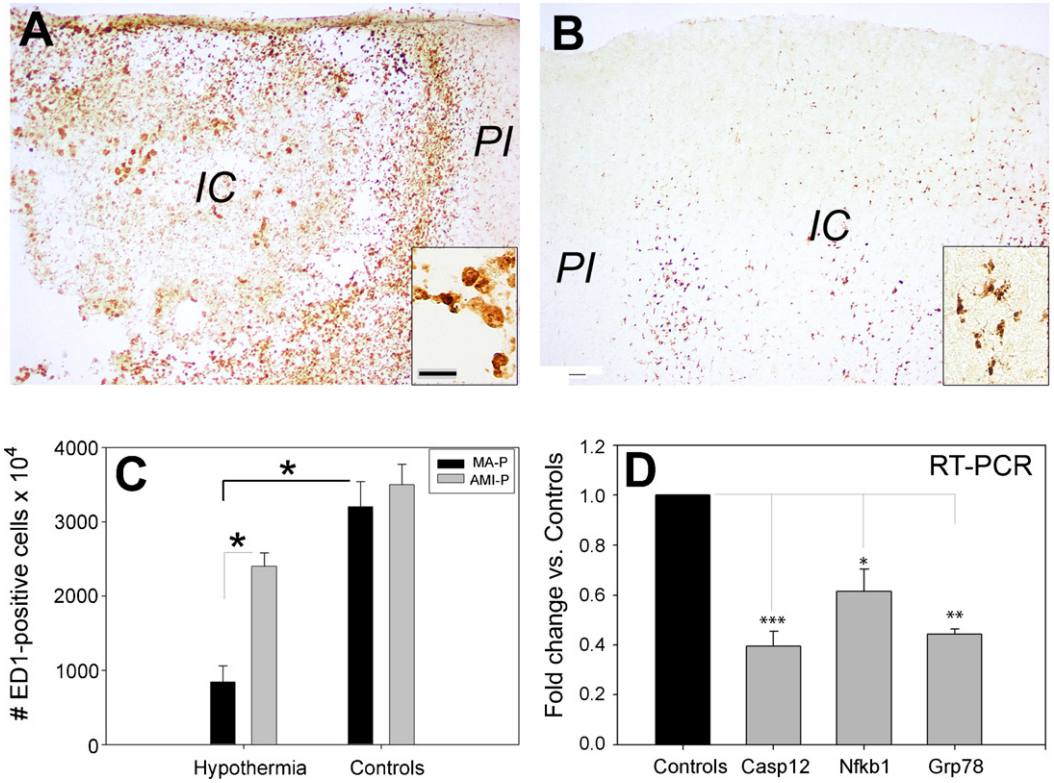


Fig. 3. Hypothermia decreases inflammatory responses to stroke. In the control group, approximately equal numbers of cells displayed activated microglia-like and macrophage-like phenotypes (A, inset), while in the hypothermia group, the activated microglia-like phenotype predominated (B, inset). The inset in (A) represents the phagocytic (macrophage-like) phenotype, and the inset in (B) represents the activated microglia phenotype. Quantitatively, the control group had 3.8 times as many macrophage-like cells as the hypothermia group (C, black-filled bars; $p < 0.01$). In the hypothermia group there were almost equal numbers of activated microglia- and macrophage-like cells (C, grey-filled bars). (D) Note the reduced levels of mRNA coding for caspase 12 (0.55-fold, $p < 0.001$), Nfkb1 (0.33-fold; $p < 0.001$) and grp78 (0.50-fold; $p < 0.001$) (grey bars) at 2 wks post-stroke in the peri-infarcted region of rats kept under hypothermia as compared to controls (black bars) ($N = 12$ for the experimental group; $N = 7$ for the control group). *Abbreviations*: *MA-P*, macrophage-like phenotype; *AMI-P*, activated microglia-like phenotype; IC, infarct core; PI, peri-infarct. Bars: 100 μm (A and B, low power images); 50 μm (insets).

size and preserves left ventricular function in an *in vivo* model of myocardial ischemia-reperfusion. This observed cytoprotection is associated with an inhibition of myocardial inflammation [11].

The effects of H_2S exposure on body temperature and oxygen consumption have been confirmed recently in a rat model of stress [17]. However, the extent to which we can extrapolate results from hypothermia studies of small mammals to large mammals is controversial. Recently, in a preclinical study of the hypothermic effect of H_2S on metabolic rate in large mammals such as pigs, 1.5 h of exposure to 80 ppm H_2S appeared to act as a hemodynamic and metabolic stimulant. Since the hypothermic effect in rodents is well established, we hypothesize that large mammals may need a longer time to manifest hypometabolic effects [15].

In humans, focal physical hypothermia, induced for a period of 1–6 h after stroke, either by intracarotid cold saline infusion (endovascular) or surface cooling, has been employed in an attempt to reduce infarct volume [7,26]. However, because of the small number of patients in these studies and the absence of matched controls, efficacy is difficult to determine. Additionally, hypothermia induced by physical means in humans is associated with body shivering, a condition that interferes with treatment [8]. Finally, the present study and others indicate that effective neuroprotection requires long-term, regulated lowering of whole body temperature. Our findings support the view that extended hypothermia has significant potential in the treatment of stroke, and should give further impetus to the evaluation of hypothermia in human clinical trials.

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Eidesstattliche Erklärung

Hiermit erkläre ich, daß ich die vorliegende Dissertation selbständig verfaßt und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät vorgelegt worden.

Ich erkläre, daß ich bisher kein Promotionsverfahren erfolglos beendet habe und daß eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

Datum
15.08.2008 Unterschrift

LEBENSLAUF

Persönliche Daten:	
Name:	**Ana-Maria Buga**
Wohnhaft in:	Ellernholzstr. 1-2; 17489 Greifswald
Geburtsdatum/-ort:	07.08.1973/Craiova/ Rumänien
Wohnort:	
Familienstand:	verheiratet; keine Kinder
Staatsangehörigkeit	rumänisch
Berufsausbildung:	
1992-1998:	Medizin-Studium an der Medizinischen Fakultät, Craiova, Rumänien
Juli 1998:	Diplom-Hauptprüfung, Gesamtnote "Sehr gut"
Berufliche Tätigkeiten:	
1998- 2000	Arzt-im-Praktikum, Krankenhaus Craiova
2001-2006	Residentin, Krankenhaus, Craiova
2006	Ernennung zum Hochschul-Assistent
an der Medizinischen Fakultät, Craiova	
Seit April 2007	Promotionsstudentin in der Klinik für Neurologie,
	Medizinische Fakultät der Ernst-Moritz-Arndt-
	Universität Greifswald
01.09.07-	Wiss. Mitarbeiterin in der Klinik für Neurologie,
	Medizinische Fakultät der Ernst-Moritz-Arndt-
	Universität Greifswald
Greifswald, 12.08.2008	Ana-Maria Buga

Publications

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Publikationsliste und Vortragsliste

1. Buga AM, Sascau M, Pisoschi C, Herndon JG, Kessler C, Popa-Wagner A.
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