Two-color Dye-swap DNA Microarray approach toward confident gene expression profiling in PMCAO mouse model for ischemia-related and PACAP38-influenced genes

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A B S T R A C T

Toward twin goals of identifying molecular factors in brain injured by ischemic stroke, and the effects of neuropeptide pituitary adenylate-cyclase activating polypeptide (PACAP) on the ischemic brain, we have established the permanent middle cerebral artery occlusion (PMCAO) mouse model and utilized the Agilent mouse whole genome 4 × 44 K DNA chip. PACAP38 (1 pmol) injection was given intracerebroventrically in comparison to a control saline (0.9% NaCl) injection, to screen genes responsive to PACAP38. Two sets of tissues were prepared, whole hemispheres (ischemic and non-ischemic) and infract core and penumbra regions at 6 and 24 h. In this study, we have detailed the experimental design and protocol used therein and explained the quality controls for the use of total RNA in the downstream DNA microarray experiment utilizing a two-color dye-swap approach for stringent and confident gene identification published in a series of papers by Hori and coworkers (Hori et al., 2012–2015).

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Direct link to deposited data

Deposited data can be found here: GSE28201 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28201), GSE37565 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37565), and GSE62884 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62884).

Data, experimental design, materials and methods

Generation of the permanent middle cerebral artery occlusion model (PMCAO)

Brain ischemia, also termed cerebral ischemia or ischemic stroke, is a major health problem worldwide [1]. Extremely debilitating to the brain tissues, it is the third most common cause of death after heart attack and cancer. The permanent middle cerebral artery occlusion (PMCAO) model was used over the transient MCAO, due to the avoidance of reperfusion injury in our research model aimed at providing new insight into the PACAP38 neuroprotective effects in the mouse brain [2–6]. PMCAO was performed on male mice (C57BL/6J), and the results of the procedure were monitored by dissecting whole brains...
and visualizing the extent of ischemia. More than 80% of the mice survived the PMCAO procedure. Post-PMCAO, the effect of neuropeptide pituitary adenylate-cyclase activating polypeptide 38 (PACAP38) treatment (injection was performed intracerebroventrically) was examined in mouse; saline injection was used as a control [2–6]. The aim was two-fold: first, to provide a detailed ischemia-related
transcriptome in mouse model, and second, was to explore the neuro-
protective effects of PACAP38 via gene inventories specific to PACAP38
effect and screening out the molecular factors and networks/pathways
being activated therein. The reason for using, DNA microarray technol-
ogy – one of the breakthrough technologies in experimental molecular
biology – was that it allows us to quickly observe the expression of
tens of thousands of genes in parallel in a single experiment. To be
emphasized, this seemingly “quick” data is the product of a good experi-
ment design, planning, sampling of tissues and proper storage, and
quality control of the total RNA prior to the use of the microarray chip
itself.

In order to achieve the above objectives, two basic approaches were
taken that involved using hemispheres, ipsilateral containing the ische-
mic or infract core (called hereafter IC), penumbra (called there after
P) and non-ischemic regions, and contralateral (non-ischemic) in the
first set of experiments with or without PACAP38 treatment, and the
second involving dissection of specific regions in the ischemic hemi-
sphere, namely the IC and the P. The model is described in [2–6] and is-
chemia was confirmed by not only visual observation of the dissected
brains but also by triphenyltetrazolium chloride (TTC) staining [7].

The gene expression data from these studies on hemispheres and
specific regions (IC and P) were deposited in the Gene Expression Om-
nibus (GEO) database under accession numbers GSE28201 (http://
www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28201) [2,3],
GSE37565 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=
GSE37565) [4], and GSE62884 (http://www.ncbi.nlm.nih.gov/geo/
query/acc.cgi?acc=GSE62884) [5,6].

Sampling sites and regimes, quality control of total RNA, and semi-
quantitative RT-PCR and gene expression data

The mouse exhibiting neurological grades G1 and G2 were used for
all experiments (Table 1) post PMCAO or after PACAP38 test solution
injection over a saline control [2–6]. We followed all animal care and ex-
perimental procedures under approval by the Institutional Animal Care
and Use Committee of Showa University (School of Medicine), Tokyo,
Japan. Two time points were selected, 6 h and 24 h post-PMCAO [2–6]
for sampling of the hemispheres and specific regions (IC and P), which
were immersed immediately post-dissection in liquid nitrogen and
thereafter transferred to a −80 °C deep freezer. The samples were

![Fig. 2](image-url)
individually ground in liquid nitrogen to prepare very fine powders for subsequent downstream gene expression analyses [2–6] and proteomic analysis as required [4]. Prior to each DNA microarray analysis, the total RNA, extracted using an optimized protocol based on the QIAGEN RNeasy Mini Kit (QIAGEN, Germantown, MD, USA) quantity and quality was measured spectrophotometrically with NanoDrop (Thermo Scientific, Wilmington, DE, USA) and re-confirmed using formaldehyde-agarose gel electrophoresis [2–6,8,9]. Briefly, the obtained total RNA quality measurements revealed good quality as demonstrated by the A260/280 values > 1.8, A260/230 > 1.8; in our experiments, we always aim for a higher value of more than 2.0 to 2.3 [2–6]. Furthermore, the total RNA was obtained in optimum quantity of > 300 ng/μl. Following the visualization for confirming pre-experimental integrity of the total RNA subunits by gel electrophoresis, an additional step comprised of cDNA synthesis was used for examining the quality of the synthesized cDNA by determining the expression of genes glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and beta-actin as positive controls. This was done by RT–PCR (AffinityScript QPCR cDNA Synthesis Kit; Stratagene, Agilent Technologies, La Jolla, CA, USA and Emerald Amp PCR Master Mix; Takara Shuzo, Shiga, Japan) on a Thermal Cycler (Applied Biosystems, Tokyo, Japan). Moreover, only gene-specific primers were used and whose sequences are shown in the articles [2–6]. Post-electrophoresis, the PCR products were visualized and quantified using a ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All the steps followed in sequence provided confidence that we had the best quality total RNA for use in the DNA microarray chip. As a last

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**Fig. 3.** Intensity scatter plot of the two colors/chips. Upper-most two graphs show the raw data at 6 and 24 h, Sham × PMCAO. Each spot on the graph refers to a single gene probe. The genes plotted on each graph represent all the genes on the chip, chip 1 and chip 2, which were used for dye-swap analysis as shown in the center four graphs (in dotted-line boxes). The cut-off for chip 1 and chip 2 has an up-regulated and down-regulated fold ratio of greater than or equal to 1.5 (genes indicated by pink color) and less than or equal to 0.75 (genes indicated by sky blue color); the blank vertical (for chip 1) and horizontal (chip 2) spaces between the sky blue and pink colors represent the no change in expression genes. For the dye-swap to take place (selection), the genes showing an up-regulated fold-change in chip 1 (pink) need to show a corresponding down-regulated value in chip 2 (blue) and vice versa for the down-regulations (pink). Then, the lower-most two graphs show the dye-swap scatter plots which reveal reliable/confident gene expression selected (common up-regulated ≥ 1.5-fold are in red; common down-regulated ≤ 0.75 are in blue) based on the chip 1 and chip 2 data analyses as described above. Genes with similar expression are on the diagonal; log-transformed expression values are provided.
step, the Agilent mouse whole genome 4 × 44 K (G4122F) DNA slide (composed of 4 chips on one slide) was used for the microarray analysis, which was performed according to the Agilent instructions (Agilent Technologies, Santa Clara, CA, USA) and detailed in our publications [2–6,8,9]. As also mentioned below, our DNA microarray analysis experiment is based on the two-color dye-swap approach [10]. Microarray and sample annotation data were deposited in the Gene Expression Omnibus (GEO) database under accession numbers GSE28201 [2,3], GSE37565 [4], and GSE62884 [5,6].

**Gene expression analysis comparison between RT-PCR and DNA microarray experiment design (dye-swap) using positive controls**

The first quality control step presented itself in examining the expression of two genes, Gapdh and beta-actin normally used as housekeeping genes or positive control genes by RT-PCR for all samples used in the study; we prefer to use the latter terminology [11]. Fig. 1 compares the expression of these two control genes using the RT-PCR analysis (A) and the dye-swap based DNA microarray experiment (B) for each sample, control and treatment in whole hemisphere and specific regions of IC and P, at both 6 and 24 h time points. To note, for RT-PCR each gene expression (Gapdh and beta-actin) analysis is performed at least twice as independent PCR reactions (at different cycles for avoiding taking the data at a plateau) and electrophoresis on gel for each cDNA generated/sample [2–6], and a representative data is presented in Fig. 1A. Similarly, the probe signal intensity level is for a single probe each for the two genes on the DNA microarray chip (Fig. 1B). Comparing these representative data, some variation of gene expression can be seen in the groups at 6 or 24 h time points, however, the expression of these genes did not dramatically vary, rather showed almost similar expression levels between the RT-PCR based analysis and the microarray experiment. In particular, similar expression levels (probe signal intensity) were seen with Cy3 and Cy5 labels. Thus the expression of positive control genes normally used in our research, and other studies, in general, are quite stable over all sample conditions and times. Based on this analysis, not only the stable expression of some positive controls is seen,

![Gene expression analysis comparison between RT-PCR and DNA microarray experiment design (dye-swap) using positive controls](image)

**Fig. 4.** Expression of two differentially expressed genes, interleukin 6 (Il6) and brain-derived neurotrophic factor (Bdnf), under DNA microarray experiment and subsequent validation by RT-PCR. A, Expression of Il6 and Bdnf genes by expressed level of probe signal intensity with Cy3 and Cy5 labels. B, RT-PCR results are presented graphically for these two genes mRNA relative abundance. Total RNA was extracted from the whole hemisphere (hemisphere) and ischemic core and penumbra in the ipsilateral (right) hemisphere at 6 and 24 h. PACAP38 was the treatment and saline served as the control (sham). DNA microarray analysis and RT-PCR were performed as in Fig. 1.
control genes but also the quality of synthesized cDNA was established. Moreover, these gene expressions were also found to be stable under the employed DNA microarray approach, namely dual-color and dye-swap. Furthermore, to check if the expression level on the microarray slide is similar across all probes plotted on the microarray chip, we examined the Cy3 and Cy5 label associated expression for all 10 probes for the beta-actin gene as an example, in the whole hemisphere and IC and P regions at both 6 h and 24 h time points (Fig. 2). Results showed a good correlation between the Cy3 and Cy5 label expression points across the 1.5-fold change ratio. This further confirms the data presented in Fig. 1, indicating that the gene expression for positive controls is stable across samples and also individual probes used in a DNA microarray design, and that can be compared with the RT-PCR-based analysis method.

Hybridization, normalization, and scatter plot distribution of intensity values

Fluorescently labeled targets of control (sham/saline) as well as treatments (PACAP38) were hybridized to the same microarray slide with 60-mer probes. Selection of differentially expressed genes was performed in a way that a gene up-regulated in chip 1 (Cy3 label for sham and Cy5 label for PMCAO) was down-regulated in chip 2 (Cy5 label for sham and Cy3 label for PMCAO) (Fig. 3). A similar data were presented for the PACAP38 versus sham (saline) treatments for the IC and P regions (Supplementary Figs. 1 and 2). The genes that were selected thus had a higher confidence, but were less than the numbers obtained by using just one dye orientation. As also elegantly explained by Dombkowski and co-workers in 2004, dye orientation has a significant influence on the measurements and inferences of differential gene expression most probably due to the labeling method, and further suggesting that until dye bias effects can be explained the dye-swap approach is important in the DNA microarray reference designs [10]. This still stands true today. Hybridization and wash processes were performed according to the manufacturer’s instructions, and hybridized microarrays were scanned using an Agilent Microarray scanner G2565BA or G2505C (Agilent Technologies). For the detection of significant differentially expressed genes between control and treated samples each slide image was processed by Agilent Feature Extraction software (version 9.5.3.1 or 11.0.1.1, Agilent Technologies). The Feature Extraction algorithms have been developed to reduce systematic errors arising from labeling bias, irregular feature morphologies, mismatched sample concentrations and cross hybridizations [12]. By this program, the Cy3 and Cy5 signal intensities of whole probes are measured. Dye-bias tends to be signal intensity dependent therefore the software selects probes using a set by rank consistency filter for dye-normalization. Said normalization is 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 log ratio. The statistical significance of the log ratio values obtained for individual microarray probes is calculated using the “most conservative error model” default function of the Feature Extraction software, by which both a propagated and a universal error model are evaluated, and the higher (more conservative estimate of error) p-value of two error models is reported [13,14; Agilent Technologies]. The propagated error model measures the error on the log ratio by propagating the pixel-level error from calculations made in the analysis (i.e., raw signal and background subtraction), whereas the universal error model measures the expected error between the red and green channels using the additive and multiplicative errors (Agilent Technologies). In these analyses, the threshold of significant differentially expressed genes was <0.01 (for the confidence that the feature was not differentially expressed). Additionally, the erroneous data generated due to artifacts were eliminated before data analysis by the software.

Validation of DNA microarray data using RT-PCR-based analysis of interleukin 6 (Il6) and brain-derived neurotrophic factor (Bdnf) gene expressions

Two differentially expressed genes, namely Il6 and Bdnf, as examples in the PMCAO (Il6) and PACAP38 influenced (Bdnf) experiments were examined and compared for their expression levels between the microarray experiment (Fig. 4 A) and RT-PCR (Fig. 4 B). A very good correlation was seen between these two experiments for both Il6 and Bdnf. A slight discrepancy was seen the case of the Bdnf gene, in the ischemic core region sample at 6 h, where a slight increase in expression under the microarray experiment (cut-off of greater than 1.5 fold) could not be confirmed by the RT-PCR analysis; it may be dependent on the probe design used for RT-PCR or the only slight induction seen at 6 h under the microarray data. Nevertheless, from this analysis as also in Fig. 1 for positive control genes, a good correlation between the Cy3 and Cy5 values from the microarray experiment could be seen. These results not only show the importance of a validation step using an independent method of gene expression analysis but also show that the dye-swap approach-based screening of gene expressions is a reliable indicator of the effect of the treatment being performed.

Discussion

We described here unique datasets of i) ischemia-related genes and ii) genes potentially related to the neuroprotective effects of the neuropeptide PACAP38 from the brains of PMCAO model mouse. Each of the three datasets is composed of genome-wide gene expression measured using the Agilent Gene Expression Microarray platform using the dye-swap approach (Agilent Technologies). We showed that the brain response to ischemia can be evaluated at the level of the gene, genome-wide, and presented the first ischemia-related transcriptome analysis of the mouse brain in a series of studies using the Agilent DNA microarray chip using both ipsilateral and contralateral hemispheres. Furthermore, as our primary interest is to clarify the role of the neuropeptide PACAP38 in protecting or as a therapy for brain ischemia, we have also comprehensively analyzed the brain responses in ischemic brain after treatment (PACAP38) over saline (sham). It is our idea that these large numbers of gene inventories obtained will provide a strong basis for further research into not only understanding the ischemic brain but also in pinpointing gene candidates potentially involved in mediating the neuroprotective effect of PACAP38.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jgdata.2015.01.007.

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