RESOURCE ARTICLE

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Unraveling the ischemic brain transcriptome in a permanent middle cerebral artery occlusion mouse model by DNA microarray analysis

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SUMMARY

Brain ischemia, also termed cerebral ischemia, is a condition in which there is insufficient blood flow to the brain to meet metabolic demand, leading to tissue death (cerebral infarction) due to poor oxygen supply (cerebral hypoxia). Our group is interested in the protective effects of neuropeptides for alleviating brain ischemia, as well as the underlying mechanisms of their action. The present study was initiated to investigate molecular responses at the level of gene expression in ischemic brain tissue. To achieve this, we used a mouse permanent middle cerebral artery occlusion (PMCAO) model in combination with high-throughput DNA microarray analysis on an Agilent microarray platform. Briefly, the right (ipsilateral) and left (contralateral) hemispheres of PMCAO model mice were dissected at two time points, 6 and 24 hours post-ischemia. Total RNA from the ischemic (ipsilateral) hemisphere was subjected to DNA microarray analysis on a mouse whole genome 4x44K DNA chip using a dye-swap approach. Functional categorization using the gene ontology (GO, MGD/AMIGO) of numerous changed genes revealed expression pattern changes in the major categories of cellular process, biological regulation, regulation of biological process, metabolic process and response to stimulus. Reverse-transcriptase PCR (RT-PCR) analysis on randomly selected highly up- or downregulated genes validated, in general, the microarray data. Using two time points for this analysis, major and minor trends in gene expression and/or functions were observed in relation to early- and late-response genes and differentially regulated genes that were further classified into specific pathways or disease states. We also examined the expression of these genes in the contralateral hemisphere, which suggested the presence of bilateral effects and/or differential regulation. This study provides the first ischemia-related transcriptome analysis of the mouse brain, laying a strong foundation for studies designed to elucidate the mechanisms regulating ischemia and to explore the neuroprotective effects of agents such as target neuropeptides.

INTRODUCTION

Brain ischemia, also known as cerebral ischemia or ischemic stroke, is the third most common cause of death worldwide, after heart attack and cancer, resulting in major negative social and economic consequences. Ischemic stroke, which results from cardiac arrest, cerebral arterial occlusion or severe vasospasm after subarachnoid ischemia, causes devastating damage to the brain and represents a serious global health problem. Briefly, brain ischemia is a condition in which there is insufficient blood flow to meet metabolic demands. It is known that an interruption of blood flow to the brain for more than 10 seconds results in a loss of consciousness, leading to ischemia and irreversible brain damage. The most common cause of stroke is the sudden occlusion of a blood vessel by a thrombus or embolism, resulting in an almost immediate loss of oxygen and glucose to the cerebral tissue. Ischemia can be classified as either focal or global. Focal ischemia is confined to a specific lesion, whereas global ischemia encompasses a wide area of the brain (see Gusev and Skvortsova, 2003).

Given the clinical importance of ischemia, it is not surprising that its causes, diagnosis and treatment are the focus of a major international research effort (see Liebeskind, 2008; Slemmer et al., 2008; Dogrukol-Ak et al., 2009; Indraswari et al., 2009; Kim et al., 2009; Chauveau et al., 2010; Gupta et al., 2010; Henninger et al., 2010; Rymner et al., 2010; Cucchiara and Kasner, 2011; Yenari and Hemmen, 2010; Fisher, 2011; Kunst and Schaefer, 2011; Leiva-Salinas et al., 2011; Molina, 2011; Ramos-Fernandez et al., 2011; Turner and Adamson, 2011; Wechsler, 2011). To provide an idea of the volume of research carried out in this area, a keyword search on May 16th, 2011 using the PubMed National Center for Biotechnology Information (NCBI) search engine revealed 78,103 articles containing the search term 'brain ischemia'. More specifically, there were 58,357 articles containing both 'brain ischemia' and 'human', and 28,253 containing both 'brain ischemia'

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and 'animal', whereas 'brain ischemia' and 'rat', and 'brain ischemia' and 'mouse' revealed 9109 and 3912 articles, respectively. The subject has also been widely reviewed, with a PubMed search using both the keywords 'brain ischemia' and 'review' resulting in a total of 9478 articles. In order to understand the pathology of stroke, targeted gene expression and proteomics studies have been carried out to investigate the role of particular genes and proteins. Decreased blood flow during ischemia activates the synthesis of various genes and proteins that regulate the ischemic process and/or are involved in the broader cellular response, depending on the extent of the injury. Among these molecular factors, we are also likely to find potentially protective genes and/or proteins.

Our group has been working on ischemic models since 1994 (Mizushima et al., 1994; Shimazu et al., 1994), with the goal of understanding the mechanisms underlying ischemia and identifying molecular targets for its prevention or amelioration, including the use of neuropeptides such as pituitary-adenylate-cyclase-activating polypeptide (PACAP). This has led to the successful characterization of certain molecular components involved in cerebral ischemia and the establishment of PACAP as a potential therapeutic agent for its prevention (Shioda et al., 1998; Ohtaki et al., 2003; Ohtaki et al., 2006; Ohtaki et al., 2007; Ohtaki et al., 2008a; Ohtaki et al., 2008b; Nakamachi et al., 2005; Nakamachi et al., 2010). The crucial issue now is to elucidate exactly how any identified neuroprotective molecules exert their effects.

Over the last decade, and particularly within the last 3 years, researchers have begun focusing their attention on genomic approaches to investigate brain ischemia and physiological responses to it (Jin et al., 2001; Büttner et al., 2008; Meschia, 2008; Qin et al., 2008; Juul et al., 2009; Grond-Ginsbach et al., 2009; Haramati, 2009; Nakajima, 2009; Popa-Wagner et al., 2009; Yao et al., 2009; Di Pietro et al., 2010; Pruissen et al., 2009; Stamova et al., 2010; Zhan et al., 2010). These studies have shown the importance of high-throughput transcript profiling, such as the DNA microarray technique (DeRisi et al., 1997), for gaining insight into the ischemic brain. In the present study, we have taken a different approach to the problem, and have adopted a whole genome DNA microarray analysis approach (Masuo et al., 2011b) as a means of providing an inventory of the time-dependent changes in global gene expression that occur at the mRNA level in mice in response to ischemia. To achieve this, we have utilized a mouse model of permanent middle cerebral artery occlusion (PMCAO) (Nakamachi et al., 2005; Nakamachi et al., 2010) (see supplementary material Fig. S1). Briefly, total RNA was extracted at 6 and 24 hours from very fine powders of post-ischemic brain tissue, then subjected to DNA microarray analysis using a mouse whole genome DNA chip (4x44K) with a dye-swap approach, followed by identification of changes in gene expression. Our results revealed a large number of highly up- and downregulated genes, whose involvement in the progression of and response to ischemia is discussed in this paper.

RESULTS AND DISCUSSION

PMCAO-generated ischemia model mice

PMCAO (supplementary material Fig. S1B,D) was performed on 13 male mice (C57/BL6J), and the results of the procedure were monitored by dissecting whole brains and visualizing the extent of ischemia. Eleven mice (84.6%) survived the PMCAO procedure (data not shown). In addition, a total of ten sham (control) mice were used. We also injected 0.9% saline intracerebroventrically in both the sham control and PMCAO model mice, consistent with our long-term goal of determining whether various neuropeptides can reverse the effects of ischemia. Injection of saline was to the left hemisphere (contralateral; supplementary material Fig. S1C). The ischemic region was visualized by 2% 2,3,5-triphenyltetrazolium chloride (TTC; Wako, Tokyo, Japan) in some mice to confirm the ischemic brain damage (supplementary material Fig. S1E). Moreover, we also checked for neurological deficiency 24 hours after PMCAO, using a routinely used methodology in our laboratory (Ohtaki et al., 2006; Dogrukol-Ak et al., 2009). Results showed neurological deficiency in 85% of these animals (PMCAO mice); these were used for dissection of the brain tissues. After perfecting the technique, mice were divided into four groups of three mice each for the control and PMCAO cohorts, and whole brains were dissected following 6 or 24 hours of ischemia. The ipsilateral (right hemisphere; non-injected) and contralateral (left hemisphere; injected with saline) hemispheres without olfactory bulb (OB) and cerebellum (supplementary material Fig. S1F,G) were quickly separated, placed in 2.0 ml Eppendorf tubes, and deep frozen in liquid nitrogen.

Overview of the brain genomic response to ischemia

Quality of total RNA and level of GAPDH and β-actin genes in the brain hemispheres

To investigate global changes in gene expression in ischemic hemispheres, we first optimized a protocol for total RNA extraction (Fig. 1; supplementary material Figs S2, S3). Here we would like to clarify that we refer to the ischemic brain hemisphere (ipsilateral) as consisting of the infarct core, penumbra and non-ischemic region under the present experimental design and sampling of the brain tissue thereof. This provides an overall picture of the ischemic brain hemisphere rather than one specific ischemic region. Nevertheless, in the future it would be interesting to examine specific ischemic regions and compare them with non-ischemic regions in the same hemisphere. The quantity and quality of the total RNA, a crucial factor in further downstream analyses, was confirmed, and this RNA was then used for synthesizing cDNA. Prior to DNA microarray analysis, we examined the expression of two commonly used house-keeping genes, namely glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and β-actin (supplementary material Table S1), in both hemispheres at 6 and 24 hours. This allowed us to subsequently use these two genes as positive controls rather than simply loading or using internal controls (see Suzuki et al., 2000). This simple test of gene expression showed that the mRNAs for *GAPDH* and β-actin were expressed almost uniformly across conditions (supplementary material Fig. S4). Following this preliminary analysis of sample quantity and quality, we proceeded to conduct a DNA microarray analysis using the ischemic (ipsilateral) brain hemisphere.

Changes in gene expressions in the ischemic brain and their functional categorization

Using the total RNA in the ischemic hemisphere and the 4x44K mouse whole genome DNA microarray chip in conjunction with a dye-swap approach (as explained in the Methods), genome-wide global gene expression profiles were obtained for ischemia-related

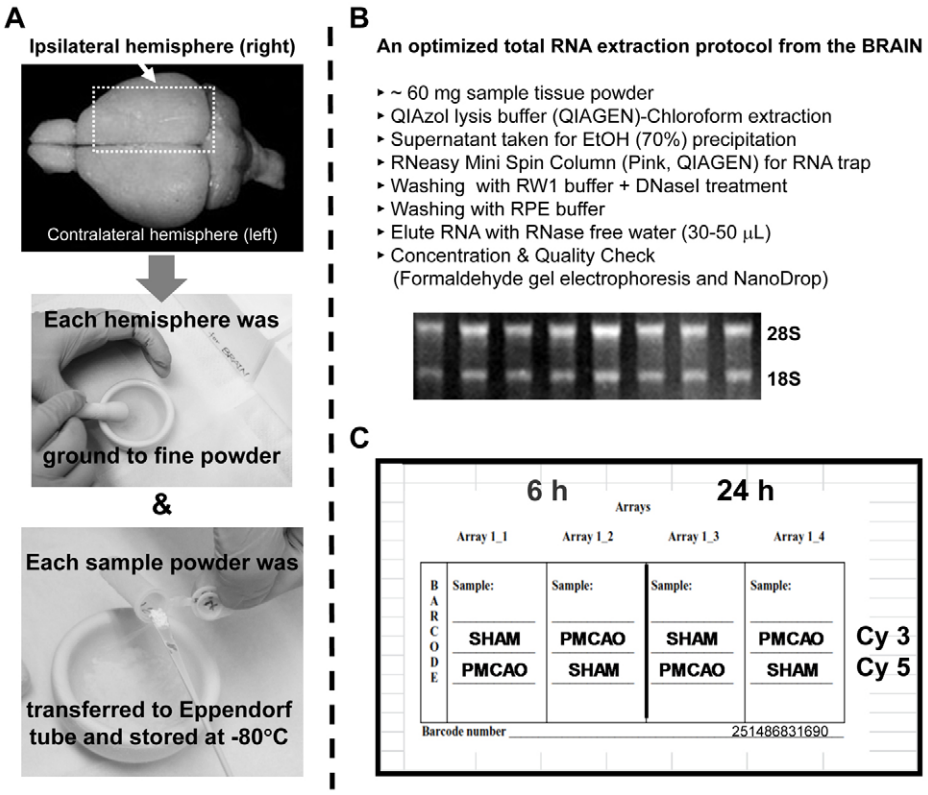

Fig. 1. Workflow from sampling and grinding of the brain hemisphere, total RNA extraction, and DNA microarray analysis of the ischemic brain (ipsilateral hemisphere). (A) Mouse whole brain showing the ipsilateral (right; boxed region) and contralateral (left) hemispheres. Brain tissues were ground to a fine powder in liquid nitrogen and stored at –80°C. (B) Total RNA extraction from the finely powdered brain tissues. Total RNA quality was confirmed by both agarose-gel electrophoresis and NanoDrop spectrophotometry. (C) DNA microarray chip showing the hybridized sample combinations (Sham × PMCAO at 6 and 24 hours) and dye-swap (Cy3 vs Cy5).

genes. The results revealed 1237 and 2759 cases of gene induction (>1.5-fold) as compared with 620 and 2102 cases of gene suppression (<0.75-fold) at 6 and 24 hours after PMCAO, respectively (Fig. 2). These genes are shown in supplementary material Tables S2-S5. For detailed information on the changed gene expression profiles, readers are referred to the total gene expression data files at the NCBI GEO data (GSE 28201) repository, submitted as part of this publication. Interestingly, the majority of these changes were unique to either the 6- or 24-hour time points, reflecting the progression of ischemia with time. Nevertheless, 792 and 167 genes were found to be commonly upregulated or downregulated at both 6 and 24 hours (Fig. 2; supplementary material Tables S6, S7).

We next categorized the affected genes on the basis of their function under gene ontology (GO) terms of biological process (3440 and 6826 induced genes, and 1398 and 3531 suppressed genes at 6 and 24 hours, respectively), molecular function (875 and 1732 induced genes, and 394 and 1021 suppressed genes at 6 and 24 hours, respectively) and cellular component (1788 and 3522 induced genes, and 762 and 2015 suppressed genes at 6 and 24 hours, respectively). The functionally categorized genes are presented graphically in Fig. 3. It should be noted that the same gene was sometimes included in different functional categories, thus making the total numbers seem inflated. In the major category of biological process, the subcategory of cellular process showed the largest number of gene changes (induction and suppression), followed by biological regulation, regulation of biological process, and metabolic process. In the second major category of molecular function, the binding activity subcategory included the most cases of changed gene expression, followed by catalytic activity, and molecular transducer activity. In the third major category of cellular component, the major gene changes were in the cell, cell part, followed by the organelle, and extracellular part subcategories. From these data, it can be seen that, in general, the change in the pattern of gene expression was time-dependent, i.e. the number of

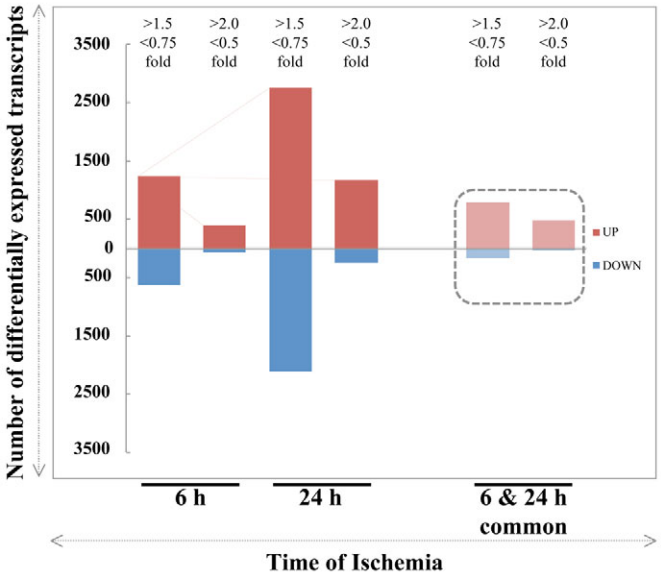

Fig. 2. Differentially expressed genes in the ipsilateral hemisphere at 6 and 24 hours. The numbers above each bar indicate the selection of genes from the total microarray datasets within a defined fold range of greater than 1.5- or 2.0-fold and less than 0.75- or 0.5-fold. The gene lists are presented in supplementary material Tables S2-S7.

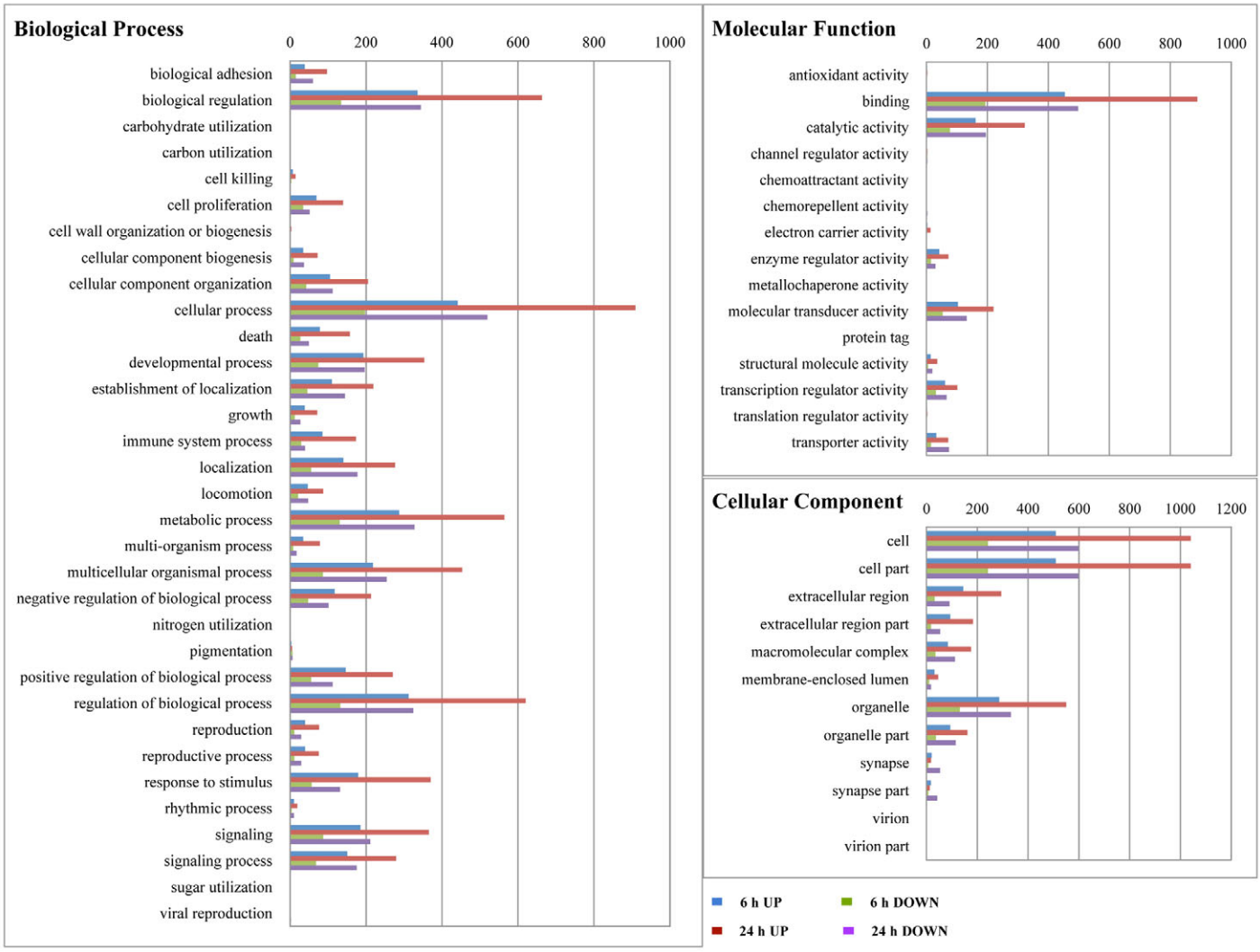

Fig. 3. Functional categorization of the differentially expressed genes based on Gene Ontology (GO). The genes were grouped into the major categories of biological function, molecular function and cellular component. Numbers above each graph represent gene numbers for each subcategory.

instances of increased and decreased gene expression was higher at 24 hours than at 6 hours. This is in line with the fact that cellular death progresses with time, i.e. following PMCAO the volume of ischemic brain tissue increases to cover almost the entire right hemisphere by 24 hours.

Changes in gene expressions reveal differential time-dependent patterns

In the ipsilateral hemisphere and at the two time points investigated in this analysis, major and minor trends were observed in relation to gene expression and/or function in response to ischemia. In a major trend, we were able to identify genes that were dramatically up- or downregulated at 6 and 24 hours; these can be considered as early (pre-ischemia)- and late (post-ischemia)-responsive genes. However, it must be emphasized that instances of upregulation were more dramatic in the ischemic brain, indicating that ischemia increases gene induction rather than gene suppression. This is understandable given the fact that ischemic damage increases with time, leading to irreversible apoptosis or cell death. For example, *S100a5* was the most highly upregulated gene (41.25-fold) at 6 hours but at 24 hours showed only a 12.97-fold induction, whereas *Mmp8* and *Mmp3* were increased 128.73- and 115.72-fold, respectively, at 24 hours but only showed a 12.44- and 6.23-fold induction, respectively, at 6 hours. This trend differentiates early (*S100a5*)- and late (*Mmp8/3*)-responsive gene expression in the ischemic brain. All the gene expression data are included in supplementary material Tables S2-S7 (see also later).

We further utilized the pathway-focused or specific-disease-states-focused gene classifications available on the QIAGEN website (SABiosciences; www.sabiosciences.com) to reveal the trend of predominant pathways affected in the ipsilateral (ischemic) hemisphere (Fig. 4). In general, it can be seen that most of the categories show an increasing trend of gene up- or downregulation with time. The up- and downregulated genes in each functional classification at 6 and 24 hours after ischemia are listed in supplementary material Table S8. However, the trends of gene

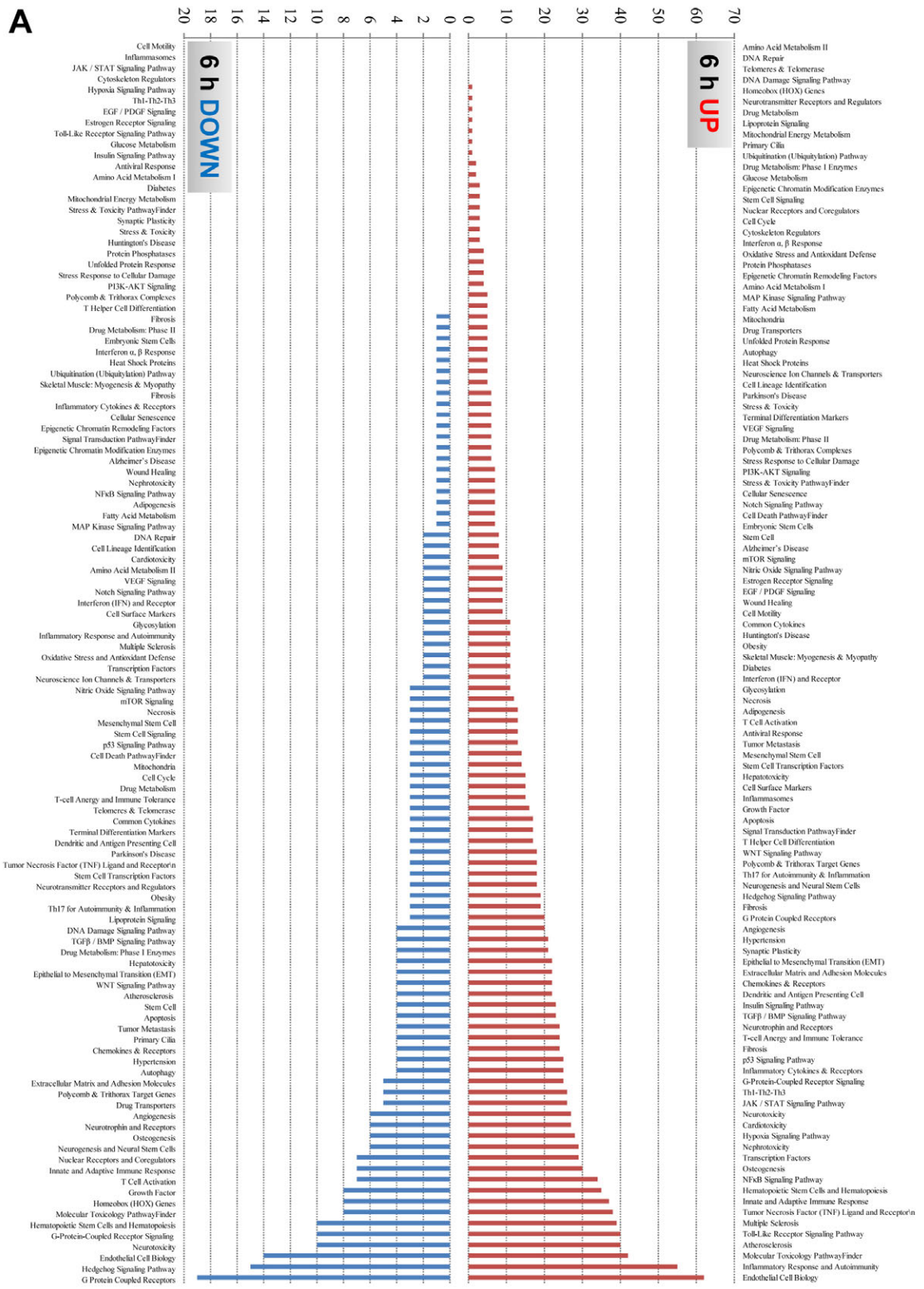

Fig. 4. See next page for legend.

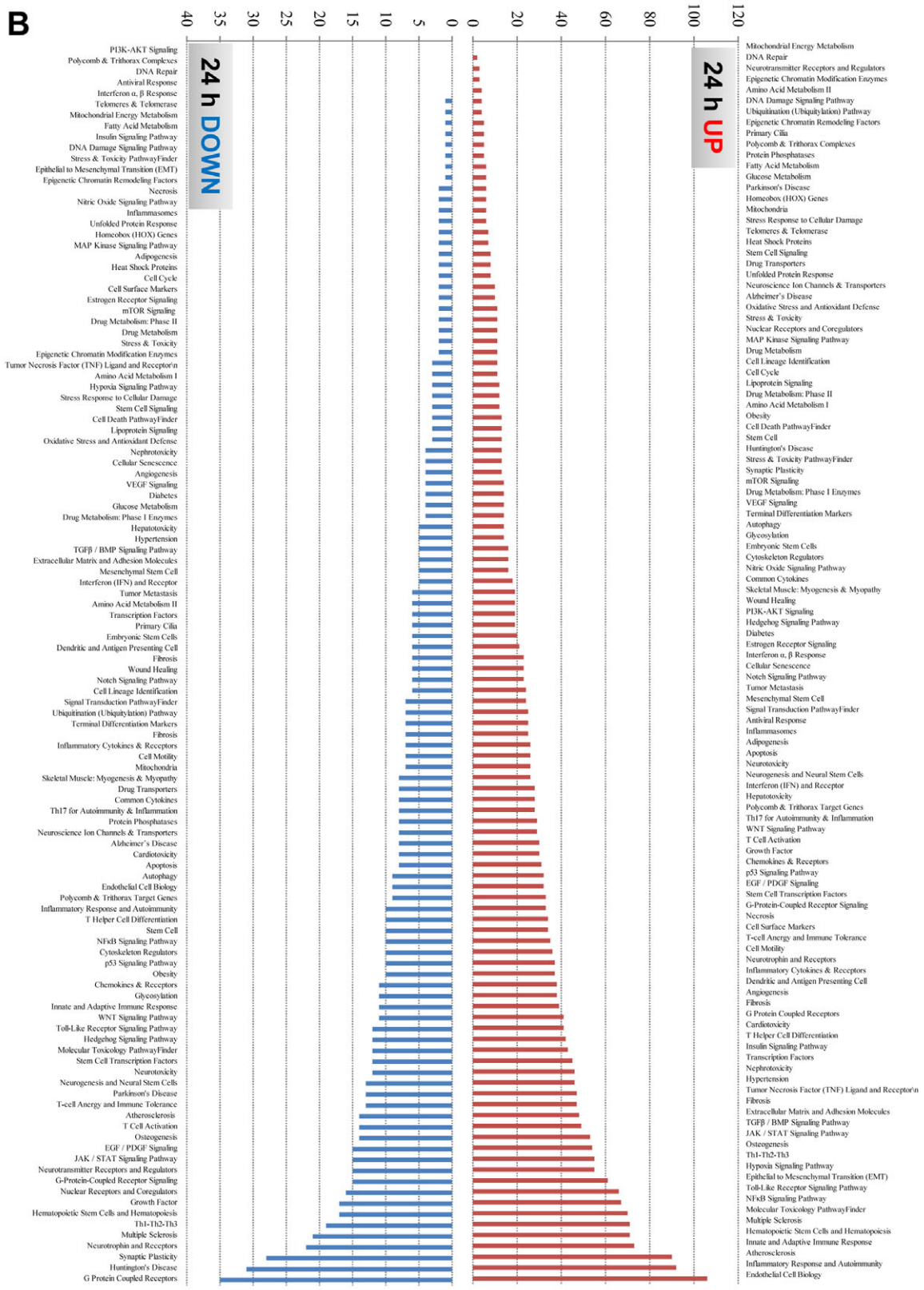

Fig. 4. Pathway- and disease-states-focused gene classification. The up- and downregulated genes at 6 (A) and 24 (B) hours after ischemia (ipsilateral hemisphere) were classified based on the available categories of more than 100 biological pathways or specific disease states in the SABiosciences PCR array list (QIAGEN; www.sabiosciences.com) for *Mus musculus*. The numbers in the *y*-axis represent number of genes in each category, which are indicated on the *x*-axis.

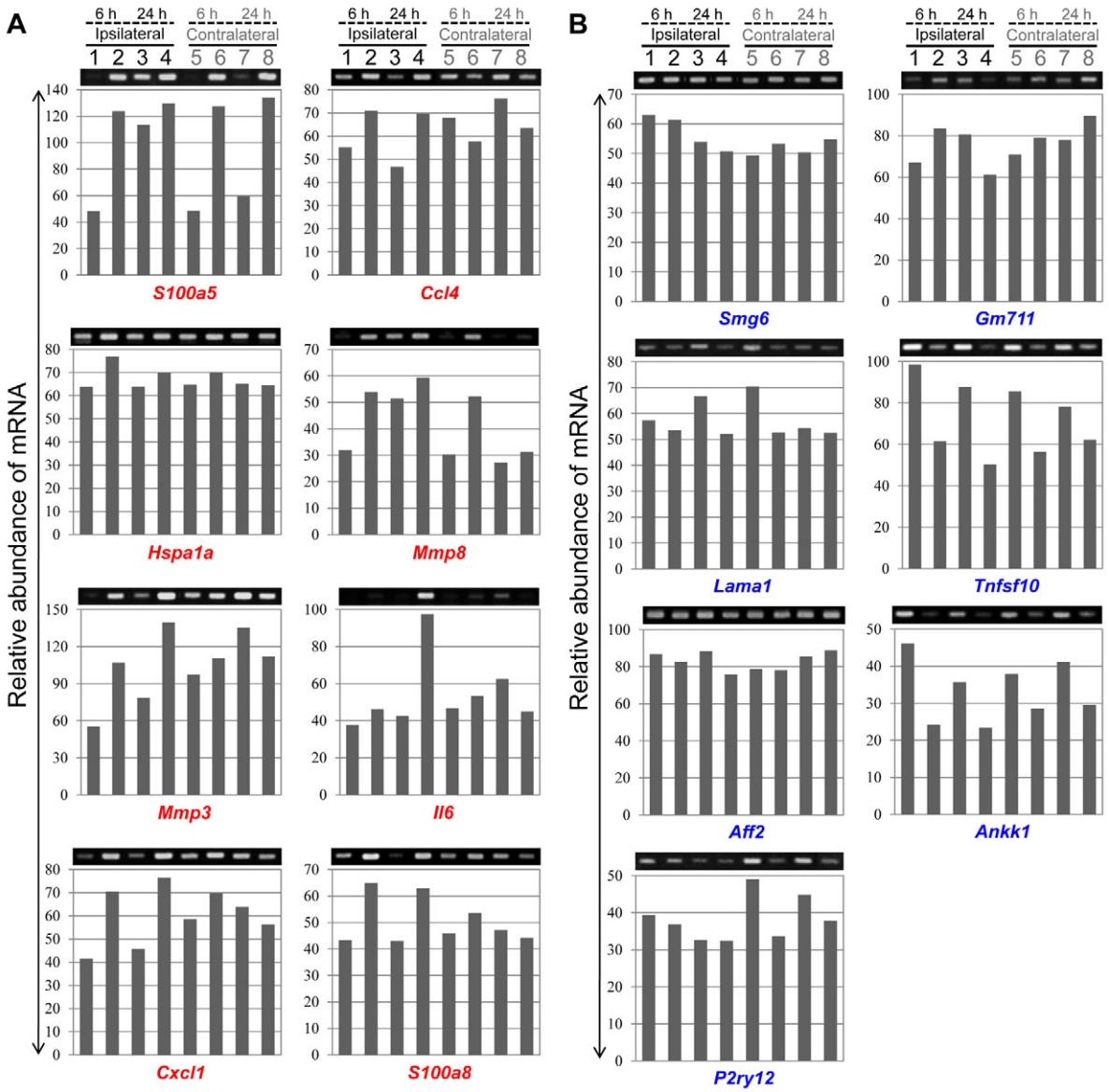

Fig. 5. mRNA expression profiles of 16 differentially expressed genes. (A) Upregulated genes; (B) downregulated genes. Gel images on top show the PCR product bands stained with ethidium bromide; the band intensities are also presented graphically below for clarity. Lane numbers 1-8 indicate sham control (lanes 1, 3, 5 and 7) and PMCAO (lanes 2, 4, 6 and 8) treatment, respectively. RT-PCR was performed as described in the Methods and the primers are detailed in supplementary material Table S1.

quantity (numbers) and quality (function) in the respective pathways varied between the two time points studied. This can be expected because the ischemic area progresses with time in the ipsilateral hemisphere. For example, genes related to endothelial cell biology, inflammatory response, autoimmunity pathways and atherosclerosis showed strongly increased expression at 24 hours. By contrast, DNA repair genes were only seen at the 24-hour but not the 6-hour time point. Some of these gene expression trends are discussed below in relation to ischemia.

S100 calcium-binding protein family

The S100 protein family constitutes the largest group of Ca^{2+}-binding proteins of the EF-hand type involved in cation homeostasis (Schafer and Heizmann, 1996; Heizmann and Cox, 1998). We found that the mRNA level corresponding to the *S100a5* gene is increased in the ischemic brain. S100a5 is known as an unusual member of the S100 protein family in that it has a high affinity for Ca^{2+}, Zn^{2+} and Cu^{2+}. S100a5 immunoreactivity has been found in neuronal dendrites, but not in neuronal soma or any glial cells in the rat brain (Schäfer et al., 2000). To the best of our knowledge, the function of S100a5 in the ischemic brain is not known, but another family member, S100b, has been shown to exhibit Cu^{2+} affinity and suppress the hemolysis of mouse erythrocytes induced by $CuCl_2$. Furthermore, when *Escherichia coli* is transformed with a vector containing *S100b* cDNA, cell damage as a result of copper-induced oxidative stress is also reduced (Nishikawa et al., 1997). It is

tempting to speculate that S100a5 might also exert such a protective role. It should be noted, however, that this gene has been previously identified as a hypoxia-inducible gene on the basis of its upregulation after 6 hours of hypoxia (Tang et al., 2006). S100a5 might protect neurons against acute ischemic damage. Around ten S100 family members were reported and reviewed in 2005 with respect to nervous system function and neurological disorders (Zimmer et al., 2005). The importance of the S100 proteins with respect to their application in clinical practice has also recently been reviewed, and there are now at least 25 members in this family (Sedaghat and Notopoulos, 2008). In our analysis, we also identified changes in the expression of other members of the S100 family, namely *S100a9* and *S100a8* (upregulated at 6 and 24 hours), and *S100a11*, *S100a14*, *S100a4*, *S100a6*, *S100a3*, *S100a15* and *S100a10* (upregulated at 24 hours only). The early and late upregulation of these genes suggests their involvement in both signal transduction processes and the inflammatory response.

Matrix metalloproteinases

Two genes belonging to the matrix metalloproteinase (MMP) enzyme family, namely *Mmp8* and *Mmp3*, showed the highest level of upregulation in response to ischemia. As the name suggests, these enzymes are known to participate in the degradation of components of the extracellular matrix. The MMPs constitute a family of more than 20 endopeptidases (Yong et al., 2001; Crocker et al., 2004; Cauwe et al., 2007). Although the induction and role of Mmp8 in ischemia has not previously been reported, this gene has been implicated in inflammatory arthritis (Cox et al., 2010), lung injury (Albaiceta et al., 2010), atherosclerosis (Laxton et al., 2009) and periodontitis (Kuula et al., 2009). Taken together with the published reports of *Mmp8* function, our finding suggests a protective role for *Mmp8* in ischemic tissues. This could occur via the processing of anti-inflammatory cytokines and chemokines by MMP8. Because MMP8 is a proteinase, it might cleave and release certain signaling factors in the extracellular matrix at a very early stage after ischemia (6 hours), and then subsequently contribute to the activation of defensive responses at a semi-acute phase (24 hours). MMP8 is also known as neutrophil collagenase and is released from activated neutrophils; this suggests that the activated neutrophil starts invasion or migration processes (Dejonckheere et al., 2011).

By contrast, the *Mmp3* gene has been associated with the pathogenesis of a number of diseases, including stroke, brain trauma and neuroinflammation (Rosenberg, 2002). The group of Rosenberg et al. also previously reported MMP3 and MMP9 immunoreactivity at 24 hours after ischemia (Rosenberg et al., 2001); MMP3 and MMP9 were found to colocalize with activated microglia and ischemic neurons. It has been previously reported that a small amount of MMP3 is needed to activate proMMP9 in endothelial cells, amplifying changes in brain blood vessel properties (Rosenberg et al., 2001). Recently, an important study regarding the role of MMP3 inside the cell was published, in which it was shown that the catalytically active, cleaved form of MMP3 (actMMP3) is produced intracellularly in oxidatively stressed neurons and plays a role in apoptotic signaling (Choi et al., 2008). The same group further demonstrated the role of MMP3 in neuronal apoptotic signaling downstream of caspase-12 during endoplasmic reticulum stress (Kim et al., 2010). MMP3 might act as an apoptosis-inducing factor as well as a protease in the ischemic brain. Interestingly, our analysis also identified a further five Mmp gene family members, including *Mmp9*, *Mmp12*, *Mmp13*, *Mmp14* and *Mmp19*. The MMP9 protein has been reported to increase after cerebral focal ischemia in rats (Romanic et al., 1998). We also found a 4.28-fold increase in *Mmp9* expression at 24 hours after ischemia, which is in line with the observation of MMP9 protein expression. It has been recently reported that MMP9 released from bone-marrow-derived cells contributes to the disruption of the blood-brain barrier that causes brain edema in stroke (Wang et al., 2009). Thus, the upregulation of MMP9 might correlate with brain edema.

In all, these results suggest another novel finding indicating the involvement of multiple MMPs in the ischemic brain. However, given the high levels of induction of *Mmp8* and *Mmp3*, we hypothesize a particularly important role for these two family members in ischemia.

Chemokines

The chemokines are chemotactic cytokines that, together with their receptors, which are expressed on leukocytes, play crucial roles in the extravasation and migration of leukocytes under inflammatory conditions (Gerard and Rollins, 2001; Semple et al., 2010). Two classes of chemokines have been identified on the basis of their structure, namely CXC and CC. The C refers to the two N-terminal cysteine residues, and the classes are defined depending on whether or not there is an amino acid between them (i.e. C-x-C versus C-C) (Rossi and Zlotnik, 2000). In addition, chemokines are reported to be important players in stroke and its pathogenesis (Semple et al., 2010; Brait et al., 2011). Brait et al., using a PCR array of 84 genes, recently reported the expression of chemokines in ischemic mouse brain at 4, 24 and 72 hours after 0.5 hours of cerebral ischemia (Brait et al., 2011).

Similarly, in our study we also identified changes in the expression of numerous chemokine-related genes, including genes encoding for chemokine (C-x-C motif and C-C motif) ligands that were highly upregulated by ischemic stress. For example, expression of the *Cxcl1* gene was increased 13.25-fold and 69.79-fold at 6 and 24 hours, respectively, whereas the *Cxcl3* gene was upregulated (117.79-fold) at 24 hours only. These trends reveal the differential and specific regulation of the Cxcl family members in the brain. Other than these two genes, we also identified *Cxcl2*, *Cxcl10*, *Cxcl4* and *Cxcl7* as being upregulated following 6 and 24 hours of ischemia, whereas *Cxcl11*, *Cxcl16* and *Cxcl13* were found to be upregulated at 24 hours only. In the C-C motif class, we identified the *Ccl4* gene as being upregulated 17.80- and 44.63-fold at 6 and 24 hours. *Ccl2*, *Ccl7*, *Ccl24*, *Ccl11*, *Ccl17*, *Ccl9*, *Ccl5*, *Ccl12* and *Ccl6* were also upregulated at both time points. However, the *Ccl19* gene was found to be upregulated specifically at 24 hours.

In addition, the chemokine receptor genes *Ccr2*, *Ccr7* and *Ccr1* were induced at 24 hours, whereas the chemokine-receptor-like gene *Ccrl2* was induced at both 6 and 24 hours. Although there has been some discussion regarding the pharmacological inhibition of chemokine ligands and receptors as a means of reducing post-ischemic neuronal damage and cell death, no chemokine has yet emerged as the most valid target (see also Brait et al., 2011). Nonetheless, our study provides information on numerous differentially expressed chemokine genes whose further analysis might shed light on their role in the ischemic brain.

Interleukins

Another example of a highly induced gene was interleukin 6 (*Il6*), which is also known to be an important inflammatory cytokine in ischemic brain tissue (Ohtaki et al., 2006). Expression of this gene was 10.35-fold and 89.23-fold upregulated at 6 and 24 hours, respectively. IL-6, which has been identified as a B-cell stimulating factor (Kishimoto, 1989), belongs to a subfamily of cytokines that include leukemia inhibitory and ciliary neurotrophic factors and use gp130 as a common receptor subunit. IL-6 has been previously shown to play a role in neurodegeneration rather than neuroprotection in cerebral ischemia (Clark et al., 2000). Another report also showed that the infarct volume after temporary MCAO is similar in wild-type and knockout mice lacking IL-6 (Pera et al., 2004). Endogenous IL-6, which transiently increases in the acute phase of cerebral ischemia, plays a crucial role in preventing damaged neurons from undergoing apoptosis; this role might be mediated by Stat3 activation. IL-6 signaling could be used as a new target for stroke therapy, but exogenous IL-6 administration is unlikely to be effective for treating brain ischemia given that excessive IL-6 sometimes generates harmful effects, such as inducing fever (Yamashita et al., 2005). Our results are in line with previous reports showing a crucial role for *Il6* in brain ischemia progression.

In contrast to *Il6*, *IL1-delta* (*Mus musculus interleukin 1 family, member 5*) expression is suppressed after ischemia (0.49-fold and 0.20-fold downregulated at 6 and 24 hours, respectively). *IL1-delta* is a subtype of the IL-1 ligand, which shows strong homology to the IL1 receptor antagonist *IL1ra* (Debets et al., 2001). Although the function of *IL1-delta* in the brain is still unclear, it was reported that *IL1-delta* blocked *IL1-epsilon* function, which activates the transcription factor *NF-kappa-B* (*NFκB*) through the orphan receptor *IL1Rrp2*. The *NFκB* signal is activated by inflammatory responses; some anti-inflammatory drugs, various non-steroidal anti-inflammatory drugs and glucocorticoids are potent inhibitors of *NFκB* (D'Acquisto et al., 2002). *NFκB* activation also contributes to the progress of brain ischemia (Harari et al., 2010). In addition, *IL1-delta* mRNA is strongly downregulated in *TNFα*-treated human adipocytes (Do et al., 2006). *IL1-delta* might act as an anti-inflammatory or cytoprotective factor in brain ischemia.

The DNA microarray analysis in the present study provided new information for the involvement of numerous interleukin family members in brain ischemia. We identified interleukin-1 receptor type 2 (*Il1r2*), *Il8rb* (β), *Il1a* (α), *Il17rb* (homolog short isoform precursor), *Il19*, *Il31ra*, *Il1b*, *Il4ra*, *Il11* and *Il1r1* as being upregulated at 6 and 24 hours, whereas *Il18rb*, *Il7r* and *Il1rap* (receptor accessory protein) were induced at 6 hours only. At 24 hours, the *Il1rn* (receptor antagonist), *Il18rap*, *Il21*, *Il1f9* (family), *Il12rb1*, *Il13ra1*, *Il2rg* (γ), *Il1rl1* (receptor-like), *Il28ra*, *Il12b*, *Il13ra2*, *Il5* and *Il6ra* genes were found to be specifically upregulated. These results again reflect the importance of global gene profiling approaches to investigate the brain.

Confirmation of gene expression by RT-PCR and differential expression of their mRNAs in the ipsilateral and contralateral hemispheres

For confirmation of alterations in gene expression by DNA microarray, we randomly selected 15 genes with annotated functions that were highly up- or downregulated in the ischemic brain (eight genes in each group, as listed in supplementary material Table S1). The mRNA expression profiles obtained by reverse-transcriptase PCR (RT-PCR) (Fig. 5) revealed that the DNA microarray data could be validated using appropriate primer design. In addition, we also examined how those genes, which displayed altered expression patterns in the ipsilateral (ischemic) hemisphere, behaved in the contralateral (non-ischemic) hemisphere. This was done (1) to confirm that these genes are indeed ischemia-related, and (2) to uncover any potential bilateral effects. We also reasoned that this parallel analysis would reveal any effects of saline injection on gene expression.

Upregulated genes

RT-PCR analysis revealed that *S100a5* mRNA was also expressed in the non-ischemic region (lanes 6 and 8, Fig. 5A). This was a surprising finding, which we are unable to fully explain. Nevertheless, it is possible that the *S100a5* gene was influenced bilaterally following ischemia, i.e. its expression was modulated by events occurring in the ischemic hemisphere. The other member of this family, *S100a8*, was primarily expressed in the ischemic hemisphere, but not (or only minimally) expressed in the non-ischemic hemisphere, suggesting that *S100a5* and *S100a8* show differential expression in the non-ischemic hemisphere. In the case of the heat-shock protein (HSP) 1a (*Hsp1a*) gene, a high level of expression was confirmed in the ischemic hemisphere at 6 hours. However, this gene was also constitutively expressed, albeit at low levels, in the brain, which is logical considering the important role of HSPs in cellular processes. Interestingly, a recent study has shown that astrocyte targeted overexpression of HSP72 (or SOD) can reduce neuronal vulnerability to forebrain ischemia (Xu et al., 2010). RT-PCR analysis revealed that *Mmp3* was more highly expressed than *Mmp8*, although both genes showed a high level of expression at 24 hours in the ischemic region. Surprisingly, *Mmp3* and *Mmp8* genes showed dramatically opposite regulation in the non-ischemic region. Considering the relatively high level of expression of *Mmp3* in the contralateral hemisphere of the sham controls, the possibility that the increase in its mRNA expression was a result of the saline injection cannot be ruled out. In a similar fashion, the *Cxcl1* gene was strongly induced in the ischemic region, but not in the non-ischemic tissue. The *Mmp3*, *CxCl1*, *Ccl4* and *Il6* genes of sham controls in the contralateral hemisphere were more highly expressed than on the ipsilateral side, suggesting that the upregulation might be a result of wounding, i.e. by the intracerebroventricular saline injection.

Downregulated genes

To date, no known brain function of the *Smg6* (Smg6-homolog, nonsense mediated mRNA decay factor, *C. elegans*) gene has been described. Our findings provide the first reported expression data of this gene in the mouse brain and its downregulation under ischemia. The *Gm711* gene is a probable protein-kinase-like protein, the expression of which has again not been previously reported in the brain. However, our results demonstrated that it is downregulated following 24 hours of ischemia. *Lama1*, which encodes for laminin, an extracellular matrix constituent similar to the MMPs, was prominently downregulated in the ischemic region. A previous report has described laminin degradation in the CA1 and CA2 areas of C57BL/6 mice subjected to 20 minutes

of global cerebral ischemia (Lee et al., 2009). Furthermore, this degradation could be reduced by the use of the tetracycline antibiotic doxycycline via the inhibition of MMP9. *Tnfsf10*, the tumor necrosis factor (ligand) superfamily member 10 gene, which is constitutively expressed in the brain, was found to be dramatically reduced in the ischemic region, and also to some extent in the non-ischemic region. Also called the proapoptotic cytokine TNF-related apoptosis-inducing ligand or TRAIL, its function in ischemia remains to be clarified. Expression of the *Aff2* gene, which has been implicated in neurodevelopmental diseases, is reduced following ischemia (Vogel and Gruss, 2009). The *Ankk1* gene is a predicted kinase shown to be expressed exclusively in astrocytes in the adult central nervous system (CNS) in humans and rodents (Hoenicka et al., 2010). Although its function in relation to ischemia remains unknown, its mRNA level was reduced in the ischemic region. Finally, the *p2ry12* gene encodes the purinergic P2Y receptor, G-protein coupled 12 protein, with a role in the vessel wall response to arterial injury and thrombosis. Downregulation of this gene was seen in both the ischemic and non-ischemic regions.

Concluding remarks

Our study provides the first inventory of ischemia-related and/or responsive genes in the mouse brain. On the basis of a high-throughput transcriptomics approach on a 44K DNA microarray chip, we have identified not only numerous genes with potential involvement in the regulation of brain ischemia, but also most of the genes that have previously been reported in targeted studies. For example, the most prominent gene with known involvement in regulating ischemia identified here was *Il6*. Apart from IL-6 and related interleukins, we also found that the Ca^{2+}-binding protein S100a and members of the matrix metalloproteinase and chemokine gene families showed the most significant changes following 6 and 24 hours of ischemia. Interestingly, many genes also showed differential expression in the non-ischemic contralateral hemisphere, revealing the identity of specific ischemia-related genes. Our study further highlights the usefulness of global gene expression profiling in searching for changes in gene expression and delineating the molecular events in a defined experimental model, in this case the ischemic brain. Elucidating the expression pattern of each differentially expressed gene in specific brain regions and determining the level of protein synthesis will be the next experimental step. These future studies will provide information on the sites of gene expression and increase our understanding of the functional role of various genes in response to an ischemic injury. Moreover, and consistent with the long-term goals of our group, the identified gene candidates will inform our investigations of the effect of target neuropeptide(s) in potentially controlling and/or reversing ischemia.

METHODS

Animals and husbandry

C57BL/6J mice purchased from Charles River (Kanagawa, Japan) were used in this study. Thirty male mice (9 weeks old; ~25-35 g body weight) were housed at the Animal Institution in Showa University in acrylic cages (eight mice per cage) at 23°C, and maintained with a standard 12-hour light/dark cycle with optimum humidity and temperature control. All animals were given access to tap water and laboratory chow ad libitum. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Showa University (School of Medicine), Tokyo, Japan.

PMCAO and sham control

To generate the PMCAO model, mice were anesthetized with 4% sevoflurane (induction) and 2% sevoflurane (maintenance) in a 30% O_2 and 70% N_2O gas mixture via a face mask. An incision was then made in the cervical skin followed by opening of the salivary gland and visualization of the right common carotid artery. The external carotid artery was exposed through a midline cervical incision. For PMCAO, we used the intraluminal filament technique, whereby a 7-0 monofilament nylon suture with its tip slightly rounded by heating was inserted into the common carotid artery, then positioned in the middle cerebral artery (supplementary material Fig. S1D), after which the wound was sutured closed. In sham control animals, the external carotid artery was exposed and then the wound was sutured. In both the sham control and PMCAO cases, 1 µl of saline (0.9% NaCl) was injected intracerebroventrically, and the animals were returned to their cages. A total of four groups were prepared: two groups of six and seven mice in the PMCAO cohorts at 6 and 24 hours after operation, respectively, and five mice each in the control (sham) groups at 6 and 24 hours after operation, respectively. We used three mice each in PMCAO groups that exhibited neurological grades G1 and G2 (Ohtaki et al., 2006) and three mice each at random in sham groups for the subsequent downstream analysis. Some of the mice were examined for ischemia by triphenyltetrazolium chloride (TTC) staining of brain sections (2 mm slices) at 37°C for 10 minutes (Ohtaki et al., 2006; Dogrukol-Ak et al., 2009) using some of the PMCAO mice brains (supplementary material Fig. S1E).

Dissection of brain and storage of samples

At 6 or 24 hours post-injection of saline, the mice were removed from their cages, decapitated and their brains carefully removed on ice. The left (contralateral) and right (ipsilateral) hemispheres were dissected and placed in 2 ml Eppendorf tubes, which were then quickly immersed in liquid nitrogen before being stored in −80°C prior to further analysis (Fig. 1A).

Grinding of the brains and total RNA extraction

The deep-frozen brain hemispheres were transferred to a pre-chilled (in liquid nitrogen) mortar and ground with a pestle to a very fine powder with liquid nitrogen. The scheme for preparation of fine tissue powders for downstream gene analysis is given in Fig. 1A (see also Masuo et al., 2011a; Masuo et al., 2011b). The powdered samples were transferred to 2 ml Eppendorf microtubes and stored in aliquots at −80°C until used for extraction of total RNA or protein. Total RNA was extracted from ~60 mg sample powder using the QIAGEN RNeasy Mini Kit (QIAGEN, Germantown, MD). The total RNA extraction protocol is briefly illustrated in Fig. 1B (see also supplementary material Fig. S2) (Masuo et al., 2011b; Ogawa et al., 2011). To verify the quality of this RNA, the yield and purity were determined spectrophotometrically (NanoDrop, Wilmington, DE) and visually confirmed using formaldehyde-agarose gel electrophoresis (Fig. 1B; supplementary material Fig. S3).

cDNA synthesis and RT-PCR

To validate the total RNA quality and subsequently synthesized cDNA, RT-PCR was performed. Two commonly used genes, namely *GAPDH* and β-actin, were used for RT-PCR. The 3′-UTR gene-specific primers are shown in supplementary material Table S1. Briefly, total RNA samples (from both the ipsilateral and contralateral hemispheres) were first DNase-treated with RNase-free DNase (Stratagene, Agilent Technologies, La Jolla, CA). First-strand cDNA was then synthesized in a 20 μl reaction mixture with an AffinityScript QPCR cDNA Synthesis Kit (Stratagene) according to the protocol provided by the manufacturer, using 1 μg total RNA isolated from each control and treated brain sample. The reaction conditions were: 25°C for 5 minutes, 42°C for 5 minutes, 55°C for 40 minutes and 95°C for 5 minutes. The synthesized cDNA was made up to a volume of 50 μl with sterile water supplied in the kit. The reaction mixture contained 0.6 μl of the first-strand cDNA, 7 pmols of each primer set and 6.0 μl of the Emerald Amp PCR Master Mix (2× premix) (TaKaRa Shuzo, Shiga, Japan) in a total volume of 12 μl. Thermal-cycling (Applied Biosystems, Tokyo, Japan) parameters were as follows: after an initial denaturation at 97°C for 5 minutes, samples were subjected to a cycling regime of 20-40 cycles at 95°C for 45 seconds, 55°C for 45 seconds and 72°C for 1 minute. At the end of the final cycle, an additional extension step was carried out for 10 minutes at 72°C. After completion of the PCR the total reaction mixture was spun down and mixed (3 μl) before being loaded into the wells of a 1.2/1.8% agarose [Agarose (fine powder) Cat no. 02468-95, Nacalai Tesque, Kyoto, Japan] gel. Electrophoresis was then performed for ~22 minutes at 100 Volts in 1× TAE buffer using a Mupid-ex electrophoresis system (ADVANCE, Tokyo, Japan). The gels were stained (8 μl of 10 mg/ml ethidium bromide in 200 ml 1× TAE buffer) for ~7 minutes and the stained bands were visualized using an UV-transilluminator (ATTO, Tokyo, Japan). The RT-PCR protocol used in this study is detailed in supplementary material Fig. S5.

DNA microarray analysis in the ipsilateral (right) hemisphere

A mouse 4x44K whole genome oligo DNA microarray chip (G4122F, Agilent Technologies, Palo Alto, CA) was used for global gene expression analysis using the ipsilateral (ischemic) hemisphere. Total RNA (900 ng; 300 ng each replicate pooled together) was labeled with either Cy3 or Cy5 dye using an Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent). Fluorescently labeled targets of control (sham) as well as treated (PMCAO) samples were hybridized to the same microarray slide with 60-mer probes (Fig. 1C). As illustrated in Fig. 1C, in this experiment we compared the PMCAO mice to sham controls, i.e. the ipsilateral brain region of the PMCAO mice was compared with the same right hemisphere of the control mice (Fig. 1A). A flip labeling (dye-swap or reverse labeling with Cy3 and Cy5 dyes) procedure was followed to nullify the dye bias associated with unequal incorporation of the two Cy dyes into cDNA (Rosenzweig et al., 2004; Altman, 2005; Martin-Magniette et al., 2005). Briefly, to select differentially expressed genes, we identified genes that were upregulated in chip 1 (Cy3/Cy5 label for control and treatment, respectively, at 6 hours) but downregulated in chip 2 (Cy3/Cy5 label for treatment and control, respectively, at 6 hours) for the ipsilateral brain hemisphere. The same selection criteria were applied for chips 3 and 4 (24 hours). The use of a dye-swap approach provides a more stringent selection condition for changed gene expression profiling than the use of a simple single/two-color approach (Hirano et al., 2007; Hirano et al., 2008; Hirano et al., 2009; Tano et al., 2010a; Tano et al., 2010b; Ogawa et al., 2011).

Hybridization and wash processes were performed according to the manufacturer's instructions, and hybridized microarrays were scanned using an Agilent Microarray scanner, G2565BA. For the detection of significantly differentially expressed genes between control and treated samples, each slide image was processed by Agilent Feature Extraction software (version 9.5.3.1). Briefly, (1) this program measured Cy3 and Cy5 signal intensities of whole probes; (2) dye-bias tends to be signal intensity dependent, and therefore the software selected probes using a set by rank consistency filter for dye normalization; (3) normalization was performed by LOWESS (locally weighted linear regression), which calculates the log ratio of dye-normalized Cy3 and Cy5 signals, as well as the final error of the log ratio; (4) the significance (*P*) value was based on the propagate error and universal error models; (5) the threshold of significance for differentially expressed genes was <0.01 (for the confidence that the feature was not differentially expressed); and (6) erroneous data

RESOURCE IMPACT

Background

The early diagnosis and treatment of patients who have suffered episodes of brain or myocardial infarction is crucial to avoid the effects of ischemia and improve survival outcomes. The obstruction of cerebral blood flow induces time-dependent pathophysiological changes in brain tissue, including tissue necrosis, and can cause death if the situation is not resolved quickly. Patients affected by ischemic brain disease can suffer from many symptoms, including neurological (e.g. palsy, speech difficulties) and psychological (e.g. decrease in memory, anxiety) problems that decrease quality of life. The most radical therapy for brain ischemia is thrombolytic reperfusion of the occluded artery, which restores blood flow to peripheral regions. However, the most important aspects of therapy for brain ischemia after onset of disease is that the patient adopts a healthy lifestyle (including diet, exercise and rest) and undergoes long-term rehabilitation to improve residual brain function.

Results

This study uses a genome-wide global gene expression approach to characterize the molecular events occurring in ischemic brain tissue in mice. Using the whole genome mouse 4x44K DNA microarray system and a dye-swap approach, the study reveals changes in the expression of several hundred genes in the mouse brain at both 6 and 24 hours following an episode of ischemia. As expected, genes linked to immune and inflammatory responses are expressed more strongly in ischemic tissue; however, numerous genes and pathways not previously linked with ischemia are also identified.

Implications and future directions

This study uncovers the identity of the many gene candidates that are up- or downregulated in the ischemic brain. Comparative and time-dependent analyses differentiate early and late changes in gene expression, providing new hypotheses regarding pathways that regulate an ischemic episode and its progression. Gene candidates identified in this study could be further investigated for their role in brain ischemia and their potential to be targeted for therapy using knockout mice. Finally, this study provides new clues about the capacity of certain neuropeptides to reverse the effects of ischemia. Further genetic and proteomic analysis of mouse models of ischemia will continue to help to unravel the molecular basis underlying this disease.

generated owing to artifacts were eliminated before data analysis using the software. The outputs of microarray analysis used in this study are available under the series number GSE 28201 at the NCBI Gene Expression Omnibus (GEO) public functional genomics data repository (http://www.ncbi.nlm.nih.gov/geo/info/linking.html).

To validate the microarray data (ipsilateral hemisphere), RT-PCR was also performed on randomly up- and downregulated genes using 3′-UTR-specific gene primers. In parallel, we also examined the gene expression profile in the contralateral (left) hemisphere by using the same genes for RT-PCR analysis.

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

M.H., T.N., K.N., Y.W., D.T. and A.Y. designed and generated the PMCAO model mice. M.H., R.R. and J.S. designed and performed the DNA microarray and related experiments, analyzed the data and wrote the paper. M.H., T.N., R.R., K.T. and S.S. checked, revised and finalized the paper.

SUPPLEMENTARY MATERIAL

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REFERENCES

Albaiceta, G. M., Gutierrez-Fernández, A., García-Prieto, E., Puente, X. S., Parra, D., Astudillo, A., Campestre, C., Cabrera, S., Gonzalez-Lopez, A., Fueyo, A. et al. (2010). Absence or inhibition of matrix metalloproteinase-8 decreases ventilator-induced lung injury. *Am. J. Respir. Cell Mol. Biol.* **43**, 555-563.

Altman, N. (2005). Replication, variation and normalisation in microarray experiments. *Appl. Bioinformatics* **4**, 33-44.

Brait, V. H., Rivera, J., Broughton, B. R., Lee, S., Drummond, G. R. and Sobey, C. G. (2011). Chemokine-related gene expression in the brain following ischemic stroke: no role for CXCR2 in outcome. *Brain Res.* **1372**, 169-179.

Büttner, F., Cordes, C., Gerlach, F., Heimann, A., Alessandri, B., Luxemburger, U., Türeci, O., Hankeln, T., Kempski, O. and Burmester, T. (2008). Genomic response of the rat brain to global ischemia and reperfusion. *Brain Res.* **1252**, 1-14.

Cauwe, B., Van den Steen, P. E. and Opdenakker, G. (2007). The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. *Crit. Rev. Biochem. Mol. Biol.* **42**, 113-185.

Chauveau, F., Cho, T. H., Berthezène, Y., Nighoghossian, N. and Wiart, M. (2010). Imaging inflammation in stroke using magnetic resonance imaging. *Int. J. Clin. Pharmacol. Ther.* **48**, 718-728.

Choi, D. H., Kim, E. M., Son, H. J., Joh, T. H., Kim, Y. S., Kim, D., Flint Beal, M. and Hwang, O. (2008). A novel intracellular role of matrix metalloproteinase-3 during apoptosis of dopaminergic cells. *J. Neurochem.* **106**, 405-415.

Clark, W. M., Rinker, L. G., Lessoy, N. S., Hazel, K., Hill, J. K., Stenzel-Poore, M. and Eckenstein, F. (2000). Lack of interleukin-6 expression is not protective against focal central nervous system ischemia. *Stroke* **31**, 1715-1720.

Cox, J. H., Starr, A. E., Kappelhoff, R., Yan, R., Roberts, C. R. and Overall, C. M. (2010). Matrix metalloproteinase 8 deficiency in mice exacerbates inflammatory arthritis through delayed neutrophil apoptosis and reduced caspase 11 expression. *Arthritis Rheum.* **62**, 3645-3655.

Crocker, S. J., Pagenstecher, A. and Campbell, I. L. (2004). The TIMPs tango with MMPs and more in the central nervous system. *J. Neurosci. Res.* **75**, 1-11.

Cucchiara, B. and Kasner, S. E. (2011). In the clinic. Transient ischemic attack. *Ann. Intern. Med.* **154**, 1-16.

D'Acquisto, F., May, M. J. and Gosh, S. (2002). Inhibition of nuclear factor kappa B (NF-B): an emerging theme in anti-inflammatory therapies. *Mol. Interv.* **2**, 22-35.

Debets, R., Timans, J. C., Homey, B., Zurawski, S., Sana, T. R., Lo, S., Wagner, J., Edwards, G., Clifford, T., Menon, S., Bazan, J. F. and Kastelein, R. A. (2001). Two novel IL-1 family members, IL-1 delta and IL-1 epsilon, function as an antagonist and agonist of NF-kappa B activation through the orphan IL-1 receptor-related protein 2. *J. Immunol.* **167**, 1440-1446.

Dejonckheere, E., Vantenbraoucke, R. E. and Libert, C. (2011). Matrix metalloproteinase8 has a central role in inflammatory disorders and cancer progression. *Cytokine Growth Factor Rev.* **22**, 73-81.

DeRisi, J. L. Iyer, V. R. and Brown, P. O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278**, 680-686.

Di Pietro, V., Amin, D., Pernagallo, S., Lazzarino, G., Tavazzi, B., Vagnozzi R., Pringle, A. and Belli, A. (2010). Transcriptomics of traumatic brain injury: gene expression and molecular pathways of different grades of insult in a rat organotypic hippocampal culture model. *J. Neurotrauma* **27**, 349-359.

Dogrukol-Ak, D., Kumar, V. B., Ryerse, J. S., Farr, S. A., Verma, S., Nonaka, N., Nakamachi, T., Ohtaki, H., Niehoff, M. L., Edwards, J. C. et al. (2009). Isolation of peptide transport system-6 from brain endothelial cells: therapeutic effects with antisense inhibition in Alzheimer and stroke models. *J. Cereb. Blood Flow Metab.* **29**, 411-422.

Do, M. S., Jeong, H. S., Choi, B. H., Hunter, L., Langley, S., Pazmany, L. and Trayhurn, P. (2006). Inflammatory gene expression patterns revealed by DNA microarray analysis in TNF-alpha-treated SGBS human adipocytes. *Yonsei Med. J.* **47**, 729-736.

Fisher. M. (2011). New approaches to neuroprotective drug development. *Stroke* **42**, S24-S27.

Gerard, C. and Rollins, B. J. (2001). Chemokines and disease. *Nat. Immunol.* **2**, 108-115.

Grond-Ginsbach, C., Horstmann, S., Hummel, M., Wiest, T., Honold, C., Pfleger, K., Hergenhahn, M., Hollstein, M., Weninger, A., Knyazev, Y. et al. (2009). Increased expression of cell-cell signaling genes by stimulated mononuclear leukocytes in patients with previous atherothrombotic stroke. A whole genome expression profile study. *Eur. Neurol.* **62**, 30-39.

Gupta, Y. K., Briyal, S. and Gulati, A. (2010). Therapeutic potential of herbal drugs in cerebral ischemia. *Indian J. Physiol. Pharmacol.* **54**, 99-122.

Gusev, E. I. and Skvortsova, V. I. (2003). *Brain Ischemia.* New York: Kluwer Academic/Plenum Publishers.

Haramati, O., Mane, R., Molczadzki, G., Perez-Polo, J. R., Chalifa-Caspi, V. and Golan, H. M. (2009). Magnesium sulfate treatment alters fetal cerebellar gene expression responses to hypoxia. *Int. J. Dev. Neurosci.* **28**, 207-216.

Harari, O. A. and Liao, J. K. (2010). NF-κB and innate immunity in ischemic stroke. *Ann. NY Acad. Sci.* **1207**, 32-40.

Heizmann, C. W. and Cox, J. A. (1998). New perspectives on S100 proteins: a multi-functional $Ca^{(2+)}$-, $Zn^{(2+)}$- and $Cu^{(2+)}$-binding protein family. *Biometals* **11**, 383-397.

Henninger, N., Kumar, R. and Fisher, M. (2010). Acute ischemic stroke therapy. *Expert Rev. Cardiovasc. Ther.* **8**, 1389-1398.

Hirano, M., Rakwal, R., Kouyama, N., Katayama, Y., Hayashi, M., Shibato, J., Ogawa, Y., Yoshida, Y., Iwahashi, H. and Masuo, Y. (2007). Gel-based proteomics of unilateral irradiated striatum after gamma knife surgery. *J. Proteome Res.* **6**, 2656-2668.

Hirano, M., Rakwal, R., Shibato, J., Sawa, H., Nagashima, K., Ogawa, Y., Yoshida, Y., Iwahashi, H., Niki, E. and Masuo, Y. (2008). Proteomics- and transcriptomics-based screening of differentially expressed proteins and genes in brain of Wig rat: a model for attention deficit hyperactivity disorder (ADHD) research. *J. Proteome Res.* **7**, 2471-2489.

Hirano, M., Shibato, J., Rakwal, R., Kouyama, N., Katayama, Y., Hayashi, M. and Masuo, Y. (2009). Transcriptomic analysis of rat brain tissue following gamma knife surgery: early and distinct bilateral effects in the un-irradiated striatum. *Mol. Cells* **27**, 263-268.

Hoenicka, J., Quiñones-Lombraña, A., España-Serrano, L., Alvira-Botero, X., Kremer, L., Pérez-González, R., Rodríguez-Jiménez, R., Jiménez-Arriero, M. A., Ponce, G. and Palomo, T. (2010). The ANKK1 gene associated with addictions is expressed in astroglial cells and upregulated by apomorphine. *Biol. Psychiatry* **67**, 3-11.

Indraswari, F., Wong, P. T., Yap, E., Ng, Y. K. and Dheen, S. T. (2009). Upregulation of Dpysl2 and Spna2 gene expression in the rat brain after ischemic stroke. *Neurochem. Int.* **55**, 235-242.

Jin, K., Mao, X. O., Eshoo, M. W., Nagayama, T., Minami, M., Simon, R. P. and Greenberg, D. A. (2001). Microarray analysis of hippocampal gene expression in global cerebral ischemia. *Ann. Neurol.* **50**, 93-103.

Juul, S. E., Beyer, R. P., Bammler, T. K., McPherson, R. J., Wilkerson, J. and Farin, F. M. (2009). Microarray analysis of high-dose recombinant erythropoietin treatment of unilateral brain injury in neonatal mouse hippocampus. *Pediatr. Res.* **65**, 485-492.

Kim, E. M., Shin, E. J., Choi, J. H., Son, H. J., Park, I. S., Joh, T. H. and Hwang, O. (2010). Matrix metalloproteinase-3 is increased and participates in neuronal apoptotic signaling downstream of caspase-12 during endoplasmic reticulum stress. *J. Biol. Chem.* **285**, 16444-16452.

Kim, G. S., Jung, J. E., Niizuma, K. and Chan, P. H. (2009). CK2 is a novel negative regulator of NADPH oxidase and a neuroprotectant in mice after cerebral ischemia. *J. Neurosci.* **29**, 14779-14789.

Kishimoto, T. (1989). The biology of interleukin-6. *Blood* **74**, 1-10.

Kunst, M. M. and Schaefer, P. W. (2011). Ischemic stroke. *Radiol. Clin. North Am.* **49**, 1-26.

Kuula, H., Salo, T., Pirilä, E., Tuomainen, A. M., Jauhiainen, M., Uitto, V. J., Tjäderhane, L., Pussinen, P. J. and Sorsa, T. (2009). Local and systemic responses in matrix metalloproteinase 8-deficient mice during *Porphyromonas gingivalis*-induced periodontitis. *Infect. Immun.* **77**, 850-859.

Laxton, R. C., Hu, Y., Duchene, J., Zhang, F., Zhang, Z., Leung, K. Y., Xiao, Q., Scotland, R. S., Hodgkinson, C. P., Smith, K. et al. (2009). A role of matrix metalloproteinase-8 in atherosclerosis. *Circ. Res.* **105**, 921-929.

Lee, H., Park, J. W., Kim, S. P., Lo, E. H. and Lee, S. R. (2009). Doxycycline inhibits matrix metalloproteinase-9 and laminin degradation after transient global cerebral ischemia. *Neurobiol. Dis.* **34**, 189-198.

Leiva-Salinas, C., Wintermark, M. and Kidwell C. S. (2011). Neuroimaging of cerebral ischemia and infarction. *Neurotherapeutics* **8**, 19-27.

Liebeskind, D. S. (2008). Aortic occlusion for cerebral ischemia: from theory to practice. *Curr. Cardiol. Rep.* **10**, 31-36.

Martin-Magniette, M. L., Aubert, J., Cabannes, E. and Daudin, J. J. (2005). Evaluation of the gene-specific dye bias in cDNA microarray experiments. *Bioinformatics* **21**, 1995-2000.

Masuo, Y., Hirano, M., Shibato, J., Nam, H. W., Fournier, I., Celine, M., Wisztorski, M., Salzet, M., Soya, H., Agrawal, G. K. et al. (2011a). Brain proteomics: sampling preparation techniques for the analysis of rat brain samples using mass spectrometry. In *Sample Preparation In Biological Mass Spectrometry* (ed. A. Ivanov and A. Lazarev). New York: Springer.

Masuo, Y., Shibato, J. and Rakwal, R. (2011b). ADHD animal model characterization: a transcriptomics and proteomics analysis. In *Psychiatric Disorders: Methods and Protocols* (ed. F. H. Kobeissy). USA: Humana Press Inc., Springer.

Meschia, J. F. (2008). Whole genome approaches in ischemic stroke. *Stroke* **40**, S61-S63.

Mizushima, H., Sasaki, K., Shimazu, M., Arai, Y., Matsumoto, K., Shioda, S. and Nakai, Y. (1994). Time-dependent changes of vasoactive substances in rat cerebral ischemia. *Brain Res. Bull.* **34**, 541-545.

Molina, C. A. (2011). Reperfusion therapies for acute ischemic stroke: current pharmacological and mechanical approaches. *Stroke* **42**, S16-S19.

Nakajima, Y., Shimazawa, M., Mishima, S. and Hara, H. (2009). Neuroprotective effects of Brazilian green propolis and its main constituents against oxygen-glucose deprivation stress, with a gene-expression analysis. *Phytother. Res.* **23**, 1431-1438.

Nakamachi, T., Endo, S., Ohtaki, H., Yin, L., Kenji, D., Kudo, Y., Funahashi, H., Matsuda, K. and Shioda, S. (2005). Orexin-1 receptor expression after global ischemia in mice. *Regul. Pep.* **126**, 49-54.

Nakamachi, T., Ohtaki, H., Yofu, S., Dohi, K., Watanabe, J., Mori, H., Sato, A., Hashimoto, H., Shintani, N., Baba, A. et al. (2010). Endogenous pituitary adenylate cyclase activating polypeptide is involved in suppression of edema in the ischemic brain. *Acta Neurochir.* **106 Suppl.**, 43-46.

Nishikawa, T., Lee, I. S., Shiraishi, N., Ishikawa, T., Ohta, Y. and Nishikimi, M. (1997). Identification of S100b protein as copper-binding protein and its suppression of copper-induced cell damage. *J. Biol. Chem.* **272**, 23037-23041.

Ogawa, T., Rakwal, R., Shibato, J., Sawa, C., Saito, T., Murayama, A., Kuwagata, M., Kageyama, H., Yagi, M., Satoh, K. and Shioda, S. (2011). Seeking gene candidates responsible for developmental origins of health and disease. *Congenit. Anom.* **51**, 110-125.

Ohtaki, H., Funahashi, H., Dohi, K., Oguro, T., Horai, R., Asano, M., Iwakura, Y., Yin, L., Matsunaga, M., Goto, N. et al. (2003). Suppression of oxidative neuronal damage after transient middle cerebral artery occlusion in mice lacking interleukin-1. *Neurosci. Res.* **45**, 313-324.

Ohtaki, H., Nakamachi, T., Dohi, K., Aizawa, Y., Takaki, A., Hodoyama, K., Yofu, S., Hashimoto, H., Shintani, N., Baba, A. et al. (2006). Pituitary adenylate cyclase-activating polypeptide (PACAP) decreases ischemic neuronal cell death in association with IL-6. *Proc. Natl. Acad. Sci. USA* **103**, 7488-7493.

Ohtaki, H., Takeda, T., Dohi, K., Yofu, S., Nakamachi, T., Satoh, K., Hiraizumi, Y., Miyaoka, H., Matsunaga, M. and Shioda, S. (2007). Increased mitochondrial DNA oxidative damage after transient middle cerebral artery occlusion in mice. *Neurosci. Res.* **58**, 349-355.

Ohtaki, H., Nakamachi, T., Dohi, K. and Shioda, S. (2008a). Role of PACAP in ischemic neural death. *J. Mol. Neurosci.* **36**, 16-25.

Ohtaki, H., Ylostalo, J. H., Foraker, J. E., Robinson, A. P., Reger, R. L., Shioda, S. and Prockop, D. J. (2008b). Stem/progenitor cells from bone marrow decrease neuronal death in global ischemia by modulation of inflammatory/immune responses. *Proc. Natl. Acad. Sci. USA* **105**, 14638-14643.

Pera. J., Zawadzka, M., Kaminska, B. and Szczudlik, A. (2004). Influence of chemical and ischemic preconditioning on cytokine expression after focal brain ischemia. *J. Neurosci. Res.* **78**, 132-140.

Popa-Wagner, A., Craitoiu, S. and Buga, A. M. (2009). Central nervous system aging and regeneration after injuries. A systematic approach. *Rom. J. Morphol. Embryol.* **50**, 155-167.

Pruissen, D. M., Kappelle, L. J., Rosendaal, F. R. and Algra, A. (2009). Genetic association studies in ischaemic stroke: replication failure and prospects. *Cerebrovasc. Dis.* **27**, 290-294.

Qin, H. P., Mei, G. H., Wei, L. and Jiang, J. Y. (2008). Effect of profound hypothermia on genomics of hippocampus following complete cerebral ischemia in rats. *Neurol. Res.* **30**, 536-541.

Ramos-Fernandez, M., Bellolio, M. F. and Stead, L. G. (2011). Matrix metalloproteinase-9 as a marker for acute ischemic stroke: a systematic review. *J. Stroke Cerebrovasc. Dis.* **20**, 47-54.

Romanic, A. M., White, R. F., Arieth, A. J., Ohlstein, E. H. and Barone, F. C. (1998). Matrix metalloproteinases expression increases after cerebral focal ischemia in rats: inhibition of matrix metalloproteinase-9 reduces infract size. *Stroke* **29**, 1020-1030.

Rosenberg, G. A. (2002). Matrix metalloproteinases in neuroinflammation. *Glia* **39**, 279-291.

Rosenberg, G. A., Cunningham, L. A., Wallace, J., Alexander, S., Estrada, E. Y., Grossetete, M., Razhagi, A., Miller, K. and Gearing, A. (2001). Immunohistochemistry of matrix metalloproteinases in reperfusion injury to rat brain: activation of MMP-9 linked to stromelysin-1 and microglia in cell cultures. *Brain Res.* **893**, 104-112.

Rosenzweig, B. A., Pine, P. S., Domon, O. E., Morris, S. M., Chen, J. J. and Sistare, F. D. (2004). Dye bias correction in dual-labeled cDNA microarray gene expression measurements. *Environ. Health Perspect.* **112**, 480-487.

Rossi, D. and Zlotnik, A. (2000). The biology of chemokines and their receptors. *Annu. Rev. Immunol.* **18**, 217-242.

Rymner, M. M., Akhtar, N., Martin, C. and Summers, D. (2010). Management of acute ischemic stroke: time is brain. *Mo. Med.* **107**, 333-337.

Schäfer, B. W. and Heizmann, C. W. (1996). The S100 family of EF-hand calcium-binding proteins: functions and pathology. *Trends Biochem. Sci.* **21**, 134-140.

Schäfer, B. W., Fritschy, J. M., Murmann, P., Troxler, H., Durussel, I., Heizmann, C. W. and Cox, J. A. (2000). Brain S100A5 is a novel calcium-, zinc-, and copper ion-binding protein of the EF-hand superfamily. *J. Biol. Chem.* **275**, 30623-30630.

Sedaghat, F. and Notopoulos, A. (2008). S100 protein family and its application in clinical practice. *Hippokratia* **12**, 198-204.

Semple, B. D., Kossmann, T. and Morganti-Kossmann, M. C. (2010). Role of chemokines in CNS health and pathology: a focus on the CCL2/CCR2 and CXCL8/CXCR2 networks. *J. Cereb. Blood Flow Metab.* **30**, 459-473.

Shimazu, M., Mizushima, H., Sasaki, K., Arai, Y., Matsumoto, K., Shioda, S. and Nakai, Y. (1994). Expression of c-fos in the rat cerebral cortex after focal ischemia and reperfusion. *Brain Res. Bull.* **33**, 689-697.

Shioda, S., Ozawa, H., Dohi, K., Mizushima, H., Matsumoto, K., Nakajo, S., Takaki, A., Zhou, C. J., Nakai, Y. and Arimura, A. (1998). PACAP protects hippocampal neurons against apoptosis: involvement of JNK/SAPK signaling pathway. *Ann. NY Acad. Sci.* **865**, 111-117.

Slemmer, J. E., Shacka, J. J., Sweeney, M. I. and Weber, J. T. (2008). Antioxidants and free radical scavengers for the treatment of stroke, traumatic brain injury and aging. *Curr. Med. Chem.* **15**, 404-414.

Stamova, B., Xu, H., Jickling, G., Bushnell, C., Tian, Y., Ander, B. P., Zhan, X., Liu, D., Turner, R., Adamczyk, P. et al. (2010). Gene expression profiling of blood for the prediction of ischemic stroke. *Stroke* **41**, 2171-2177.

Suzuki, T., Higgins, P. J. and Crawford, D. R. (2000). Control selection for RNA quantitation. *Biotechniques* **29**, 332-337.

Tang, Y., Pacary, E., Fréret, T., Divoux, D., Petit, E., Schumann-Bard, P. and Bernaudin, M. (2006). Effect of hypoxic preconditioning on brain genomic response before and following ischemia in the adult mouse: identification of potential neuroprotective candidates for stroke. *Neurobiol. Dis.* **21**, 18-28.

Tano, K., Hamamoto, H., Ito, T., Sumiya, E., Rakwal, R., Shibato, J., Masuo, Y., Ijiri, K., Sekimizu, K. and Akimitsu, N. (2010a). Reduced expression of *Sytl 1* and *Ccdc21* and impaired induction of *Mt I* by oxidative stress in SII-K1 knockout mice. *Drug Discov. Ther.* **4**, 368-372.

Tano, K., Mizuno, R., Okada, T., Rakwal, R., Shibato, J., Masuo, Y., Ijiri, K. and Akimitsu, N. (2010b). MALAT-1 enhances cell motility of lung adenocarcinoma cells by influencing the expression of motility-related genes. *FEBS Lett.* **584**, 4575-4580.

Turner, D. A. and Adamson, D. C. (2011). Neuronal-astrocyte metabolic interactions: understanding the transition into abnormal astrocytoma metabolism. *J. Neuropathol. Exp. Neurol.* **70**, 167-176.

Vogel, T. and Gruss, P. (2009). Expression of leukaemia associated transcription factor Af9/Mllt3 in the cerebral cortex of the mouse. *Gene Expr. Patterns* **9**, 83-93.

Wang, G., Guo, Q., Hossain, M., Fazio, V., Zeynalov, E., Janigro, D., Mayberg, M. R. and Namura, S. (2009). Bone marrow derived cells are the major source of MMP-9 contributing to blood-brain barrier dysfunction and infarct formation after ischemic stroke in mice. *Brain Res.* **1294**, 183-192.

Wechsler, L. R. (2011). Imaging evaluation of acute ischemic stroke. *Stroke* **42**, S12-S15.

Xu, L., Emery, J. F., Ouyang, Y. B., Voloboueva, L. A. and Giffard, R. G. (2010). Astrocyte targeted overexpression of Hsp72 or SOD2 reduces neuronal vulnerability to forebrain ischemia. *Glia* **58**, 1042-1049.

Yamashita, T., Sawamoto, K., Suzuki, S., Suzuki, N., Adachi, K., Kawase, T., Mihara, M., Ohsugi, Y., Abe, K. and Okano, H. (2005). Blockade of interleukin-6 signaling aggravates ischemic cerebral damage in mice: possible involvement of Stat3 activation in the protection of neurons. *J. Neurochem.* **94**, 459-468.

Yao, H., Nakahara, T., Nakagawa, N., Hashimoto, K. and Kuroki, T. (2009). Regional and temporal changes in proteomic profile after middle cerebral artery occlusion with or without reperfusion in rats. *Neurochem. Res.* **34**, 1999-2007.

Yenari, M. A. and Hemmen, T. M. (2010). Therapeutic hypothermia for brain ischemia: where have we come and where do we go? *Stroke* **41**, S72-S74.

Yong, V. W., Power, C., Forsyth, P. and Edwards, D. R. (2001). Metalloproteinases in biology and pathology of the nervous system. *Nat. Rev. Neurosci.* **2**, 502-511.

Zhan, X., Ander, B. P., Jickling, G., Turner, R., Stamova, B., Xu, H., Liu, D., Davis, R. R. and Sharp, F. R. (2010). Brief focal cerebral ischemia that simulates transient ischemic attacks in humans regulates gene expression in rat peripheral blood. *J. Cereb. Blood Flow Metab.* **30**, 110-118.

Zimmer, D. B., Chaplin, J., Baldwin, A. and Rast, M. (2005). S100-mediated signal transduction in the nervous system and neurological diseases. *Cell. Mol. Biol. (Noisy-le-grand)* **51**, 201-214.