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 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' and 'clinical.'

INTRODUCTION

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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/BL6f), 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 intracerebroventriculally 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...
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...
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.
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
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 (Albaceta 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 protease, 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; Bra¨ıt et al., 2011). Bra¨ıt 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 (Bra¨ıt 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 *Ccl3* gene was upregulated (117.79-fold) at 24 hours only. These trends reveal the differential and specific regulation of the Ccl family members in the brain. Other than these two genes, we also identified *Cxc12*, *Cxc10*, *Cxc4* and *Cxl7* as being upregulated following 6 and 24 hours of ischemia, whereas *Cxc11*, *Cxc16* and *Cxc13* 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 *Ccr12* 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 posts ischemic neuronal damage and cell death, no chemokine has yet emerged as the most valid target (see also Bra¨ıt 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, IL11-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). IL11-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 IL11-delta in the brain is still unclear, it was reported that IL11-delta blocked IL1-epsilon function, which activates the transcription factor NF-kappa-B (NFkB) through the orphan receptor IL1Rrp2. The NFkB signal is activated by inflammatory responses; some anti-inflammatory drugs, various non-steroidal anti-inflammatory drugs and glucocorticoids are potent inhibitors of NFkB (D’Acquisto et al., 2002). NFkB activation also contributes to the progress of brain ischemia (Harari et al., 2010). In addition, IL11-delta mRNA is strongly downregulated in TNFalpha-treated human adipocytes (Do et al., 2006). IL11-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), Il17r (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, Il2r (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
Afk2 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 Ca2+-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
central 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%
O2 and 70% N2O 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 intracerebroventricularly,
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...
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., T.R., K.T. and S.S. checked, revised and finalized the paper.

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