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## Research Report

# Genomic response of the rat brain to global ischemia and reperfusion

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### ABSTRACT

To identify genes that are involved in ischemia response of the brain, we have evaluated changes of gene expression in rat cerebrum after 15 min complete global ischemia, followed by reperfusion for 1 h, 6 h or 24 h. The expression profiles of ~30,000 transcripts from three subjects in each group (including sham-operated controls) were monitored employing oligonucleotide microarrays. About 20,000 transcripts were detectable in rat brains. The levels of 576 transcripts (~2.9%) were significantly altered in response to experimental ischemia. 419 transcripts were up- and 157 downregulated; 39 transcripts changed after 1 h reperfusion, 174 after 6 h and 462 after 24 h. Results from quantitative real-time reverse transcription PCR of 18 selected genes showed excellent agreement with the microarray data. There is surprisingly little overlap between gene regulation patterns at different reperfusion times (only seven genes displayed significant changes in transcript levels at all reperfusion times). Several genes that were previously unknown to be involved in ischemia-response have been identified. Analyses of gene ontology patterns and the most strongly regulated transcripts showed that the immediate response to an ischemia/reperfusion is mediated by the induction of specific transcription factors and stress genes. Delayed gene expression response is characterised by inflammation and immune-related genes. These results support the hypothesis that the brain's response to ischemia is an active, specific and coordinated process.

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Abbreviations: CBF, cerebral blood flow; lCBF, local cerebral blood flow; MABP, mean arterial blood pressure; MCAO, middle cerebral artery occlusion; PCR, polymerase chain reaction; qPCR, quantitative realtime reverse transcriptase-polymerase chain reaction

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## 1. Introduction

Cerebral ischemia describes a temporal or permanent reduction of blood flow that leads to an impaired delivery of oxygen and glucose (for review, see: Dirnagl et al., 1999; Haddad and Jiang, 1993; Hansen, 1985). Global as well as focal cerebral ischemia comprise the third most common cause of death in Western countries, with an incidence for stroke of approximately 250–400 in 100,000 and a mortality rate of around 30% (Dirnagl et al., 1999). The permanent and often lethal damage of the brain is mainly the result of the death of neurons, which are particularly susceptible to any shortage of supply with metabolic energy. The immediate effect of oxygen and glucose depletion is the decrease of ATP levels, which impairs ion transport, causes the loss of membrane potential and eventually leads to the depolarisation of neurons and glia (Katsura et al., 1994; Martin et al., 1994). Depolarisation of neurons causes the release of glutamate and other excitatory amino acids into the extracellular space, which e.g. activates the NMDA receptors and leads to accumulation of  $\text{Ca}^{2+}$  in the cytosol.  $\text{Ca}^{2+}$  is at least partly responsible for long-term pathogenic effects, which include inflammation processes and programmed cell death (Dirnagl et al., 1999). After restoration of the blood flow, reoxygenation causes the generation of reactive oxygen species (ROS) which brings about additional brain damage.

DNA microarray technology has enabled the large scale analyses of gene expression patterns. This technique has been applied to study the effects of permanent or transient ischemia on total brain or distinct regions (Jin et al., 2001; Kawahara et al., 2004; Keyvani et al., 2002; Lu et al., 2004; Rickhag et al., 2006; Schmidt-Kastner et al., 2002; Sonninen et al., 2006; Soriano et al., 2000; Tang et al., 2002). The aim of the present study was to delineate the molecular response to injury of cerebral ischemia on the RNA level at different stages after the insult.

We applied a global ischemia regime to trace the expression of ischemia/reperfusion-responsive genes. We expected that global ischemia allows detecting the effects of ischemia/reperfusion without interferences by penumbra pathophysiology. Expression profiling of transcripts in the rat brain was carried out by a recently available whole-genome DNA microarray (“GeneChip® Rat Genome 230 2.0 Array” by Affymetrix), which allows the detection of about 30,000 transcripts and transcript variants of about 28,700 genes. We detected a total of 576 regulated transcripts. To the best of our knowledge, no similar large scale approach has been applied so far to identify genes involved in ischemia/reperfusion response of the brain.

## 2. Results

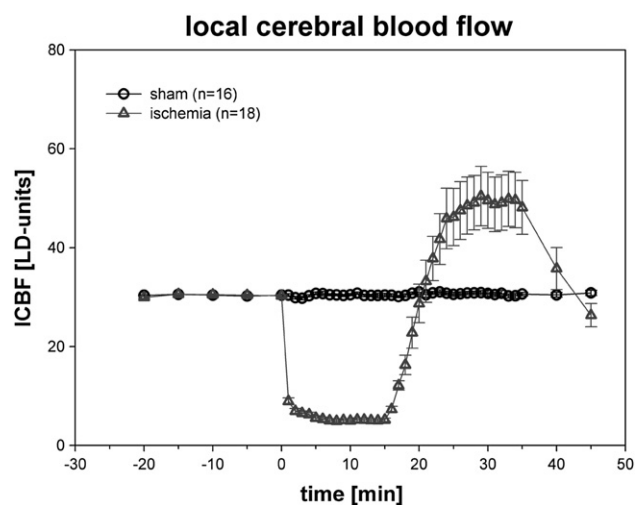
### 2.1. Physiological data

Transient global cerebral ischemia was induced in adult male rats for 15 min by bilateral occlusion of the carotid artery, followed by reperfusion for 1 h, 6 h or 24 h (six animals per group). Five or six sham operated animals that had been treated under the same conditions served as controls. The

physiological data show no statistical differences during baseline conditions neither between sham operated animals and the ischemia group, nor between the groups designated to the different survival times before brain harvest (see [Supplemental Table 1](#)). During baseline blood pressure ranged between  $83.07 \pm 1.10$  (sham) and  $84.66 \pm 0.78$  mm Hg (ischemia) and was significantly reduced to  $35.30 \pm 0.68$  mm Hg by hypotension during the ischemic period ( $82.80 \pm 1.21$  mm Hg sham) and normalized after transient ischemia ( $76.38 \pm 2.33$  mm Hg). The induction of global cerebral ischemia caused a significant decrease of CBF to ischemic values from  $30.32 \pm 0.23$  to  $5.67 \pm 0.49$  LD-units (Fig. 1). Five minutes after reperfusion CBF normalized to  $33.19 \pm 4.27$  LD-units and showed a typical hyperperfusion between 14th and 25th min ( $p < 0.05$  vs. sham) before CBF returned to baseline values at 25 and 30 min reperfusion. CBF remained unchanged in the sham operated animals. Likewise, the superfusion temperature was constant during the acute experiment ranging between  $37.06 \pm 0.12$  °C in the sham operated and  $36.96 \pm 0.11$  °C in the ischemic animals. In the sham group  $37.13 \pm 0.13$  °C were measured during baseline,  $37.03 \pm 0.11$  °C during sham-ischemia and  $37.06 \pm 0.12$  °C during reperfusion and  $37.25 \pm 0.11$  °C (baseline),  $36.82 \pm 0.11$  °C (ischemia) and  $36.99 \pm 0.09$  °C (reperfusion) in the ischemic group, respectively (all physiological data for individual groups are shown in [Supplemental Table 1](#)).

### 2.2. Gene expression profiles deduced by microarray analyses

After extraction of RNA from the left brain hemisphere, we first analysed the transcript levels of seven selected genes by means of qPCR: Arbp, Ppia, Hmox1, Hspb1, Vegf, Ngf and Cygb. Standard deviations were low in these experiments, being less than 10% within each group of six animals for most genes except for the strongly upregulated Hmox1 and Hspb1.



**Fig. 1 – Local cortical blood flow during baseline, ischemia and reperfusion. Data of the three outcome groups (1/6/24 h survival) have been pooled for demonstration of the time course during the acute phase of the experiments. For data of individual groups see [Supplemental Table 1](#). Cortical blood flow shows a typical hyperperfusion during reperfusion.**

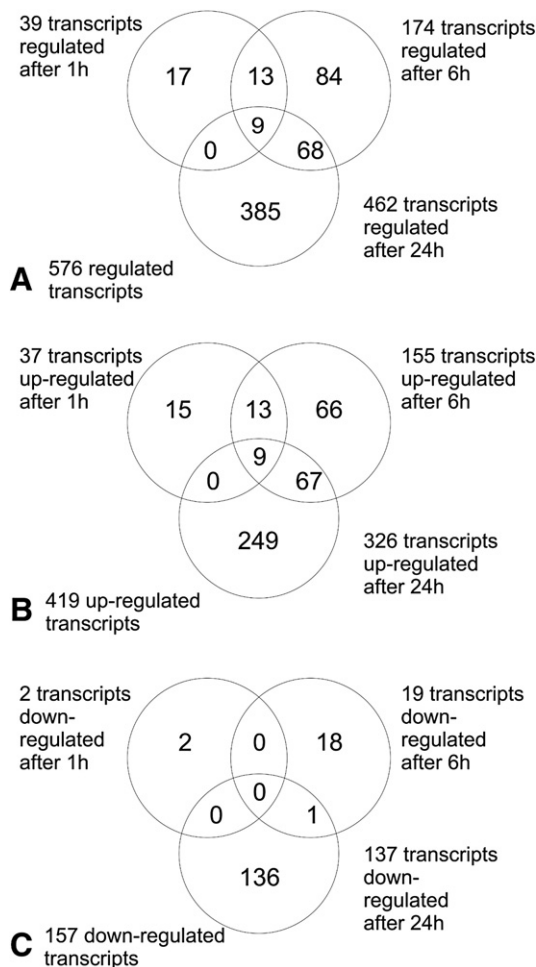
On the basis of these data, three experimental and three sham controls were selected per group and used for microarray studies; animals that showed the strongest divergence from the mean expression level for any of these genes were excluded.

The expression levels of genes in rat cerebrum were analysed employing the “GeneChip® Rat Genome 230 2.0 Array” by Affymetrix UK Ltd. (High Wycombe, UK), which represents more than 30,000 transcripts and transcript variants of about 28,700 genes. Only a part of the genome is expressed in a specific tissue such as the brain. 20,089 transcripts (about 66%) were detected in the rat cerebrum of at least five of the nine of the sham control group, applying the criteria defined by the ArrayAssist software.

The microarray data from the experimental animals were analysed in comparison to sham controls that had been treated for the same time. We found that after 1 h, 6 h or 24 h reperfusion the levels of a total of 576 transcripts were significantly altered more than or equal to twofold in response to ischemia (Fig. 2A). The complete list of transcripts that displayed expression changes in our studies can be found in Supplemental Table 2. The levels of 39 transcripts (represent-

**Table 1 – Genes most strongly regulated by brain ischemia at all times of reperfusion**

Differentially expressed genes	Gene symbol	Function	Fold change		
			1 h	6 h	24 h
Heat shock 70 kD protein 1	Hsp70-1	Chaperone	68.7	250.4	148.4
Growth arrest and DNA-damage-inducible 34	Gadd34	Cell death	3.0	6.5	6.6
v-jun sarcoma virus 17 oncogene homolog	Jun	Transcription factor	2.5	4.0	3.7
CCAAT/enhancer binding protein (C/EBP), beta	Cebpb	Transcription factor	2.5	2.6	5.7
Prostaglandin-endoperoxide synthase 2	Ptgs2	Inflammation	2.4	3.9	5.1
Protein COQ10 B	COQ10 B	Mitochondrial protein	2.1	2.3	3.0
Heat shock 40kD protein B	Hsp40-B	Chaperone	2.1	2.6	3.4



**Fig. 2 – Venn diagrams showing the total numbers of regulated genes (A), only upregulated genes (B) and only downregulated genes (C) after 15 min ischemia and 1, 6 or 24 h reperfusion.**

ing 33 distinct genes) had changed after 1 h reperfusion, 174 transcripts (158 genes) after 6 h and 462 transcripts (415 genes) after 24 h. A total number of 419 transcripts were found upregulated (Fig. 2B), 157 transcripts were downregulated (Fig. 2C). Each time of reperfusion used in this study produced a distinct genomic response in the brain: 17 transcripts had changed expression levels only after 1 h reperfusion, 84 only after 6 h and 385 after 24 h. Nine transcripts were found to have changed their levels of expression at all experimental times. These transcripts in fact represent seven distinct genes, which all showed enhanced expression (Table 1), whereas no gene was downregulated at all selected times of reperfusion. As illustrated by the Venn diagram, 13 genes showed significant expression changes after 1 h as well as after 6 h (but not after 24 h) reperfusion, 68 genes were found to be regulated only at 6 and 24 h (Fig. 2; Supplemental Table 3). There is no gene that significantly changed its expression only after 1 and 24 h, but not after 6 h reperfusion.

The maximum expression change at all times of reperfusion was found for Hspa1a (heat shock 70 kDa protein 1; note that the probe sets do not discriminate between the closely related genes Hspa1a and Hspa1b), which increased ~69- to 250-fold. The other six genes code for Gadd34 (growth arrest and DNA damage 34; also known as MyD116), the transcription factors Jun and Cebpb (CCAAT/enhancer binding protein, beta), the enzyme Ptgs2 (prostaglandin-endoperoxide synthase 2), the putative coenzyme Q10 binding protein CoQ10 B (Protein Q10 homolog B), and Dnajb1 (heat shock 40 kDa protein B). With the exception of Hspa1a and Jun, which peaked at 6 h, the transcript levels of the genes continuously increased with reperfusion time.

Other most-strongly upregulated genes (>10-fold increase; cf. Table 2) after different times of reperfusion were the transcription factors Fos (~46-fold increase after 1 h, ~13-fold increase after 6 h reperfusion; cf. Table 2) and Atf3 (12.5-fold, 6 h), Hspb1 (heat shock 27 kDa protein; ~16-fold, 6 h; ~47-fold

**Table 2 – Most strongly up-regulated genes by brain ischemia after 1 h (A), 6 h (B) and 24 h reperfusion (C)**

	Gene symbol	Function	Fold change
<b>A. Most strongly up-regulated genes after 1 h</b>			
Heat shock 70 kD protein 1	Hsp70-1	Chaperone	68.7
FBJ murine osteosarcoma viral oncogene homolog	Fos	Transcription factor	45.5
FBJ murine osteosarcoma viral oncogene homolog B	FosB	Transcription factor	7.1
Nuclear receptor subfamily 4, group A, member 1	Nr4a1	Transcription factor	6.8
Kruppel-like factor 4	Klf4	Transcription factor	4.0
Early growth response 2	Egr2	Transcription factor	4.0
B-cell translocation gene 2	Btg2	Cell cycle arrest	3.9
Jun-B	Jun-B	Transcription factor	3.9
Activity regulated cytoskeletal-associated protein	Arc	Cytoskeleton	3.1
A disintegrin-like and metalloprotease with thrombospondin type 1 motif, 1	Adamts1	Extracellular matrix	3.1
<b>B. Most strongly up-regulated genes after 6 h</b>			
Heat shock 70 kD protein 1	Hsp70-1	Chaperone	250.4
Heat shock 27 kDa protein 1	Hsp27-1	Chaperone	16.2
FBJ murine osteosarcoma viral oncogene homolog	Fos	Transcription factor	13.1
Activating transcription factor 3	Atf3	Transcription factor	12.5
Early growth response 2	Egr2	Transcription factor	10.0
Chemokine ligand 2	Ccl2	Inflammation	9.0
Growth arrest and DNA-damage-inducible 45 gamma	GADD45g	Cell death	8.5
Tumor necrosis factor receptor superfamily, 12a	Tnfrsf12a	Angiogenesis	7.9
FBJ murine osteosarcoma viral oncogene homolog B	FosB	Transcription factor	7.6
Heme oxygenase 1	Hmox1	Stress defence	7.5
<b>C. Most strongly up-regulated genes after 24 h</b>			
Heat shock 70 kD protein 1	Hsp70-1	Chaperone	148.4
Heat shock 27 kDa protein 1	Hsp27-1	Chaperone	46.6
Chemokine ligand 2	Ccl2	Inflammation	43.9
Secreted phosphoprotein 1	Spp1	Inflammation	34.6
Tissue inhibitor of metalloproteinase 1	Timp1	Extracellular matrix	33.3
Lipocalin 2	Lcn2	Inflammation	27.1
Colony stimulating factor 2 receptor, beta 1	Csf2rb1	Inflammation	20.3
Heme oxygenase 1	Hmox1	Stress defence	19.8
Growth arrest and DNA-damage-inducible 45 gamma	GADD45g	Cell death	15.4
Suppressor of cytokine signalling 3	Socs3	Inflammation	14.6

24 h), the zinc-finger protein EGR-2 (early growth response 2; ~10-fold, 6 h), the monocyte chemotactic protein Ccl2 (chemokine ligand 2; ~9-fold, 6h; ~44-fold, 24 h), secreted phosphoprotein 1 (Spp1; also known as osteopontin; ~35-fold, 24 h), the collagenase inhibitor Timp1 (tissue inhibitor of metalloproteinase 1; ~33-fold, 24 h), the modulator of inflammation Lcn2 (lipocalin 2; ~27-fold, 24 h), the interleukin 5 receptor subunit Csf2rb1 (colony stimulating factor 2 receptor, beta 1; ~20-fold, 24 h), heme oxygenase 1 (Hmox1; 8-fold, 6 h; ~20-fold, 24 h), the growth suppressor Gadd45 (growth arrest and DNA-damage-inducible 45 gamma; 9-fold, 6 h; ~15-fold, 24 h), and the SH2-containing protein Socs3 (suppressor of cytokine signalling 3; ~15-fold, 24 h).

Only two genes showed significant (about twofold) down-regulation after 1 h reperfusion (Table 3): Gnb1 (beta 1 subunit of guanine nucleotide binding protein) and the hypothetical protein LOC310764, which shows significant similarities to the *Xenopus laevis* protein Inca ("induced in neural crest by AP2"; Luo et al., 2007). After 6 h reperfusion, the most strongly downregulated gene is MEF2C (MADS box transcription enhancer factor 2, polypeptide C), for which only a tenth of the transcripts could be discovered compared to the sham control. Other downregulated genes decreased in expression by a factor of 2.5 or more (Table 3). After 24 h reperfusion, the

amount of transcript of Nufip2 (nuclear fragile X mental retardation protein interacting protein 2) was 20-fold lower compared to the sham controls. Two members of the solute carrier organic anion transporter family (Slco1a4 and Slco1c1) exhibited four- to fivefold downregulation, followed by P2ry12 (purinergic receptor P2Y, G-protein coupled 12; 3.9-fold down-regulation). Several transcripts showed an about threefold decrease: the transcription factor Neurod6 (neurogenic differentiation 6), an unknown transcribed locus (Affymetrix identifier 1396339\_at), Cml5 (putative N-acetyltransferase camello-like 5), Timp4 (tissue inhibitor of metalloproteinase 4), Camk4 (calcium/calmodulin-dependent protein kinase IV) and Dact2 (antagonist of  $\beta$ -catenin dapper).

### 2.3. Hierarchical cluster analysis

The expression patterns of the 576 transcripts that showed significant changes were hierarchically clustered. The dendrogram shows two major branches, consisting of the up- and down-regulated genes (Fig. 3A; see also: Supplemental Fig. 1). Several subclusters were observed, which contain genes that display similar kinetics of expression changes. We evaluated gene ontology of selected subsets (Figs. 3B–E). Three clusters include genes which are up-regulated in response to ischemia

**Table 3 – Most strongly down-regulated genes by brain ischemia after 1 h (A), 6 h (B) and 24 h reperfusion (C)**

	Gene symbol	Function	Fold change
<b>A. Most strongly down-regulated genes after 1 h</b>			
Guanine nucleotide binding protein, beta 1 (transducin)	Gnb1	Signalling	0.45
Hypothetical LOC310764 (Inca)	Inca	Cytoskeleton	0.50
<b>B. Most strongly down-regulated genes after 6 h</b>			
Myocyte enhancer factor 2C	Mef2c	Transcription factor	0.10
Transcribed locus	(1379711_at)	Unknown	0.37
Cyclin-dependent kinase-like 4	Cdkl4	Signalling	0.40
Transcribed locus	(1390740_at)	Unknown	0.42
Solute carrier organic anion transporter family, 1a4	Slco1a4	Transporter	0.43
Glutamate receptor, metabotropic 5	Grm5	Signalling	0.44
Hairy and enhancer of split 5	Hes5	Transcription factor	0.44
Coiled-coil domain containing 77	Ccdc77	Unknown	0.45
Potassium channel, subfamily V, member 1	Kcnn1	Transporter	0.45
Zinc finger, DHHC domain containing 21	Zdhhc21	Transcription factor (?)	0.46
<b>C. Most strongly down-regulated genes after 24 h</b>			
Nuclear fragile X mental retardation protein interacting protein 2	Nufip2	RNA-binding protein	0.05
Solute carrier organic anion transporter family, 1a4	Slco1a4	Transporter	0.19
Solute carrier organic anion transporter family, 1c1	Slco1c1	Transporter	0.24
Purinergic receptor P2Y, G-protein coupled 12	P2ry12	Signalling	0.26
Neurogenic differentiation 6	Neurod6	Transcription factor	0.28
Family with sequence similarity 19, member A1	Fam19a1	chemokine	0.28
Camello-like 5	Cml5	Enzyme	0.29
Tissue inhibitor of metalloproteinase 4	Timp4	Extracellular matrix	0.29
Calcium/calmodulin-dependent protein kinase IV	Camk4	Enzyme	0.30
Dapper homolog 2, antagonist of beta-catenin	Dact2	Signalling	0.31

(Figs. 3B–D). Most of the genes (>50%) in Fig. 3B are involved in development, others in metabolic processes. The cluster shown in Fig. 3C mainly comprises transcription factors and regulators of nucleic acid metabolism. The third cluster (Fig. 3D) comprises genes that regulate apoptosis as well as the typical stress response genes (e.g., heat shock proteins, Hmox1). The fourth subset (Fig. 3E) contains transcripts that are down-regulated after response to ischemia. These genes mainly code for proteins that are integrated in the membrane, and mediate transport processes or are involved in cell adhesion.

#### 2.4. Quantitative real-time RT-PCR

To verify the gene expression profiles as deduced by Affymetrix microarrays, we performed qPCR analyses of 18 selected transcripts. We chose up- and downregulated genes, differentially regulated genes, and non-regulated genes on the basis of the microarray data, as well as genes of particular interest (CygB, Ngb, Vegf). qPCR was performed on the same RNA samples that had been applied in microarray studies. Transcript levels were estimated on the basis of total cDNA quantities, which had been measured by PicoGreen. Brain samples from all experimental and all sham operated rats were applied; i.e., all values resulted from the analyses of three ischemic rats were compared to three sham animals. We observed excellent agreement between qPCR and microarray data (Fig. 4; Supplemental Table 4). In all samples expression changes were identical in terms of direction of variation and very similar in the extent of alteration. Only for Fos we found much stronger upregulation by qPCR, which may be due to low basic levels of expression. Standard errors of the mean of the

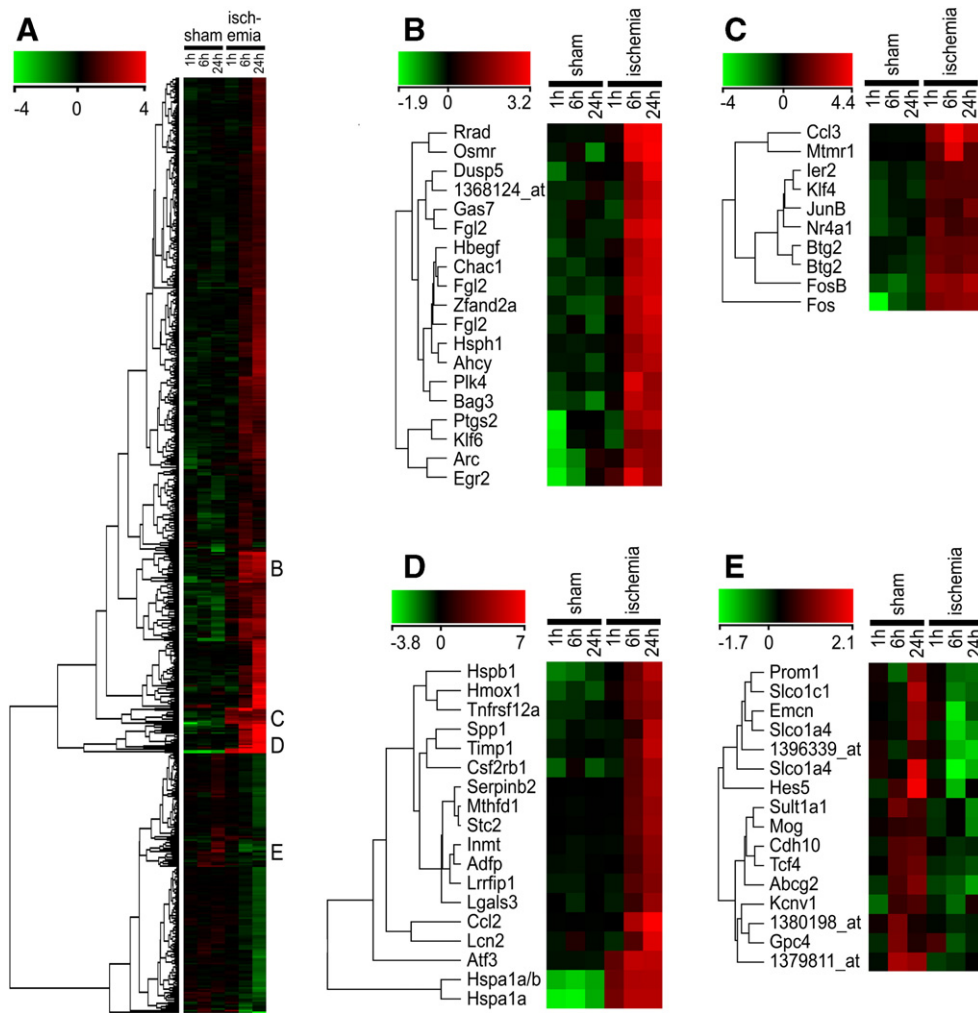
microarray data tend to be lower than those of the qPCR data. No reaction was obtained in two of the 54 qPCR assays, indicating low transcript levels or technical problems.

#### 2.5. Gene ontology of ischemia-regulated genes

We categorized the transcripts that displayed significant expression increase according to gene ontology categories (Fig. 5). We found that most of the genes with enhanced expression levels code for genes that are involved in primary metabolism (29 to 31%), followed by transcription regulators (8 to 16%), genes that are involved in morphogenesis (12 to 13%) and organ development (10 to 13%). As expected, we also observed expression changes in genes that are involved in oxidative stress (1 to 3%), cell death (6 to 10%), wound healing (5 to 6%) and immune response (5 to 10%). Although the total number of differentially expressed transcripts increased from 40 (1 h reperfusion) to 463 (24 h reperfusion), the percentages of gene ontology patterns of the three samples were similar. Some minor differences were observed, however. For example, the percentage of mRNAs that code for proteins involved in transcription regulation decreased from 16% to 8%, oxidative stress genes decreased from 3% to 1%, cell growth-related genes increased from 0% to 2%, immune response genes increased to 10%, and cell death related genes peaked after 6 h reperfusion.

### 3. Discussion

Global or focal cerebral ischemia triggers a variety of changes in brain function, metabolism and morphology (Dirnagl et al.,

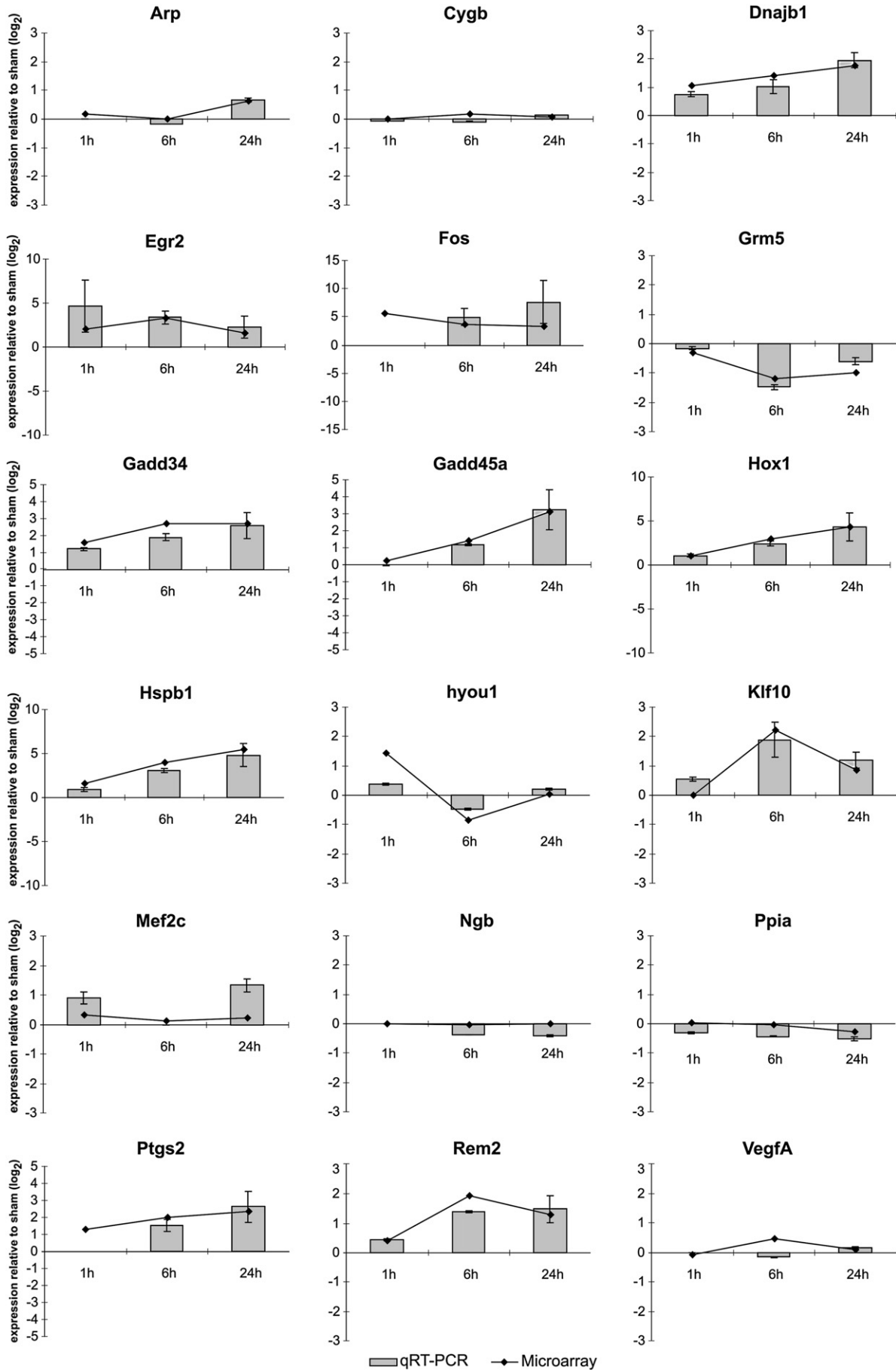


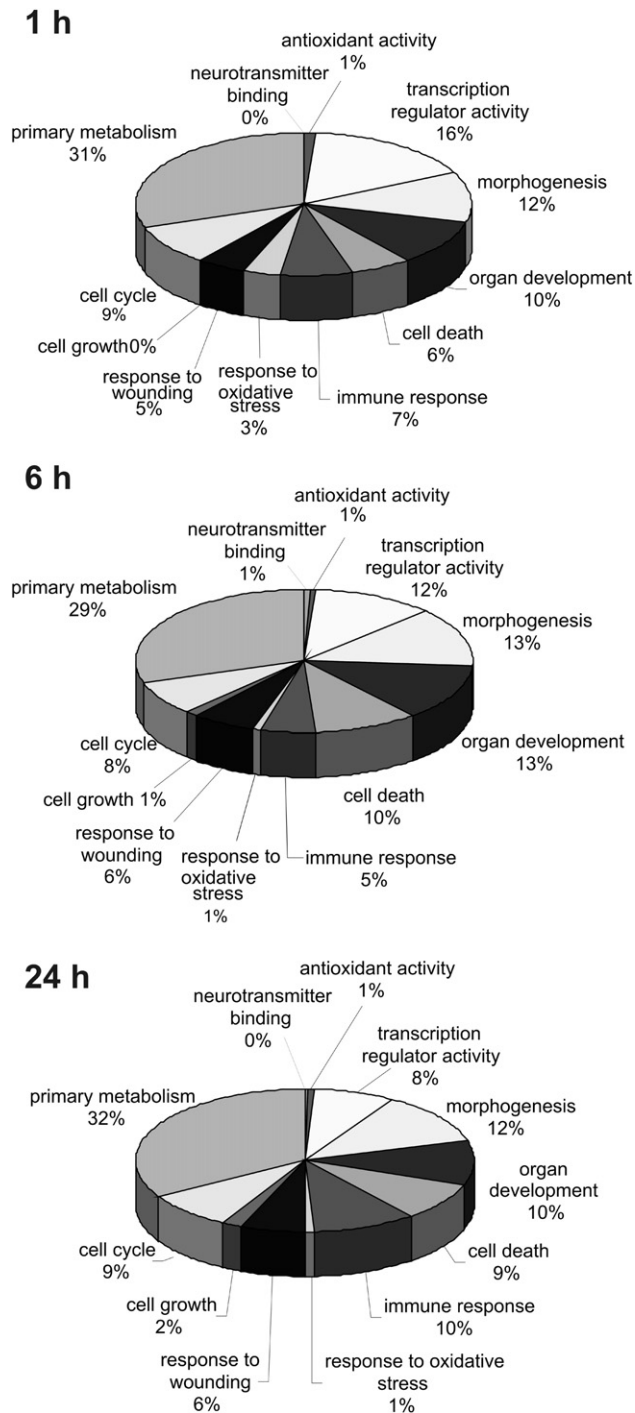
**Fig. 3** – Hierarchical clustering of the 576 differentially expressed transcripts. The red colour indicates upregulation, the deep green colour downregulation. The scale bar shows the degree of regulation in the range of the given values. Transcripts that display similar expression patterns cluster together (A). The complete cluster analysis is provided as [Supplemental Fig. 1](#). Selected subclusters are displayed in B–E. Subcluster (B) includes mainly transcripts that are upregulated after 6 and 24 h reperfusion. Gene ontology analyses indicate involvement in development and metabolic processes. Subcluster (C) shows increased expression of the genes after 1 h of ischemia, which are mainly involved in transcription regulation. The most strongly upregulated genes of this study are related to stress response (heat shock proteins and heme oxygenase) and regulation of apoptosis (subcluster D). Subcluster (E) includes transcripts that are most strongly downregulated after ischemia. These genes code for proteins that are part of membranes and mediate cell adhesion.

1999; Haddad and Jiang, 1993; Hansen, 1985). These modifications are supposedly associated with induction and repression of specific genes, of which some code for proteins that may help the brain cells to better survive the insult. Numerous studies have investigated the expression changes of single or few selected genes in response to ischemia. The microarray technology allows the genome-wide survey of gene expression changes. Previous studies applied focal ischemia induced by middle cerebral artery occlusion (MCAO) (cf. [Supplemental Table 5](#)). Focal ischemia induced by permanent MCAO were applied by [Soriano et al. \(2000\)](#) and [Tang et al. \(2002\)](#), who

analysed the changes of 750 or 8,000 transcripts, respectively, in the rat cerebrum. [Schmidt-Kastner et al. \(2002\)](#) investigated expression changes after 2 h MCAO and 3 h reperfusion employing a microarray with 9,596 transcripts, while ([Lu et al. 2004](#)) followed the time course of 1,322 genes up to seven days after 30 min or 2 h MCAO. [Rickhag et al. \(2006\)](#) used a cDNA array to investigate regional and temporal gene expression differences in rat cerebrum after transient MCAO. [Kawahara et al. \(2004\)](#) and [Sonninen et al. \(2006\)](#) focussed on expression changes in rat hippocampus. Here we used a well reproducible model of temporary global forebrain ischemia under

**Fig. 4** – Microarray results compared to real-time RT-PCR results for 18 selected genes relative to the sham control. Expression changes are depicted as  $\log_2$  values (y-axis). Error bar indicates the standard error of the mean ( $n=3$ ).





**Fig. 5 – Ontology of genes upregulated by ischemia after 1 h , 6 h , and 24 h reperfusion. The total numbers of analysed genes were 40 1 h, 175 6 h, and 463 24 h. Sequences were classified according to <http://www.geneontology.org>. Note that some genes are included in more than one class and that a single ontology group may include different genes at different times of ischemia/reperfusion.**

stringently controlled physiological conditions. This model allows the evaluation of gene expression changes induced by ischemia/reperfusion-related genes without interference by changes due to penumbra pathophysiology. However, our

method only identifies global gene expression changes in the forebrain. Cellular or local variations, which certainly contribute to the differential survival of brain regions after stroke, were not detected.

mRNA levels from ischemic rat cerebrum were monitored using the high-density microarray by Affymetrix (GeneChip Rat Genome 230 2.0 Array), which allows the detection of over 30,000 rat transcripts. To the best of our knowledge, no similar large scale study on brain ischemia has been performed so far, which is reflected by the unparalleled large number of regulated genes. While several of these genes have been identified before in previous studies with MCAO techniques, we discovered a large number of transcripts that have not been linked before to ischemia response of the brain (cf. Supplemental Table 5). Only a few genes that had been identified in previous studies were not recovered by our approach. While those genes probably reflect technical differences (e.g. operation method, reperfusion times), the additionally discovered ischemia/reperfusion regulated genes most likely also reflect the application of the high-density microarray.

By employing qPCR we carried out *a priori* standardisation of the RNA samples, which allowed the exclusion of strongly diverging samples. Together with the closely controlled physiological parameters, this strategy increased the accuracy of the microarray data with a limited sample size. The validity of our approach was further confirmed by *a posteriori* qPCR controls of 18 selected genes, which were in excellent agreement with the microarray data (Fig. 4). This view is reinforced by the fact that independent probe sets of single genes as well as distinct genes with similar functions group in cluster analyses (Supplemental Fig. 1).

### 3.1. Constitutive response to ischemia: stress proteins and transcription regulation

Seven genes are constitutively upregulated by ischemia/reperfusion, but no gene was found permanently downregulated, suggesting that the ischemia/reperfusion response of the rat brain is an active process (Fig. 2). Consistent with previous microarray studies, heat shock proteins are among the most prominently upregulated genes after the ischemic insult (Lu et al., 2004; Schmidt-Kastner et al., 2002; Tang et al., 2002). For example, Hspa1a and Dnajb1 act as molecular chaperones that promote protein folding, prevent protein aggregation and protect them from degradation (Houry, 2001). Expression induction of heat shock proteins is not restricted to ischemic insults, but had been observed in response to a variety of stresses. Hspb1 has anti-apoptotic properties because it inhibits the caspase pathway (Concannon et al., 2003).

Hmox1 is a typical stress protein with delayed transcription response (Fig. 4; Table 2). It codes for an oxidoreductase that catalyses the degradation of free heme (Ryter and Choi, 2005). Hmox-1 protects the cells from various oxidative stresses, although the exact molecular mechanism is still unknown. It has been speculated that bilirubin scavenges reactive oxygen species (ROS) (Sedlak and Snyder, 2004) and that the generated CO plays an important role as second messenger (Fujita et al., 2001). Gadd34 (constitutively induced) and Gadd45g (delayed response) are stress proteins responsive



to ER and DNA damage (Doutheil et al., 1999). Gadd34 interacts with a kinesin and Gadd45g activates a mitogen-activated protein (MAP-) kinase, thereby causing growth arrest and apoptosis.

CoQ10B is another constitutively ischemia-induced gene, which has not been linked to ischemia by previous studies. It codes for a putative coenzyme Q10 binding protein. It may be assumed that the protein is involved in the control of mitochondrial respiration, although the exact function must remain elusive.

Immediate and constitutive changes in gene expression of brain after ischemia/reperfusion are largely mediated by transcription regulation. The common response of tissues to low oxygen levels is mainly mediated by stabilization of the hypoxia-inducible transcription factor HIF-1 $\alpha$ , which then dimerizes with HIF-1 $\beta$  (Sharp and Bernaudin, 2004). These post-translational changes cannot be detected by microarray or qPCR methods. However, it can be assumed that HIF controls the transcription of a large number of genes detected in this study. Proteins belonging to the Jun and Fos families constitute the dimeric AP-1 transcription factor. While JunB, Fos and FosB were found upregulated in the early phases of reperfusion (see below), Jun was constitutively highly expressed. Jun interacts with HIF to induce hypoxia-related transcription (Alfranca et al., 2002) and has been implicated to be involved in neuronal apoptosis (Raivich and Behrens, 2006). The other permanently upregulated transcription factor was Cebpb, which is involved in control of the cell cycle and is required for cell proliferation (Greenbaum et al., 1998). Similar to the results presented here, focal ischemia was found to cause an increase of Cebpb expression in the brain (Keyvani et al., 2002; Raghavendra Rao et al., 2002). In Cebpb deficient knockout-mice, focal ischemia leads to decreased apoptosis and smaller infarcts, resulting in less neurological deficits, suggesting that Cebpb plays an important role in post-ischemic inflammation and brain damage (Kapadia et al., 2006).

### 3.2. Immediate response to ischemia is mainly mediated by transcription regulation

Gene ontology analyses showed that a significant fraction (16%) of the genes found upregulated after 1 h reperfusion code for transcription factors (Fig. 5). The three AP-1-forming proteins JunB, Fos and FosB were upregulated only in the early phases of reperfusion, suggesting their involvement in immediate response to ischemia. AP-1 is known to activate the transcription of various cytokines and many other genes that may play a role in signalling processes after ischemia. Other transcription factors upregulated in immediate response include Klf4, Egr1, Egr2, Egr4, Nr4a1, Nr4a2 and Nr4a3. Klf4, which functions as transcriptional repressor (Yet et al., 1998), had not been identified in ischemia response previously. Egr1 is a transcription factor commonly induced by hypoxia or ischemia (Yan et al., 2000). Egr1 knockout mice subjected to MCAO show smaller infarcts, improved neurological function, and less induction of inflammatory genes (Tureyen et al., 2008). Nr4a1 to 3 are nuclear proteins that belong to the steroid hormone receptor family, but may be involved in apoptotic cell death (Li et al., 2000). When Nr4a1 is

translocated into the cytoplasm, it induces cytochrome c release from mitochondria and thus may enhance apoptosis after ischemia.

### 3.3. Delayed gene expression: inflammation and apoptosis

At 6 h reperfusion, expression of genes that are involved in inflammatory reactions and immune response had commenced to increase (Table 2). Acute inflammation processes are known to start within hours after the ischemic insult and persist for days (Barone and Feuerstein, 1999; Yi et al., 2007). Macrophage and microglia-mediated inflammation are required for the removal of apoptotic and necrotic cells after ischemia (Danton and Dietrich, 2003). On the other hand, the inflammation processes increase the generation of ROS and other free radicals, which lead to additional cell death. Therefore, a balanced expression of inflammation-promoting and anti-inflammatory genes is required. In fact, we found increased expression of both groups of genes.

The strongest induction of inflammation-related genes was found after 24 h reperfusion. For example, Ccl2 was found to have increased ~9-fold after 6 h and ~44-fold after 24 h. Ccl2, which is also known as monocyte chemoattractant protein-1, plays a role in the attraction of monocytes to sites of injury and infection (Lu et al., 1998). Lcn2 is thought to bind small lipophilic substances and may function as a modulator of inflammation (Goetz et al., 2002). The Csf2rb1 gene encodes a high-affinity subunit which is shared by the receptors for interleukin-3, interleukin-5 and the granulocyte-macrophage colony-stimulating factor (Kitamura et al., 1991). Csf2rb1 is involved in the activation of macrophages and possibly other leukocytes. The Socs3 protein suppresses cytokine signalling by inhibiting the activity of Janus kinases. It has been suggested that Socs3 blocks interleukin-6 signalling, thereby repressing the activation of macrophages (Yasukawa et al., 2003). Spp1 is a key cytokine for macrophage-mediated immune response (Ashkar et al., 2000). Beck et al. (2000) demonstrated that Spp1 is induced by increasing phosphate levels, thereby providing a mechanistic explanation how it is upregulated in damaged tissue. CD44 is a transmembrane glycoprotein involved in endothelial cell recognition, trafficking of lymphocytes and regulation of cytokine gene expression. It is induced in microglia, macrophages, and microvessels after ischemia (Wang et al., 2001). MHC molecules are upregulated on microglia/macrophages after ischemia (Kato et al., 1996; Matsuoka et al., 1998) and may play a crucial role in the immune-mediated elimination of necrotic and apoptotic cells.

Other genes that showed delayed upregulation upon ischemia/reperfusion can be linked to a variety of functions, such as cell signalling, tissue remodelling or apoptosis. Notably, we found upregulation of pro-apoptotic genes like p53 and caspase-3 (Chen et al., 1998; Schmidt-Kastner et al., 2000), as well as increased expression of apoptosis-suppressing genes such as Ciapin1 (cytokine induced apoptosis inhibitor 1) and Aatf (apoptosis antagonizing transcription factor). It is very unlikely that these genes are upregulated in the same brain cells. Therefore, while cells that show irreversible damage may undergo apoptosis, the survival of other brain cells is promoted by an increased expression of anti-apoptotic genes.

### 3.4. Downregulated genes after ischemia may be associated with loss of neurons and metabolism changes

The downregulated transcripts in response to ischemia correspond to a variety of functions and their association with particular processes in the brain is not straightforward. Fewer genes were found down- than upregulated and the degrees of changes were lower. This observation corroborates the notion that brain ischemia is an active process which rather involves the induction than the repression of genes. Only two genes were found significantly downregulated after 1 h reperfusion. The putative gene *Inca* may be involved in restructuring cytoskeletal organization and in the regulation of cell adhesion (Luo et al., 2007). *Gnb1*, also known as transducin, is a  $\beta$ -subunit of G proteins that transmit extracellular signals to effector proteins. While its function in the visual process is well established, its role in brain signal processing is hitherto unknown.

Other signalling proteins were found downregulated in later stages of reperfusion, including *P2ry12*, *Grm5*, *Mtmr1*, *TAF1A-1* and *Dact2*. For example, *Grm5* is a glutamate-binding metabotropic G protein-coupled receptor that has been associated with spatial learning in the hippocampus (Lu et al., 1997). Interestingly, overexpression of metabotropic glutamate receptors protects neurons from apoptosis (Copani et al., 1995). Downregulation of *Gmr5* may therefore be associated with the loss of hippocampus neurons after ischemia. We also observed the downregulation of some transcription factor genes in later stages of reperfusion. For example, *Mef2c* and *Hes5* can be linked to neuronal development. *Mef2c* is essential for survival of newly differentiated cerebral neurons (Verzi et al., 2007). *Hes5* expression is high in the developing mouse brain but decreased upon neuronal differentiation (Takebayashi et al., 1995). The functions of *Mef2c* and *Hes5* in the adult brain are yet unknown, but one may speculate that their downregulation may either contribute to the loss of neurons upon ischemia or may be the result of this process.

Another group of downregulated genes encode for members of the solute carrier family 1 (*Slc1*), which function as organic anion exchangers for the sodium-independent transport. We also found other solute carrier genes downregulated, such as the GABA-transporter *Slc6a13* or the fatty acid transporter *Slc27a1*. Notably, still other solute carriers (inositol transporter; glucose transporter *Slc2a1*) were found upregulated (Supplemental Table 2), suggesting modified uptake of nutrients, ions and signalling molecules, and thus a profound change in the forebrain's primary metabolism.

### 3.5. Respiratory proteins do not contribute to ischemia response of the brain

Respiratory proteins such as myoglobin may enhance the supply of tissues with oxygen. Therefore, it was tempting to assume that *Ngb* and *Cygb* may be involved in the ischemia response of the brain (Burmester et al., 2000, 2002). In fact, studies by Sun et al. (2001, 2003) suggested that *Ngb* mRNA increase after an ischemic insult. However, we found no changes of *Ngb* mRNA levels in response to ischemia (see also:

Schmidt-Kastner et al., 2006). While *Cygb* expression was found enhanced by hypoxia in brain (Burmester et al., 2007), there was essentially no response of *Cygb* to ischemia. Thus it is unlikely that these proteins play a significant role in ischemia adaptation *in vivo*, in contrast to what was proposed for *Ngb* by Sun et al. (2001, 2003).

Surprisingly, neither in microarray data nor by qPCR we found a significant increase in the expression of vascular endothelial growth factor A (*VegfA*) at any time of reperfusion. *VegfA* promotes the growth of capillaries. This finding is in contrast to previous studies that identified *VegfA* as a main indicator for brain ischemia (e.g., Kovacs et al., 1996). The only possible explanation is technical differences, which may e.g. result in dissimilar induction kinetics.

### 3.6. Conclusions: coordinated gene expression response to ischemia

Lack of oxygen is a well-known threat to an organism and many animals show a well-defined hypoxia response, which comprises behavioural, physiological, biochemical and other molecular changes (for review, see e.g. Lutz and Nilsson, 2004; Nilsson and Lutz, 2004). During global ischemia of the rat brain, there is surprisingly little overlap between the regulated genes after distinct times of reperfusion (Fig. 2), i.e. most genes are up- or downregulated only in one specific time interval. This result provides support for the concept that the brain's response to ischemia is a dynamic but well-coordinated process that comprises distinct phases (Dirnagl et al., 1999). Any of these phases is characterised by the specific and coordinated expression of distinct sets of genes, which determine cell death or allow cell survival. Further characterisation of these genes will help to understand their role in normal neuronal metabolism and ischemia. Therapeutic strategies that aim at the improvement of the neurological outcome of brain ischemia must consider the temporal differences of the molecular basis of brain pathology.

## 4. Experimental procedures

### 4.1. Animals

The study was conducted according to the current animal protection legislation and was reviewed by the regional ethics committee (Bezirksregierung Rheinhessen-Pfalz, Germany). A total of 34 male Wistar R rats, weighing 280–350 g, were used for the experiments.

### 4.2. Surgery

Surgery was performed as previously described (Brambrink et al., 1999; Soehle et al., 1998). In brief, animals were anaesthetised by intraperitoneal chloral-hydrate injection (36 mg/kg/hr), orally intubated, and ventilated (100% oxygen saturation). The left common carotid artery was catheterised (PE-50 tube) for blood sampling and continuous monitoring of arterial blood pressure. The right common carotid artery was loosely looped with a nylon thread (6-0, Ethicon, Germany) for later induction of transient cerebral

global ischemia. The head of the rat was fixed in a stereotaxic frame and a small cranial window (approximately 3 mm diameter) was carefully drilled over the left hemisphere (dura intact) in order to measure local cerebral blood flow (LCBF) using a stationary laser-Doppler flow probe (BPM 2, Vasamedics, St. Paul, MN). LCBF measurements were used to confirm the onset, the quality and the persistence of ischemia during the insult. CBF measurements are expressed in laser-Doppler units (LD-units). During the acute experiment the dura was superfused with warmed saline (0.9%) and the superfusion temperature was monitored continuously within the craniotomy by a temperature probe (Licox, Integra LTD, UK). In addition the temperature was measured in the left auricular tube and the rectum (Brambrink et al., 1999). After a 20 min postsurgical stabilization period, cerebral ischemia was induced by occlusion of the right common carotid artery (tightening of the previously placed nylon thread by attaching a defined weight and by the simultaneous reduction of the mean arterial blood pressure (MABP) to 35 mm Hg by vacuum-induced venous blood pooling using an airtight chamber around the lower part of the animal's body (for hypobaric hypotension technique see: (Dirnagl et al., 1993; Heimann et al., 1994). After 15 min the nylon thread was removed and hypobaric hypotension was terminated to allow reperfusion of the brain. Physiologic variables (cerebral blood flow, MABP, temperatures) were monitored for 30 min of reperfusion. Arterial blood gases, pH, base excess, hematocrit, hemoglobin, blood glucose, and lactate levels were measured 10 min before brain ischemia (baseline) and at the end of observation period at 20 min of reperfusion. Subsequently, catheters were removed, incisions were closed and after extubation the rats were returned to their housing cages which were continuously warmed to avoid heat loss (warming lamp) for the next 1, 6 or 24 h, respectively. The following exclusion criteria were defined for baseline: MABP < 60 mm Hg, arterial oxygen tension < 99% and LCBF < 20 LD-units. During ischemia MABP had to be within 34–36 mm Hg and LCBF had to be lower than 9 LD-units, otherwise animals were excluded.

After the defined survival time rats were anesthetized deeply (Isofluran plus chloralhydrate) and the brains were harvested within 90 s. To reduce the contamination by exogenic RNase sterile instruments and gloves were used. The brain was divided into two hemispheres. The left hemisphere was frozen immediately in liquid nitrogen without brainstem and cerebellum. The samples were stored at  $-80^{\circ}\text{C}$  until further processing. Thirty four animals were randomized to sham ( $n=16$ ) and to 15 min of global cerebral ischemia ( $n=18$ ). Sham-operated animals underwent the same experimental procedures, omitting hypobaric hypotension and brain ischemia. In both groups (sham/ischemia) animals were allowed to survive 1 h ( $n=6/n=6$ ), 6 h ( $n=5/n=6$ ) or 24 h ( $n=5/n=6$ ) before sacrifice. Five brain samples per removal time point were included for qPCR analyses as well as 3 animals for the analysis by microarrays. Data are expressed as mean  $\pm$  SEM. For analysis and comparison of MABP, LCBF and temperatures areas under the curve were calculated. Then baseline-, ischemia- and reperfusion-periods were compared within and between the different groups (1 h/6 h/24 h survival time) as well as between sham and

ischemia using one way ANOVA followed by Kruskal–Wallis post hoc tests, if appropriate. Blood gases were tested using a paired or unpaired t-test (SigmaStat 3.5; Systat Software, Inc.). Differences were considered statistically significant at  $p < 0.05$ .

#### 4.3. RNA preparation

Total RNA was extracted from the left brain hemisphere without cerebellum and medulla oblongata (0.5 to 0.7 g) employing the “RNeasy Midi kit” by Qiagen (Hilden, Germany) according to the manufacturer's instructions, including an on-column digestion with RNase-Free DNase (Qiagen). The resulting RNA was precipitated with ethanol and stored at  $-80^{\circ}\text{C}$  until use. The quality and integrity of RNA was verified by gel electrophoresis and by the Agilent Bioanalyzer (Böblingen, Germany).

#### 4.4. Microarray studies

Probes were prepared according to the manufacturer's instructions (One-Cycle Target Labeling protocol/Affymetrix, Santa Clara, CA, USA). In brief, 5  $\mu\text{g}$  total RNA per sample were reverse transcribed to single stranded cDNA using T7-dT(24) promoter primer (5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG (dT)<sub>24</sub>) (Operon Biotechnologies, Cologne, Germany) and subsequently converted into double-stranded cDNA. The double-stranded cDNA product was cleaned up and in vitro transcribed to biotinylated cRNA. The IVT product was purified, fragmented and hybridized to the GeneChip Rat Genome 230 2.0 Array. Washing and staining of hybridized arrays was carried out using the automatic Gene Chip Fluidics 450 station (Affymetrix) according to the procedure protocol EukGE-WS2v5 first with nonstringent buffer (6 $\times$  SSPE: 0.9 M NaCl, 60 mM sodium phosphate, 6 mM EDTA, pH 7.4, with NaOH; 0.01% Tween 20) and thereafter with stringent buffer (100 mM MES, 0.1 M Na<sup>+</sup>, 0.01% Tween 20). For signal amplification a first staining with R-phycoerythrin streptavidin (SAPE, Molecular Probes, P/N S-866) was followed by goat biotinylated anti-streptavidin antibody (Vector, P/N BA-0500) and a second staining with SAPE. Signals were scanned by the Gene Chip Scanner 3000 7G (Affymetrix) and primary analysis was performed using Gene Chip Operating Software (GCOS) Version 1.4 (Affymetrix). For quality assurance positive controls supplied as Eukaryotic Poly-A RNA Control Kit (Affymetrix) were spiked in.

#### 4.5. Array data analysis

Data were analysed using the “ArrayAssist<sup>®</sup> 4.0 Expression Software” (Stratagene, Heidelberg, Germany) applying the GC-RMA algorithm (Wu et al., 2004). Unpaired Student's t-tests without correction for multiple testing were used for significance analysis for differential gene expression. The thresholds were 2.0 for absolute fold change and  $p < 0.05$  for the p-value. Genes identified according to these criteria as differentially expressed in ischemia versus sham for all time points were listed. Those genes that changed their expression levels most strongly up or down for each time point after ischemia compared to sham operated controls were selected. Genes

were designated according to the Affymetrix identifiers. When no known gene had been annotated by Affymetrix, own BLAST searches were performed with the ESTs provided with the identifiers. Genes were named according to the OMIM (<http://www.ncbi.nlm.nih.gov/omim>) database. Gene ontology analyses were performed using the ArrayAssist® 4.0 and classes were selected manually. The number of genes in each class was divided by the total number of genes. Hierarchical cluster analyses (Eisen et al., 1998) were performed using GeneSpring GX 9.0.6 software (Agilent Technologies, Waldbronn, Germany), applying the Euclidean centroid algorithm.

#### 4.6. Quantitative real time RT-PCR

Quantitative real-time RT-PCR was performed with a two-step protocol. First, total RNA was converted into cDNA employing Superscript II RNase H<sup>-</sup> Reverse Transcriptase (Invitrogen, Karlsruhe) and an oligo-(dT)<sub>16</sub>-primer according to manufacturer's instructions. The samples were diluted with the same volume of DNase-free water. Real-time RT-PCR experiments were carried out on ABI Prism 7000 or 7300 realtime PCR cyclers (Applied Biosystems, Darmstadt, Germany) employing the Power SYBR® Green PCR Master Mix (Applied Biosystems). Levels of acidic ribosomal phosphoprotein P0 (gene symbol: Arbp), cytoglobin (Cygbl), early growth response 2 (Egr2), FBJ osteosarcoma viral oncogene (Fos), glutamate receptor, metabotropic 5 (Grm5), growth arrest and DNA-damage-inducible 34 (Gadd34), growth arrest and DNA-damage-inducible 45 alpha (Gadd45a), heat shock 27 kDa protein 1A (Hspb1), heat shock 40 kDa protein 1 (Dnaja1), heme oxygenase 1 (Hmox1), hypoxia upregulated 1 (Hyou1), kruppel-like factor 10 (Klf10), myocyte enhancer factor 2C (Mef2C), neuroglobin (Ngb), peptidylprolyl isomerase A (Ppia), also known as cyclophilin, prostaglandin-endoperoxide synthase 2 (Ptgs2), rad and gem related GTP binding protein 2 (Rem2), vascular endothelial growth factor A (VegfA) were evaluated. The relevant cDNAs were cloned into the pCR4-TOPO-TA vector (Invitrogen) and used as standards for qPCR. Amplification of genomic DNA was avoided by using primer pairs that included one intron-spanning oligonucleotide or which consisted of intron-flanking primers. Oligonucleotide primers were obtained from SigmaGenosys (Hamburg, Germany) (see Supplemental Table 6). Reactions were run in triplicates, using 1 µl of diluted cDNA as template in a reaction volume of 30 µl. Primer concentrations were 0.13 µM for each oligonucleotide. The Taq-polymerase was activated for 10 min at 95 °C, followed by 40 cycles of a standard PCR protocol (95 °C 15 s, 60 °C 15 s, 72 °C 30 s). Efficiency of reaction was measured by the slope of a standard curve, amplification specificity by means of melting curve analysis. For visualization of qPCR, microarray, and gene ontology results we used the Microsoft Excel spreadsheet program.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.brainres.2008.10.045](https://doi.org/10.1016/j.brainres.2008.10.045). The data shown in the paper are part of the inaugural dissertation of Christian Cordes.

#### REFERENCES

- Alfranca, A., Gutierrez, M.D., Vara, A., Aragonés, J., Vidal, F., Landazuri, M.O., 2002. c-Jun and hypoxia-inducible factor 1 functionally cooperate in hypoxia-induced gene transcription. *Mol. Cell. Biol.* 22, 12–22.
- Ashkar, S., Weber, G.F., Panoutsakopoulou, V., Sanchirico, M.E., Jansson, M., Zawaideh, S., Rittling, S.R., Denhardt, D.T., Glimcher, M.J., Cantor, H., 2000. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. *Science* 287, 860–864.
- Barone, F.C., Feuerstein, G.Z., 1999. Inflammatory mediators and stroke: new opportunities for novel therapeutics. *J. Cereb. Blood Flow Metab.* 19, 819–834.
- Beck Jr., G.R., Zerler, B., Moran, E., 2000. Phosphate is a specific signal for induction of osteopontin gene expression. *Proc. Natl. Acad. Sci. U. S. A.* 97, 8352–8357.
- Brambrink, A.M., Kopacz, L., Astheimer, A., Noga, H., Heimann, A., Kempfski, O., 1999. Control of brain temperature during experimental global ischemia in rats. *J. Neurosci. Methods* 92, 111–122.
- Burmester, T., Weich, B., Reinhardt, S., Hankeln, T., 2000. A vertebrate globin expressed in the brain. *Nature* 407, 520–523.
- Burmester, T., Ebner, B., Weich, B., Hankeln, T., 2002. Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. *Mol. Biol. Evol.* 19, 416–421.
- Burmester, T., Gerlach, F., Hankeln, T., 2007. Regulation and role of neuroglobin and cytoglobin under hypoxia. *Adv. Exp. Med. Biol.* 618, 169–180.
- Chen, J., Nagayama, T., Jin, K., Stetler, R.A., Zhu, R.L., Graham, S.H., Simon, R.P., 1998. Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. *J. Neurosci.* 18, 4914–4928.
- Concannon, C.G., Gorman, A.M., Samali, A., 2003. On the role of Hsp27 in regulating apoptosis. *Apoptosis* 8, 61–70.
- Copani, A., Bruno, V.M., Barresi, V., Battaglia, G., Condorelli, D.F., Nicoletti, F., 1995. Activation of metabotropic glutamate receptors prevents neuronal apoptosis in culture. *J. Neurochem.* 64, 101–108.
- Danton, G.H., Dietrich, W.D., 2003. Inflammatory mechanisms after ischemia and stroke. *J. Neuropathol. Exp. Neurol.* 62, 127–136.
- Dirnagl, U., Thoren, P., Villringer, A., Sixt, G., Them, A., Einhaupl, K.M., 1993. Global forebrain ischaemia in the rat: controlled reduction of cerebral blood flow by hypobaric hypotension and two-vessel occlusion. *Neurol. Res.* 15, 128–130.
- Dirnagl, U., Iadecola, C., Moskowitz, M.A., 1999. Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci.* 22, 391–397.
- Doutheil, J., Althausen, S., Gissel, C., Paschen, W., 1999. Activation of MYD116 (gadd34) expression following transient forebrain ischemia of rat: implications for a role of disturbances of endoplasmic reticulum calcium homeostasis. *Brain Res. Mol. Brain Res.* 63, 225–232.
- Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D., 1998. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. U. S. A.* 95, 14863–14868.
- Fujita, T., Toda, K., Karimova, A., Yan, S.F., Naka, Y., Yet, S.F., Pinsky, D.J., 2001. Paradoxical rescue from ischemic lung injury

- by inhaled carbon monoxide driven by derepression of fibrinolysis. *Nat. Med.* 7, 598–604.
- Goetz, D.H., Holmes, M.A., Borregaard, N., Bluhm, M.E., Raymond, K.N., Strong, R.K., 2002. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol. Cell* 10, 1033–1043.
- Greenbaum, L.E., Li, W., Cressman, D.E., Peng, Y., Ciliberto, G., Poli, V., Taub, R., 1998. CCAAT enhancer-binding protein beta is required for normal hepatocyte proliferation in mice after partial hepatectomy. *J. Clin. Invest.* 102, 996–1007.
- Haddad, G.G., Jiang, C., 1993. O<sub>2</sub> deprivation in the central nervous system: on mechanisms of neuronal response, differential sensitivity and injury. *Prog. Neurobiol.* 40, 277–318.
- Hansen, A.J., 1985. Effect of anoxia on ion distribution in the brain. *Physiol. Rev.* 65, 101–148.
- Heimann, A., Kroppenstedt, S., Ulrich, P., Kempfski, O.S., 1994. Cerebral blood flow autoregulation during hypobaric hypotension assessed by laser Doppler scanning. *J. Cereb. Blood Flow Metab.* 14, 1100–1105.
- Houry, W.A., 2001. Chaperone-assisted protein folding in the cell cytoplasm. *Curr. Protein Pept. Sci.* 2, 227–244.
- Jin, K., Mao, X.O., Eshoo, M.W., Nagayama, T., Minami, M., Simon, R.P., Greenberg, D.A., 2001. Microarray analysis of hippocampal gene expression in global cerebral ischemia. *Ann. Neurol.* 50, 93–103.
- Kapadia, R., Tureyen, K., Bowen, K.K., Kalluri, H., Johnson, P.F., Vemuganti, R., 2006. Decreased brain damage and curtailed inflammation in transcription factor CCAAT/enhancer binding protein beta knockout mice following transient focal cerebral ischemia. *J. Neurochem.* 98, 1718–1731.
- Kato, H., Kogure, K., Liu, X.H., Araki, T., Itoyama, Y., 1996. Progressive expression of immunomolecules on activated microglia and invading leukocytes following focal cerebral ischemia in the rat. *Brain Res.* 734, 203–212.
- Katsura, K., Kristian, T., Siesjo, B.K., 1994. Energy metabolism, ion homeostasis, and cell damage in the brain. *Biochem. Soc. Trans.* 22, 991–996.
- Kawahara, N., Wang, Y., Mukasa, A., Furuya, K., Shimizu, T., Hamakubo, T., Aburatani, H., Kodama, T., Kirino, T., 2004. Genome-wide gene expression analysis for induced ischemic tolerance and delayed neuronal death following transient global ischemia in rats. *J. Cereb. Blood Flow Metab.* 24, 212–223.
- Keyvani, K., Witte, O.W., Paulus, W., 2002. Gene expression profiling in perilesional and contralateral areas after ischemia in rat brain. *J. Cereb. Blood Flow Metab.* 22, 153–160.
- Kitamura, T., Sato, N., Arai, K., Miyajima, A., 1991. Expression cloning of the human IL-3 receptor cDNA reveals a shared beta subunit for the human IL-3 and GM-CSF receptors. *Cell* 66, 1165–1174.
- Kovacs, Z., Ikezaki, K., Samoto, K., Inamura, T., Fukui, M., 1996. VEGF and flt. Expression time kinetics in rat brain infarct. *Stroke* 27, 1865–1872 discussion 1872–3.
- Li, H., Kolluri, S.K., Gu, J., Dawson, M.I., Cao, X., Hobbs, P.D., Lin, B., Chen, G., Lu, J., Lin, F., Xie, Z., Fontana, J.A., Reed, J.C., Zhang, X., 2000. Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. *Science* 289, 1159–1164.
- Lu, Y.M., Jia, Z., Janus, C., Henderson, J.T., Gerlai, R., Wojtowicz, J.M., Roder, J.C., 1997. Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP) but normal CA3 LTP. *J. Neurosci.* 17, 5196–5205.
- Lu, B., Rutledge, B.J., Gu, L., Fiorillo, J., Lukacs, N.W., Kunkel, S.L., North, R., Gerard, C., Rollins, B.J., 1998. Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J. Exp. Med.* 187, 601–608.
- Lu, X.C., Williams, A.J., Yao, C., Berti, R., Hartings, J.A., Whipple, R., Vahey, M.T., Polavarapu, R.G., Woller, K.L., Tortella, F.C., Dave, J.R., 2004. Microarray analysis of acute and delayed gene expression profile in rats after focal ischemic brain injury and reperfusion. *J. Neurosci. Res.* 77, 843–857.
- Luo, T., Xu, Y., Hoffman, T.L., Zhang, T., Schilling, T., Sargent, T.D., 2007. Inca: a novel p21-activated kinase-associated protein required for cranial neural crest development. *Development* 134, 1279–1289.
- Lutz, P.L., Nilsson, G.E., 2004. Vertebrate brains at the pilot light. *Respir. Physiol. Neurobiol.* 141, 285–296.
- Martin, R.L., Lloyd, H.G., Cowan, A.I., 1994. The early events of oxygen and glucose deprivation: setting the scene for neuronal death? *Trends Neurosci.* 17, 251–257.
- Matsuoka, Y., Kitamura, Y., Okazaki, M., Sakata, M., Tsukahara, T., Taniguchi, T., 1998. Induction of heme oxygenase-1 and major histocompatibility complex antigens in transient forebrain ischemia. *J. Cereb. Blood Flow Metab.* 18, 824–832.
- Nilsson, G.E., Lutz, P.L., 2004. Anoxia tolerant brains. *J. Cereb. Blood Flow Metab.* 24, 475–486.
- Raghavendra Rao, V.L., Bowen, K.K., Dhodda, V.K., Song, G., Franklin, J.L., Gavva, N.R., Dempsey, R.J., 2002. Gene expression analysis of spontaneously hypertensive rat cerebral cortex following transient focal cerebral ischemia. *J. Neurochem.* 83, 1072–1086.
- Raivich, G., Behrens, A., 2006. Role of the AP-1 transcription factor c-Jun in developing, adult and injured brain. *Prog. Neurobiol.* 78, 347–363.
- Rickhag, M., Wieloch, T., Gido, G., Elmer, E., Krogh, M., Murray, J., Lohr, S., Bitter, H., Chin, D.J., von Schack, D., Shamloo, M., Nikolic, K., 2006. Comprehensive regional and temporal gene expression profiling of the rat brain during the first 24 h after experimental stroke identifies dynamic ischemia-induced gene expression patterns, and reveals a biphasic activation of genes in surviving tissue. *J. Neurochem.* 96, 14–29.
- Ryter, S.W., Choi, A.M., 2005. Heme oxygenase-1: redox regulation of a stress protein in lung and cell culture models. *Antioxid. Redox Signal.* 7, 80–91.
- Schmidt-Kastner, R., Truettner, J., Zhao, W., Belayev, L., Krieger, C., Busto, R., Ginsberg, M.D., 2000. Differential changes of box, caspase-3 and p21 mRNA expression after transient focal brain ischemia in the rat. *Brain Res. Mol. Brain Res.* 79, 88–101.
- Schmidt-Kastner, R., Zhang, B., Belayev, L., Khoutorova, L., Amin, R., Busto, R., Ginsberg, M.D., 2002. DNA microarray analysis of cortical gene expression during early recirculation after focal brain ischemia in rat. *Brain Res. Mol. Brain Res.* 108, 81–93.
- Schmidt-Kastner, R., Haberkamp, M., Schmitz, C., Hankeln, T., Burmester, T., 2006. Neuroglobin mRNA expression after transient global brain ischemia and prolonged hypoxia in cell culture. *Brain Res.* 1103, 173–180.
- Sedlak, T.W., Snyder, S.H., 2004. Bilirubin benefits: cellular protection by a biliverdin reductase antioxidant cycle. *Pediatrics* 113, 1776–1782.
- Sharp, F.R., Bernaudin, M., 2004. HIF1 and oxygen sensing in the brain. *Nat. Rev., Neurosci.* 5, 437–448.
- Soehle, M., Heimann, A., Kempfski, O., 1998. Postischemic application of lipid peroxidation inhibitor U-101033E reduces neuronal damage after global cerebral ischemia in rats. *Stroke* 29, 1240–1246 discussion 1246–7.
- Sonninen, R., Virtanen, T., Sivenius, J., Jolkonen, J., 2006. Gene expression profiling in the hippocampus of rats subjected to focal cerebral ischemia and enriched environment housing. *Restor. Neurol. Neurosci.* 24, 17–23.
- Soriano, M.A., Tessier, M., Certa, U., Gill, R., 2000. Parallel gene expression monitoring using oligonucleotide probe arrays of

- multiple transcripts with an animal model of focal ischemia. *J. Cereb. Blood Flow Metab.* 20, 1045–1055.
- Sun, Y., Jin, K., Mao, X.O., Zhu, Y., Greenberg, D.A., 2001. Neuroglobin is up-regulated by and protects neurons from hypoxic–ischemic injury. *Proc. Natl. Acad. Sci. U. S. A.* 98, 15306–15311.
- Sun, Y., Jin, K., Peel, A., Mao, X.O., Xie, L., Greenberg, D.A., 2003. Neuroglobin protects the brain from experimental stroke in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 100, 3497–3500.
- Takebayashi, K., Akazawa, C., Nakanishi, S., Kageyama, R., 1995. Structure and promoter analysis of the gene encoding the mouse helix–loop–helix factor HES-5. Identification of the neural precursor cell-specific promoter element. *J. Biol. Chem.* 270, 1342–1349.
- Tang, Y., Lu, A., Aronow, B.J., Wagner, K.R., Sharp, F.R., 2002. Genomic responses of the brain to ischemic stroke, intracerebral haemorrhage, kainate seizures, hypoglycemia, and hypoxia. *Eur. J. Neurosci.* 15, 1937–1952.
- Tureyen, K., Brooks, N., Bowen, K., Svaren, J., Vemuganti, R., 2008. Transcription factor early growth response-1 induction mediates inflammatory gene expression and brain damage following transient focal ischemia. *J. Neurochem.* 105, 1313–1324.
- Verzi, M.P., Agarwal, P., Brown, C., McCulley, D.J., Schwarz, J.J., Black, B.L., 2007. The transcription factor MEF2C is required for craniofacial development. *Dev. Cell* 12, 645–652.
- Wang, H., Zhan, Y., Xu, L., Feuerstein, G.Z., Wang, X., 2001. Use of suppression subtractive hybridization for differential gene expression in stroke: discovery of CD44 gene expression and localization in permanent focal stroke in rats. *Stroke* 32, 1020–1027.
- Wu, Z., Irizarry, R.A., Gentleman, R., Murillo, F.M., Spencer, F., 2004. A model based background adjustment for oligonucleotide expression arrays. *J. Am. Stat. Assoc.* 99, 909–917.
- Yan, S.F., Fujita, T., Lu, J., Okada, K., Shan Zou, Y., Mackman, N., Pinsky, D.J., Stern, D.M., 2000. Egr-1, a master switch coordinating upregulation of divergent gene families underlying ischemic stress. *Nat. Med.* 6, 1355–1361.
- Yasukawa, H., Yajima, T., Duplain, H., Iwatate, M., Kido, M., Hoshijima, M., Weitzman, M.D., Nakamura, T., Woodard, S., Xiong, D., Yoshimura, A., Chien, K.R., Knowlton, K.U., 2003. The suppressor of cytokine signaling-1 (SOCS1) is a novel therapeutic target for enterovirus-induced cardiac injury. *J. Clin. Invest.* 111, 469–478.
- Yet, S.F., McA’Nulty, M.M., Folta, S.C., Yen, H.W., Yoshizumi, M., Hsieh, C.M., Layne, M.D., Chin, M.T., Wang, H., Perrella, M.A., Jain, M.K., Lee, M.E., 1998. Human EZF, a Kruppel-like zinc finger protein, is expressed in vascular endothelial cells and contains transcriptional activation and repression domains. *J. Biol. Chem.* 273, 1026–1031.
- Yi, J.H., Park, S.W., Kapadia, R., Vemuganti, R., 2007. Role of transcription factors in mediating post-ischemic cerebral inflammation and brain damage. *Neurochem. Int.* 50, 1014–1027.