Psychophysiological stress-regulated gene expression in mice

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Abstract

Eight genes showed significant changes in expression in mice under psychophysiological stress provided by cage-restraint and water-immersion. The transcription level of most of these genes was affected in all the tissues analyzed, and some of them were responsive genes in several different stress systems. Peculiarly, the expression level of one gene, cdc2-like kinase 1 (CLK1), was reduced only in the brain, while the balance of partially- and alternatively-spliced CLK1 mRNA species changed in all the tissues including the brain. These results suggest that some stress-response mechanisms, including transcriptional and post-transcriptional events, are coordinated in the whole body in mice under psychophysiological stress.

Key words: Psychophysiological stress; Microarray; Quantitative RT-PCR; CLK1; Alternative splicing

Abbreviations: Q-PCR, quantitative RT-PCR; CLK1, cdc2-like kinase 1; SGK1, serum/glucocorticoid-regulated kinase 1; Ct, threshold cycle
1. Introduction

Stress responses are ubiquitous among organisms. Studies on such responses have been conducted using cultured cells or whole organisms, including a shift of temperature, exposure to chemicals, irradiation and lack of nutrients. Here, we report the responses to stress occurring in the brain of mice subjected to cage-restraint and water-immersion, which are methods that have been used widely in psychophysiological analyses [1,2]. Recently, Hayashi et al. reported that diazepam, an anti-depressant drug, blocked the induction of a stress-response marker in mouse brain under psychophysiological stress, suggesting a psychological component of the stress [3]. The relationship between stress and psychiatric disorders, including depression, has been studied for several decades [4], but the exploration of differential gene expression under psychological or psychophysiological stresses is a more recent area of study [5,6]. We examined the brain tissue-specific and ubiquitous gene expression induced by psychophysiological stress using the microarray method, and compared the stress-responsive genes in the brain and other tissues using quantitative RT-PCR.

We noticed the unique behavior of the cdc2-like kinase 1 (CLK1) gene, which codes for a dual specific kinase and is also referred to as STY (serine, threonine and tyrosine) kinase. The CLK1 protein is responsible for phosphorylation of serine and
arginine-rich (SR) proteins, a family of non-snRNP-type splicing factors, and is believed to be involved in regulation of mRNA splicing and/or alternative splicing events, because some SR proteins regulate the efficiency of alternative splicing [7,8]. However, the networks of alternative splicing in vertebrates with the SR proteins, including their signaling pathways and biological functions, are largely unknown [9].

Transcription of the CLK1 gene leads to two mature mRNA splice variants (Clk1.8 and Clk1.7) and also to accumulation of two partially-spliced forms (P-I and P-II) [10]. The Clk1.8 transcript includes 13 exons and codes for the full-size protein, which has a binding domain for SR proteins (also called the regulatory domain) and a catalytic kinase domain [11]. In contrast, exon 4 is missing from the Clk1.7 mRNA, introducing an in-frame stop codon that consequently produces a truncated protein without a kinase domain, but still including the regulatory domain. This protein is suspected to act as a factor dominant-negative to the full-size protein [7], and the difference in sub-nuclear localization between them has been reported [12]. Spatial and temporal regulation of CLK1 and/or SR protein activities in alternative splicing events remains controversial [9]. P-I is a 5.6 Kb partially-spliced mRNA that includes several introns from the 12 Kb unspliced mRNA, and P-II is a 3.2 Kb partially-spliced mRNA with two introns flanking exon 4.
2. Materials and Methods

2.1. Materials

A mouse brain cDNA microarray was made from about 7,000 clones from five libraries of normal adult brain regions: cerebrum, cerebellum, hippocampus, interbrain and olfactory bulb. In addition, an oligo-microarray covering 30,000 mouse genes (AceGene Mouse 30K; DNA Chip Research Inc. and Hitachi Software Engineering Co., Ltd., Yokohama, Japan) was also used; the protocol is available at http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf. The primers (supplemental data) were designed by using Web-based Primer 3 software [13] and synthesized by FASMAC. CO., LTD., Atsugi, Japan.

2.2. Animals

Male C57BL/6J mice aged seven weeks were restrained in a cage and immersed up to their shoulders for six hours in water maintained at 22 to 25°C [14]. They were then decapitated, and the brain regions from seven animals were isolated for microarray and quantitative RT-PCR experiments. Several other tissues from three animals were isolated for validation experiments. Controls from non-stressed animals were prepared
similarly. All studies reported here were reviewed by the Animal Care Committee of Taisho Pharmaceutical Co., Ltd., and meet the Japanese Experimental Animal Research Association Standards, as defined in the Guidelines for Animal Experiments (1987).

2.3. Microarray experiments

Total RNAs (n=7) were extracted individually using TRIzol (Invitrogen, Carlsbad CA), and then mixed and labeled by a standard cDNA post-labeling method for cDNA microarrays [15] or by using an Amino Allyl MessageAmp aRNA kit (Ambion, Austin, TX) for oligo-microarrays (AceGene). Total RNA from the cerebrum of adult mice (a mixed population of males and females, six-weeks old) was used as the reference throughout the microarray experiments. A sample and the reference were labeled with Cy3 and Cy5, respectively, mixed, and hybridized on a microarray. A two-color dye-swap experiment was performed, and the microarrays were then scanned using ScanArray Lite and signal values were calculated using QuantArray (PerkinElmer, Boston, MA). Following background subtraction, the gene-spot signals were adjusted to compensate for excitation differences between the two dyes. Genes showing highly inconsistent data between the dye-swap experiments were discarded, and the expression levels of the remaining genes were represented as the average for each gene spot. The
complete data set of microarrays is available at http://www.dna-chip.co.jp/Murata_Data/index.html.

2.4. Quantitative RT-PCR (Q-PCR)

Total RNAs were treated with DNase I using an RNeasy Micro kit (QIAGEN K.K., Tokyo, Japan). cDNAs were then synthesized from 2 µg of total RNA by SuperScript II (Invitrogen), followed by dilution up to 100 µl. Q-PCR reactions were performed using a QuantiTect SYBR Green PCR kit (QIAGEN K.K.) with 500 nM of each primer and 0.5 µl of cDNA template in each reaction. Each cycle was performed at 94°C for 15 sec, 55°C for 20 sec and 72°C for 20 sec, with analysis using an Mx4000 system (Stratagene Japan K. K., Tokyo, Japan). For tenfold dilutions, the threshold cycle (Ct) value differed from 3.3 to 3.6, compared to 3.32 calculated theoretically. Two ribosomal proteins, Mrpl37 and rps3, were used as references to correct the amount of mRNA in each reaction by calculating ΔCt value, that is, subtracting the difference of their reference Ct values in each reaction from the Ct value with a specific primer set. Every experiment with each individual was performed in triplicate and the mean was used as the value of that individual.

Long-range PCR experiments were performed for 35 cycles with an extension
time of three minutes using platinum Taq DNA polymerase high fidelity (Invitrogen) and the primers exon_3F and exon_5R (supplemental data).

3. Results

3.1. Genes affected by psychophysiological stress in mouse tissues

Genes that showed a clear-cut difference in their expression level between psychophysiological stress-exposed samples and untreated controls were identified from the frontal cortex and hippocampus. Four and ten such genes were detected in the cDNA microarray and oligo-microarray experiments, respectively, and nine of these, including the four found in the cDNA microarray, were verified by quantitative RT-PCR (Q-PCR) experiments using several different tissues, in addition to the brain. One gene was found in both microarray experiments, and finally eight genes were identified (Table 1).

Six of the eight genes were more than two-fold up-regulated under psychophysiological stress in all the tissues examined: the serum/glucocorticoid-regulated kinase 1 (SGK1), the MAP kinase phosphatase 1 (MKP-1 or Dusp1), the early growth response gene 1 (Egr-1), the NF-kappaB inhibitor alpha (Nfkbia or IkBa), the cysteine-rich angiogenic inducer 61 (Cyr61) and the hypoxia-inducible factor 1 responsive gene (RTP801). In contrast, the cdc2-like kinase
1 (CLK1) in the brain and a zinc finger DNA-binding protein (Kin17) were down-regulated in most tissues. In some other tissues, CLK1 and Kin17 did not show significant changes in expression under the stress (p>0.05).

3.2. Brain-specific transcriptional regulation of CLK1 under psychophysiological stress

The CLK1 gene showed down-regulation in both cDNA and oligo-microarray experiments. In Q-PCR experiments using the exon_2F and exon_3R primer set, which can detect all the CLK1 mRNA species (see below), a three-fold stress-dependent decrease was observed with this gene only in the frontal cortex and hippocampus with very low individual SD values (Fig. 1 and Table 1). Other brain regions, including the hypothalamus and amygdala, showed a similar decrease (data not shown). On the other hand, the CLK1 expression in other tissues examined did not change significantly under the stress.

3.3. Splicing variants of CLK1

We examined the levels of the four CLK1 mRNA species: in descending order of size, two partially-spliced forms (P-I and P-II), the full-size Clk1.8 transcript, and the exon-4-deleted Clk1.7 transcript [10]. To analyze stress-induced alternative splicing
events, we used primers corresponding to exon 3 and exon 5 sequences, which
discriminated between Clk1.8 and Clk1.7, but did not allow unique identification of P-I
and P-II. Analysis of the long-range PCR products by agarose gel electrophoresis (Fig.
2a) showed a stress-induced increase in the Clk1.7 level, but decrease in the level of
full-size Clk1.8 and of the P-I / P-II mixture (referred to as P). All the tissues examined
showed similar behavior.

This observation was confirmed by Q-PCR experiments with specific primer sets,
as shown schematically in Fig. 2b. Although the quantitative changes in RNA levels
differed among tissues, the general profile of the transcripts was the same in all tissues,
including the brain: stress-induced increase in the relative level of Clk1.7 and decrease
in Clk1.8 and P-II, but no tendency for P-I.

3.4. Individual analysis of SGK1 gene expression

The serum/glucocorticoid-regulated kinase 1 (SGK1) expression level increased
two-fold in the brain and up to eight-fold in other tissues by Q-PCR (Table 1),
demonstrating behavior completely different from that of CLK1. Interestingly, the
expression level of SGK1 in control individuals showed large differences between
animals (Fig. 3a), in contrast to the CLK1 data in Fig. 1 using the same individuals.
Expression levels of the control animal No. 6 in frontal cortex and Nos. 5, 6 and 7 in hippocampus were similar to the constant expression levels of stressed animals. Other tissues did not exhibit such characteristics in individual animals (Fig. 3b).

4. Discussion

Significant changes in the expression levels of eight genes were observed under psychophysiological stress (Table 1). These genes appear to be associated with several different stress-inducible systems (Table 2), although the genes identified herein are only a subset of those in various stress-responsive networks in mammals [16-19]. The fold-change values obtained in the microarray experiments in Table 2 were in general smaller than those obtained in the quantitative RT-PCR (Q-PCR) experiments in Table 1, partly because non-specific signals in hybridization may mask specific signals on microarray gene spots. Consistent changes of seven genes among tissues, other than CLK1, may be caused by the physiological part of the stress affecting the whole body, which might be controlled through the circulation or the autonomic nervous systems. The remaining gene, CLK1, is likely to reflect a unique psychological stress-response in the brain as the effect is different in other tissues. Incoherent behavior in the response (+/-) shown with Kin17 and Cry61 in Table 2 might reflect unique responses to different...
types of stresses in cultured cells, or the duration of those effects. Our experimental condition using the whole animal is likely to reveal several different response systems to resist or surrender to a given stress.

Unique three-fold down-regulation of CLK1 gene expression was observed in the brain under psychophysiological stress (Fig. 1), which is consistent with the weak down-regulation of CLK1 (about 1.2-fold) observed in the hippocampus of tree shrew under chronic psychosocial stress [6]. Further experiments are needed to understand the nature of the stress given to the brain tissues, as well as the relationships among the genes identified here and the contributions of other factors in the stress response networks.

Apart from the brain-specific down-regulation of CLK1, comprehensive alterations of splicing under psychophysiological stress were observed in different tissues. There is a possibility that the “residual expression” of this gene in a limited class of cells in the brain, such as non-neural cells, might cause this effect, although this hypothesis needs to be tested. Splicing regulation in the levels of the full-size Clk1.8 and exon-4-deleted Clk1.7 transcripts may lead to subsequent changes in regulatory networks of mRNA processing, together with the activities of specific SR proteins. Further studies on SR protein networks are needed to understand the roles of the
splicing regulation of CLK1 under the stress [9]. Some stress-responsive alternative splicing events have been reported in mammals: activating transcription factor 3 (ATF3) [20], serum response factor (SRF) [21] and neurofibromatosis type 1 protein (NF1) [22]. However, their splicing patterns did not change under our stress condition (data not shown). Thus, the alternative splicing behavior of CLK1 is likely to be a response peculiar to psychophysiological stress.

In normal tissues, partially-spliced forms of CLK1 mRNA (P-I and P-II) tend to accumulate [10], whereas similar transcription products of other genes are usually short-lived. The fact that about one-third of the CLK1 ESTs in mouse databases are derived from the partially-spliced products, as was the CLK1 clone in our cDNA collection, also provides evidence for the stability of the partially-spliced CLK1 transcripts. Hence, the behavior of the CLK1 gene in our report is the first example of a specific stress response through a regulatory combination of gene expression, partially-spliced mRNA storage, and an alternative splicing preference in a single gene.

It is interesting to note that, in the brain, some individuals under normal conditions showed an expression level of SGK1 similar to that in the individuals under stress (Fig. 3a). This variation was not seen in other tissues (Fig. 3b). Tsai et al. have proposed that the high SGK1 expression in rat hippocampus may be correlated with
individuals with good learning ability [23], suggesting some correlation between the psychophysiological stress-response in the nervous system and learning performance under a new circumstance.

The present findings pave the way to further studies on gene expression in whole animals under various stress conditions, leading to better understanding of the correlation among specific effects of stress on unique genes in unique cells.
References


45651-45660.


Figure Legends

Fig. 1. Levels of CLK1 transcripts measured by quantitative RT-PCR (Q-PCR) analysis in the frontal cortex and hippocampus (n=7), and in four other tissues (n=3). The data are the same as those in Table 1. Expression levels were calculated as fold changes (sample or control/control), following correction using the expression level of the ribosomal proteins Mrpl37 and rps3. The primers used were exon_2F and exon_3R (see text). Bars indicate the standard deviation (SD) values among individuals. **: p<0.01 by t-test on mean difference between samples and controls. #: p>0.05 by t-test. C: controls. S: stressed samples.

Fig. 2. Quantification of CLK1 mRNA splicing variants. a) Long-range PCR using the exon_3F and _5R primer set, followed by agarose gel electrophoresis. PCR products, indicated by arrowheads, are drawn schematically on the right. Exons are indicated by squares and identified by their numbers, and introns are depicted as horizontal bars between exons. Splicing events are represented by dotted lines. b) Expression level of each mRNA species determined by Q-PCR analysis and shown as a fold change (sample/control). Increased values under stress are circled. The primers used are
indicated schematically. P-I: 5.6 Kb partially-spliced mRNA. P-II: 3.2 Kb partially-spliced mRNA. P: P-I and P-II mixture. Clk1.8: 1.8 Kb full-size mRNA. Clk1.7: 1.7 Kb exon-4-deleted mRNA. C: controls. S: stressed samples. M: 100-bp ladder DNA marker (Invitrogen, Carlsbad, CA). arrow: PCR primer.

Fig. 3. Expression analyses for the SGK1 transcript, measured by quantitative RT-PCR (Q-PCR). a) Individual expression levels shown as the ∆Ct values, corrected by reference genes, in the brain regions (n=7). Bars indicate experimental SD values in individuals. Numbers of control (standard style) or stressed (italic style) sample exhibit the same individuals. b) Individual expression levels in other tissues than the brain (n=3). Bars indicate experimental SD values. **: p<0.01 by t-test on mean difference between samples and controls.
### Table 1. Stress-response in tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Up-regulated</th>
<th>Down-regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SGK1</td>
<td>MKP-1</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Heart</td>
<td>8.0</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Kidney</td>
<td>8.0</td>
<td>8.6</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>5.3</td>
<td>3.0</td>
</tr>
<tr>
<td>Blood</td>
<td>2.3</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

Data by quantitative RT-PCR are shown as the fold change (sample/control).

N=7 for the samples or controls of frontal cortex and hippocampus, n=3 for those of other tissues.

#: p>0.05 by t-test on mean difference between samples and controls.

*: p<0.05, and others are p<0.01.

nd: no data, because of very low expression level.
Table 2. List of psychophysiological stress-responsive genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>fold change</th>
<th>+/-</th>
<th>Ref. #</th>
<th>Stress type</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLK1 a,b</td>
<td>0.5 x</td>
<td>-</td>
<td>[6]</td>
<td>Chronic psychosocial</td>
<td>-</td>
</tr>
<tr>
<td>SGK1 a</td>
<td>2.0 x</td>
<td>+</td>
<td>[24]</td>
<td>Hypertension, UV, etc.</td>
<td>+</td>
</tr>
<tr>
<td>MKP-1 a</td>
<td>1.5 x</td>
<td>+</td>
<td>[25,26]</td>
<td>UVC, hypoxia, etc.</td>
<td>+</td>
</tr>
<tr>
<td>Egr-1 a</td>
<td>1.5 x</td>
<td>+</td>
<td>[27,28]</td>
<td>Stretch, oxidative, etc.</td>
<td>+</td>
</tr>
<tr>
<td>Nfkbia b</td>
<td>1.8 x</td>
<td>+</td>
<td>[29]</td>
<td>UVC $^\ddagger$</td>
<td>+</td>
</tr>
<tr>
<td>Kin17 b</td>
<td>0.6 x</td>
<td>-</td>
<td>[29]</td>
<td>UVC $^\ddagger$</td>
<td>+</td>
</tr>
<tr>
<td>Cyr61 b</td>
<td>1.8 x</td>
<td>+</td>
<td>[30]</td>
<td>Hypoxia $^\ddagger$</td>
<td>+</td>
</tr>
<tr>
<td>RTP801 b</td>
<td>1.8 x</td>
<td>+</td>
<td>[31]</td>
<td>Shear $^\ddagger$</td>
<td>-</td>
</tr>
</tbody>
</table>

Fold change (sample/control) is shown as data by microarray experiments, not equal to Q-PCR data in Table 1.

+: induction, -: reduction in each stress type.

a: identified from cDNA microarray, b: from oligo-microarray.

$^\ddagger$: stress given to cultured cells.
Fig. 1. Murata et al.
Fig. 2. Murata et al.
Fig. 3. Murata et al.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction; position</th>
<th>5'</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLK1</td>
<td>Forward; exon_2F</td>
<td>AGAAGCAGCAGCAGTCACAA</td>
</tr>
<tr>
<td></td>
<td>Reverse; exon_3R</td>
<td>ATCTGCTTCAGGTTCTCCA</td>
</tr>
<tr>
<td></td>
<td>Forward; exon_3F</td>
<td>ATGGAGAACCTGGAAGCAGA</td>
</tr>
<tr>
<td></td>
<td>Reverse; exon_5R</td>
<td>CCGAAAGCACCCTCACCTAA</td>
</tr>
<tr>
<td></td>
<td>Forward; intron_4F</td>
<td>CATTGGTCTCATTCTCTGTTATGAA</td>
</tr>
<tr>
<td></td>
<td>Reverse; intron_6R</td>
<td>GGGCTTACAAATTCAAACCTCTC</td>
</tr>
<tr>
<td></td>
<td>Reverse; exon_6-7R*</td>
<td>TGGACACAACGGAAAGTACTATG</td>
</tr>
<tr>
<td></td>
<td>Reverse; exon_4R</td>
<td>TGACCCTCCTCATCATCCTC</td>
</tr>
<tr>
<td></td>
<td>Forward; exon_3-5F</td>
<td>ATTCACACGGGATGAAATTGT</td>
</tr>
<tr>
<td></td>
<td>Reverse; exon_6R</td>
<td>TGTGACCTCGATGCTCAAAC</td>
</tr>
<tr>
<td>SGK1</td>
<td>Forward</td>
<td>GCCAAGGATGACTTTATGGAGATTA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGAATCCACAGGAGGATGATA</td>
</tr>
<tr>
<td>MKP-1</td>
<td>Forward</td>
<td>GAGCTGTGACGCAAACAGTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTTCCGAGAAGCGTGATAGG</td>
</tr>
<tr>
<td>Egr-1</td>
<td>Forward</td>
<td>GAGCGAACAACCCCTATGAGC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGGCCACTGACTAGGCTGAA</td>
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</table>
Nfkbia Forward TCGCTCTTGTGGAAATGTGG
Reverse TGGCCTCIAACACACACAGT
Kin17 Forward GGCACAAAGGTCCACAA
Reverse TACCAGCTTTCTGGGTCTC
Cyr61 Forward AGACCAGGATCTGTGAAGTG
Reverse TTCTGGTCTGCAGAGGTGTG
RTP801 Forward CTTCGTCTCGTCTCGAACT
Reverse GCACACAGGTGCTCATCCTC
Mrpl37 Forward CTGGAGCCCATTACCTACGA
Reverse TCTTGGGAGGCTTCTATT
rps3 Forward CAGGCAGAGTCTCTACGCTACA
Reverse TCTCCATAATGAACCGAAGCA

*: exon 6-7 corresponds to the junction between exons 6 and 7.