overlap in neuronal circuits that are involved (Joels and Baram 2009). These studies suggest that the experimental stress exerts relatively great influences on distinct neuronal circuits and functions. To avoid possible errors or artifacts caused by experimental stress, stress-reduction techniques were applied, and control groups were established under the exactly same experimental conditions. However, the experimental stress sometimes still caused experiment bias and resulted in incorrect conclusion (McEwen 2002). For example, when laboratory animals accepted electroacupuncture treatment accompanied with restraint stress, plasma corticosterone levels increased significantly, and assessment of electroacupuncture-induced c-*Fos* expression was confounded by restraint stress in many encephalic regions (Liu *et al.* 2005). Therefore, investigations of experimental stress not only help us better understand the interaction of experiment effects and experimental stress comprehensively, but also eliminate the effects produced by stress from the main effect caused by experimental maneuvers.

Gene expression in the CNS, comprising the measurable indicator of the interaction between the genome and the stimulation, may decipher the molecular basis of specialized function and behavior comprehensively (Robinson 2004). Therefore, it is important to elucidate the gene expression changes in CNS after stress. cDNA microarray technology offers a powerful approach to bridge the gap between the physiological responses and the molecule level alterations by simultaneously monitoring genome-wide expression profiling, and gives insights into mechanism and feature of behavior or stimulation (Geschwind 2000).

As restraint stress may produce analgesic or hyperalgesic effects (Porro and Carli 1988; Imbe *et al.* 2006), we selected the CNS regions of arcuate nucleus (Arc), periaqueductal gray (PAG) and spinal dorsal horn (DH), all of which are closely related to stress and pain regulation, for gene expression profiling after stress. The changes in gene expression profiles of the different CNS anatomical regions after stress were dissected and the effects of the moderate restraint stress were discussed.

Materials and methods

Animals

All experiments were performed on male Sprague–Dawley rats, obtained from the Experimental Animal Center, Peking University, weighing 200–220 g at the beginning of the experiment. Animals were housed in a 12 h light/dark cycle with food and water available *ad libitum*. The room temperature was maintained at 22 ± 1°C and relative humidity at 45–50%. Four to five animals were housed in each cage. The rats were habituated to the environment and handled daily during the first 3 days after arrival. All experimental procedures were approved by the Animal Care and Use Committee of the Peking University Health Science Center.

Moderate restraint stress treatment

The rats were treated and divided into five groups: control group (C): being killed without receiving any restraint and nociceptive testing; restraint group 1 (R1): received restraint and returned to home cages for 1 h before being killed; restraint group 2 (R24): received restraint and returned to home cages to recover 24 h before being killed; the restraint and the nociceptive test group 1 (RT1): received restraint, nociceptive testing and returned to home cages for 1 h before being killed; the restraint and the nociceptive test group 2 (RT24): received restraint, nociceptive testing and returned to home cages 24 h before being killed (Table S1).

The rats were placed in well-ventilated Plexiglas restraint barrel and received 50 min moderate restraint stress with hind limbs and tail extending out (Fig. S1). Before and after the restraint, nociceptive threshold of RT1 and RT24 groups were measured. Rats were returned to their home cages after the restraint stress (R1 and R24 groups), or after the restraint stress and pain testing (RT1 and RT24 groups).

Nociceptive testing and statistical analysis

Nociceptive threshold to acute thermal stimulation was measured by the tail-flick test (D'Amour and Smith 1941). Focused light (3 mm diameter) from a 12.5 W projection bulb was applied directly to the middle of the tail. The projection bulb was turned off as soon as the rats flicked its tail, and a digital timer measured the tail-flick latency (TFL) to an accuracy of 0.1 s. The voltage was adjusted to 12 V and room temperature was carefully monitored to be 22 ± 1°C to minimize the possible influence of ambient temperature on TFLs (Tjolsen and Hole 1993) during the test. We used a cut-off latency of 12 s in order to avoid possible tissue damage to the superficial tail. Results were presented as mean ± SEM and were analyzed with Mann–Whitney Rank Sum Test.

RNA extraction and cDNA microarray hybridization

Rats were killed by decapitation and the tissue of Arc, PAG and the DH of the fifth and sixth lumbar (L5 and L6) spinal cord were quickly removed and stored immediately in cold RNAlater (Qiagen, Hilden, Germany), stored at −80°C until later experiment.

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified with RNeasy column (Qiagen, Valencia, CA, USA). RNA quality was assessed with Lab-on-chip Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Homemade cDNA microarrays containing 11 444 rat genes/ESTs (Expressed Sequence Tags) were as previously described (Li *et al.* 2008; Gu *et al.* 2009). The microarray platform was submitted to the GEO database with the accession number GPL3498. Microarray experiments and data extraction strategy were performed as previously described (Xie *et al.* 2009). Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) was used for RNA linear amplification following the manufacturer's protocol. Equal amounts of RNA from the same CNS regions (Arc, PAG or DH) of five control rats were pooled and labeled with Cy3, as a reference. RNA samples from different CNS regions of groups RT1 ($n = 5$) and RT24 ($n = 5$) were individually labeled with Cy5. Cy3- and Cy5-labeled cRNA pools were mixed to hybridize to the microarrays.

Bioinformatics analysis

Normalization of each microarray was accomplished by using intensity-dependent locally weighted scatter plot smoothing regression analysis (LOWESS) of GeneSpring 6.1 software package (Agilent Technologies). The spots with low signal noise ratio (< 2) were automatically eliminated, and only those genes present in > 3 samples (60%) in each group were applied in further analysis. Regulated genes were selected out by significance analysis of microarrays (SAM) (Tusher *et al.* 2001) with false discovery rate < 0.01, and average regulation of the gene was no less than 1.5-fold against the control group. Principal components analysis (PCA) was used to summarize the gene expression profiles between groups.

We applied the online gene ontology (GO) terms annotation software, GOTree Machine (http://www.genereg.ornl.gov/gotm/) (Zhang *et al.* 2004; Lee *et al.* 2006) to elucidate the biological process of the regulated genes. The significance of category enrichment of genes was set to $p < 0.01$ as determined by the hypergeometric test implemented in GOTree Machine tool (Zhang *et al.* 2004).

Quantitative RT-PCR

RTs were primed with oligo(dT). Quantitative RT-PCR (qRT-PCR) was conducted in duplicate with *Gapdh* as internal control using Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR green I qRT-PCR kit (Toyobo, Osaka, Japan). The primer sequences are listed in Table S2. Data are presented as mean ± SD and was analyzed with the unpaired *t*-test.

Western blot

Experiment was performed as before (Xie *et al.* 2009). After removing RNAlater, frozen tissues were homogenized in cold lysis buffer (Beyotime Biotechnology Co., Haimen, Jiangsu, China) and centrifuged at 13 200 *g* for 15 min at 4°C, the supernatant total protein was quantify by Lowry method (Lowry *et al.* 1951). Samples (30 μg total protein per loading) were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and electro-transfered onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk overnight at 4°C, and then incubated with the primary antibodies recognizing β-actin (mouse monoclonal, 1 : 5000, Abcam, Cambridge, UK) or SGK1 antibody (rabbit polyclonal, 1 : 1000, Cell Signaling Technology, Danvers, MA, USA) for 2 h at 22°C. Then, the membranes were incubated with a horseradish peroxidase-conjugated anti-mouse (1 : 5000, Abcam) or goat anti-rabbit (1 : 5000, Abcam) secondary antibodies. The signal was visualized with ECL Plus reagent (GE healthcare, Buckinghamshire, UK) and exposed onto x-ray film (Kodak, Rochester, NY, USA).

Results

The pain threshold of restraint stress-treated animals

In this study, our manipulation as temporary moderate restraint treatment did not cause significant changes in pain threshold of the rats (Mann–Whitney U Statistic = 397, $p = 0.58$, $n = 27$) (Fig. S2).

Transcriptome modulation after moderate stress exposure

Gene expression profiles were investigated across three CNS regions (Arc, PAG, and DH) at two time points after restraint and nociceptive test (RT1/RT24). In RT1 group, we identified 130, 154, and 231 genes/ESTs to be regulated in the Arc, PAG, and DH, respectively, and 422, 807, and 249 in each tissue of RT24 group (Fig. 1a). Quantitative RT-PCR results showed good correlation with the microarray data and demonstrated reliability of microarray results (Fig. S3).

As time elapsed after restraint stress, gene expression profiles diversified in all three tissues. More genes were regulated in RT24 group than in RT1 group, the regulation profiles were basically different in the RT1- and RT24-groups. PCA plot with all the detected genes among the three tissues revealed more divergence in RT24 group than in RT1 group (Fig. 1b).

Differentially expressed profiling in Arc

In Arc tissue, after restraint and nociceptive test, 130 and 422 genes/ESTs from the arrays were identified to be regulated in

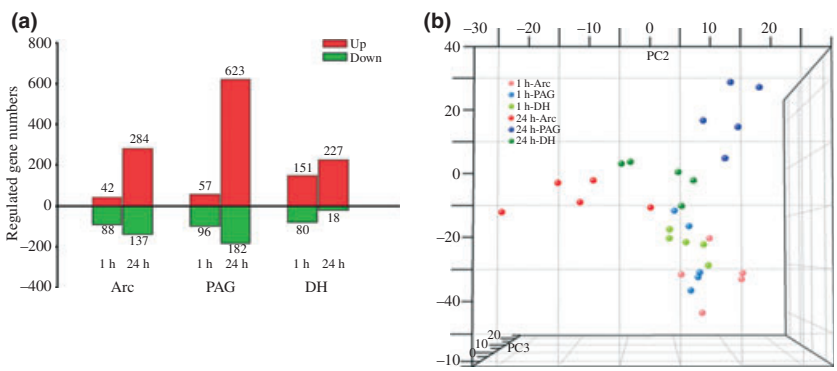

Fig. 1 Temporary moderate restraint-induced gene expression was revealed by significance analysis of microarrays (a) and PCA (b) analysis. (a) Numbers of differentially expressed genes/ESTs in Arc, PAG, and DH at two time points by significance analysis of microarrays analysis. List of the complete regulated genes for each group is accessible in Table S3. (b) The first three principle components of the filtered gene/ESTs of all samples from all arrays were analyzed by PCA analysis to show their separation.

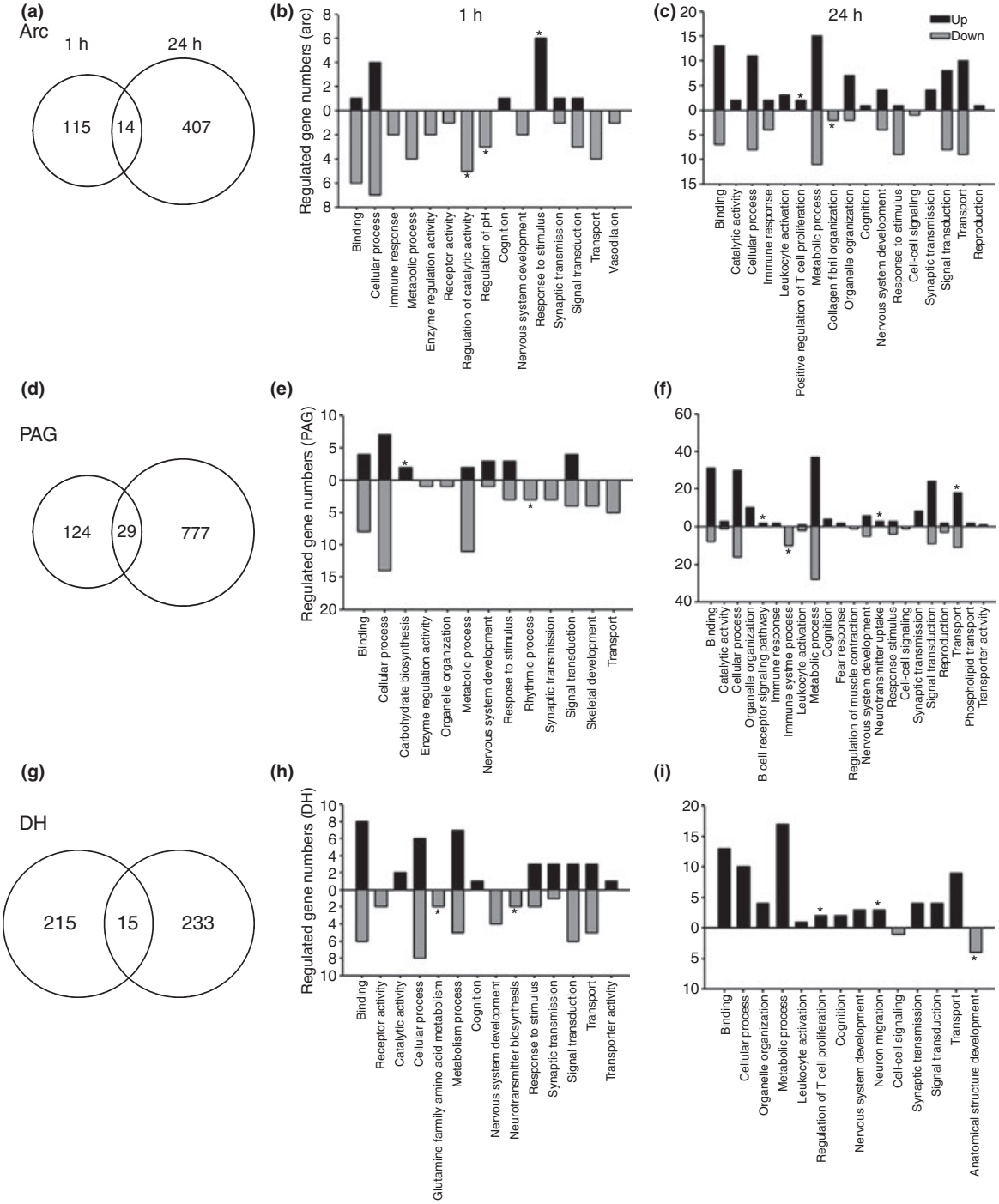

Fig. 2 Overlapped and non-overlapped gene expression and GO categories in each Arc, PAG and DH regions at 1 h/24 h. (a, d, g) Region-dependent expression patterns for Arc, PAG and DH at 1 h/24 h are represented as circles. The diagram shows the number of genes with the indicated expression patterns. (b, c) GO annotations of regulated genes with known in Arc at 1 h/24 h. (e, f) GO annotations of regulated genes with known in PAG at 1 h/24 h. (h, i) GO annotations of regulated genes with known in DH at 1 h/24 h. *The significant enriched GO categories by GO Tree Machine analysis ($p < 0.01$).

RT1 and RT24 group respectively, while 15 of them overlapped in the both groups (Fig. 2a). In RT1 group, 14 genes with GO annotation according to GenBank database had up-regulated expression, and 41 had down-regulation, while 84 annotated genes had up- and 65 annotated ones had down-regulations in RT24 group (Table S3).

Based on the GO annotations, the regulated genes involved in 15 GO categories in RT1 group, and 17

categories involved in RT24 group, but the gene profiles revealed differences (Fig. 2b and c). GOTree Machine enriched seven up-regulated genes to be presented in the 'response to stimulus' category in RT1 group, but in RT24 group there were six genes of this category had down-regulation (Fig. 2b and c; Tables S3 and S4). Immune-related biological process GO categories, including immune response, leukocyte activation, and positive regulation of T cell proliferation, were affected in RT24 group (Fig. 2c) and 'positive regulation of T cell proliferation' also enriched by GOTree Machine (Table S4). But only two genes in 'immune response' had down-regulation in 1-h time point (Fig. 2b; Table S3). In addition, at both RT1 and RT24 groups, GO categories were linked with nervous system functions, including nervous system development, synaptic transmission, and cognition (Fig. 2b and c; Table S3).

Differentially expressed profiling in PAG

In PAG tissue, 154 and 807 genes/ESTs from the arrays were identified to be regulated in RT1 and RT24 group respectively, while 30 of them overlapped in the both groups, both group possessed the most regulated genes among the three tissues explored (Fig. 2d). In RT1 group, 25 genes with GO annotation had up-regulated expression, and 58 had down-regulation, while 189 had up- and 99 had down-regulation in RT24 group (Table S3).

Gene ontology annotation revealed that the regulated genes involved in 13 GO categories in RT1 group, and 22 GO categories in RT24 group. In RT1 group, the GO categories of 'carbohydrate biosynthesis' and 'rhythmic process' were regulated (Fig. 2e) and enriched by GOTree Machine (Table S4), but no regulations of them were found in RT24 group (Fig. 2f). Immune-related GO categories, including B cell receptor signaling pathway, immune response, immune system process and leukocyte activation, had regulations in RT24 group but not in RT1 group (Fig. 2e and f). GO categories of 'B cell receptor signaling pathway' and 'immune system process' were also enriched by GOTree Machine (Table S4). GO categories linked with nervous system function and behaviors, including nervous system development, fear response, regulation of muscle contraction, and neurotransmitter uptake, had regulations in both RT1 and RT24 groups (Fig. 2e and f).

Differentially expressed profiling in DH

In DH tissue, 231 and 249 genes/ESTs from the arrays were identified to be regulated in RT1 and RT24 group respectively, and the both groups shared 16 of them (Fig. 2g). In RT1 group, 37 genes with GO annotation had up-regulated expression, and 43 had down-regulation, while RT24 group had 72 up- and five down-regulated ones (Table S3).

In RT1 or RT24 group, the respective regulated genes with GO annotation were involved in 14 GO categories. Nervous system functions-related GO categories of 'glutamine family amino acid metabolism', 'neurotransmitter biosynthesis', 'synaptic transmission', and 'nervous system development' were regulated in RT1 group (Fig. 2h), and 'cognition', 'nervous system development', 'neuron migration', and 'synaptic transmission' were regulated in RT24 group (Fig. 2i). Immune-related GO categories of 'immune response' had regulation in RT1 group (Fig. 2h), 'leukocyte activation' and 'regulation of T cell proliferation' had regulations in RT24 group (Fig. 2i). GOTree Machine revealed that 'glutamine family amino acid metabolism', 'neurotransmitter biosynthesis', and 'immune response' were enriched in RT1 group, and 'neuron migration', 'microtubule organizing center organization and biogenesis', 'morphogenesis', and 'positive regulation of T cell proliferation', were enriched in RT24 group (Table S4).

The simultaneously regulated genes in the three regions in response to moderate stress

To explore the common characteristics in CNS involved in stress response, the genes that were simultaneously regulated at the Arc, PAG and DH after moderate stress were analyzed. Fourteen and 123 genes/ESTs were identified as being simultaneously regulated in response to moderate restraint in the three CNS regions in RT1 and RT24 groups (Fig. 3a and b; Tables S5 and S6), respectively. Importantly, these co-regulated genes showed similar regulated directions (up- or down-) among the three CNS regions after moderate stress at respective time points. Among the 14 and 123 co-regulated genes, 6 and 38 were categorized according to GO annotation at 1-h and 24-h time point, respectively (Fig. 3c and d). The complete GO categories gene list is accessible at Table S7. For example, microarray analysis indicated that the expression of the serum and glucorticoid-regulated kinase 1 (*Sgk1*) was significantly up-regulated at 1-h time point in the three CNS regions after moderate restraint and returned to basal level at 24-h time point (Fig. 4a–c).

To validate the differential expression of *Sgk1*, qRT-PCR and western blot were performed. Consistent with microarray, qRT-PCR showed the expression of *Sgk1* was increased at 1-h time point after moderate restraint stress and the expression was decreased at 24-h time point compared with 1-h time point (Fig. 4d–f). Western blot analysis showed that Sgk1 protein level gradually increased among three regions after temporary moderate restraint treatment (Fig. 4g–i).

Different gene expression between restraint-only and restrain-nociceptive test

In pain related research, experimental animals were subjected to nociceptive test which were often accompanied by restraint stress. TFL, as one of the most widely adapted nociceptive tests, results in an acute thermal stimulation to the tail of the animals. In order to determine whether TFL test

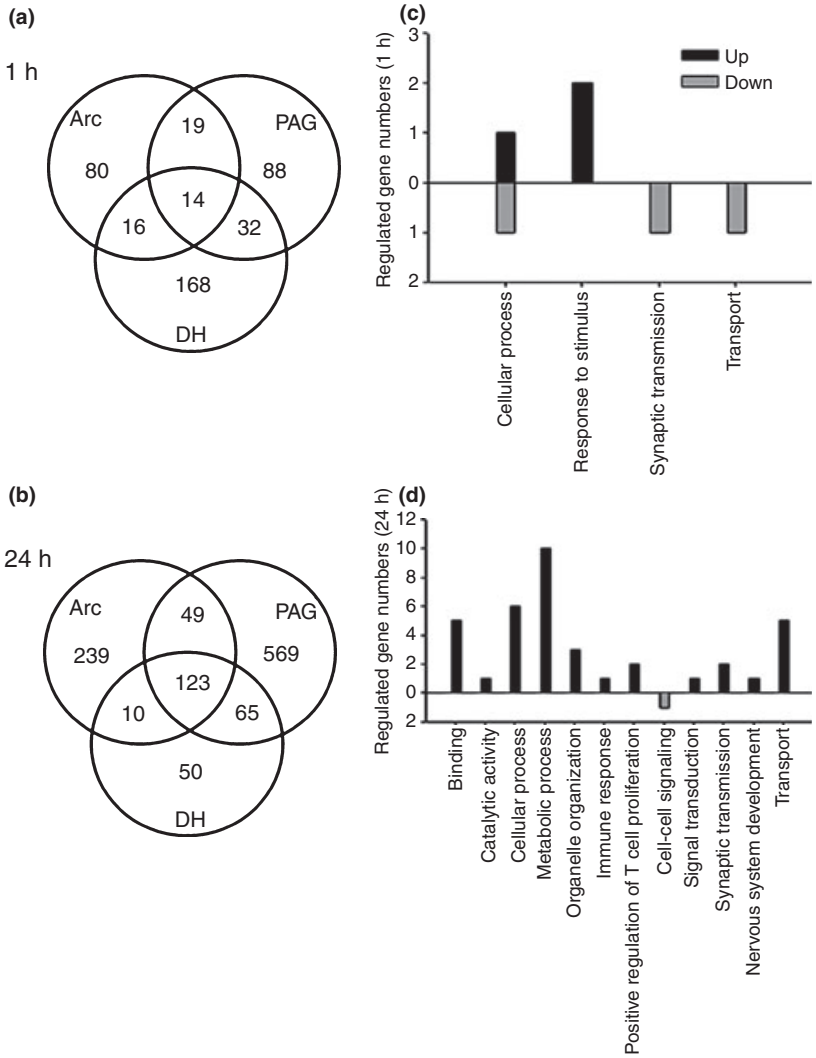

Fig. 3 Overlapped and non-overlapped gene expression and GO categories of co-regulated genes among three regions at each time point. (a, b) Region-dependent expression patterns for Arc, PAG and DH at 1 h/24 h are represented as circles. The diagram shows the number of genes with the indicated expression patterns. (c, d) GO annotations of co-regulated genes among three regions at 1 h/24 h.

would affect gene expression, the gene expression between restraint-only group and restraint-nociceptive test groups were compared. The genes that were previously selected for verifying the reliability of the microarray results were selected to compare the gene expression difference between R1, R24 groups and RT1, RT24 groups at the Arc, PAG and DH by real-time RT-PCR. As shown in Fig. 5, approximately 50% of genes showed some different regulation by TFL measurement.

Discussion

Restraint is regarded as a common experimental stress in investigating stress-related physiological or pathologic phenomena in many studies (Glavin *et al.* 1994). Restraint may affect the activities and functions of nervous systems through transcriptional and non-transcriptional mechanisms. Therefore, cDNA microarray was used to examine the gene expression in the three CNS regions at the time of 1 h and 24 h after the moderate restraint stress.

In this study, the nociceptive threshold of rats was not changed before and after 50 min restraint, as measured by TFL test (Fig. S2). It is still a controversial issue whether restraint stress would change pain sensitivity. On one hand, studies have shown that exposure to restraint stress may lead to change of the pain sensitivity (Porro and Carli 1988; Imbe *et al.* 2006). On the other hand, there are reports showing that restraint did not change the pain sensitivity (Maier 1986; Sun *et al.* 2004). The discrepancy may result from the differences in intensity, duration and frequency of the stress (Pare and Glavin 1986). Restraint with high-intensity or long duration may cause aversive or irritable behaviors, leading to analgesic or hyperalgesic effect (Imbe *et al.* 2004). In contrast, our experiment revealed that the temporary moderate restraint may not change the pain sensitivity in experimental animals.

Compared to the relatively inert nociceptive threshold measurement, the gene expression regulation in response to stress is much more sensitive. Thus, gene expression profiling revealed robust changes in gene expression

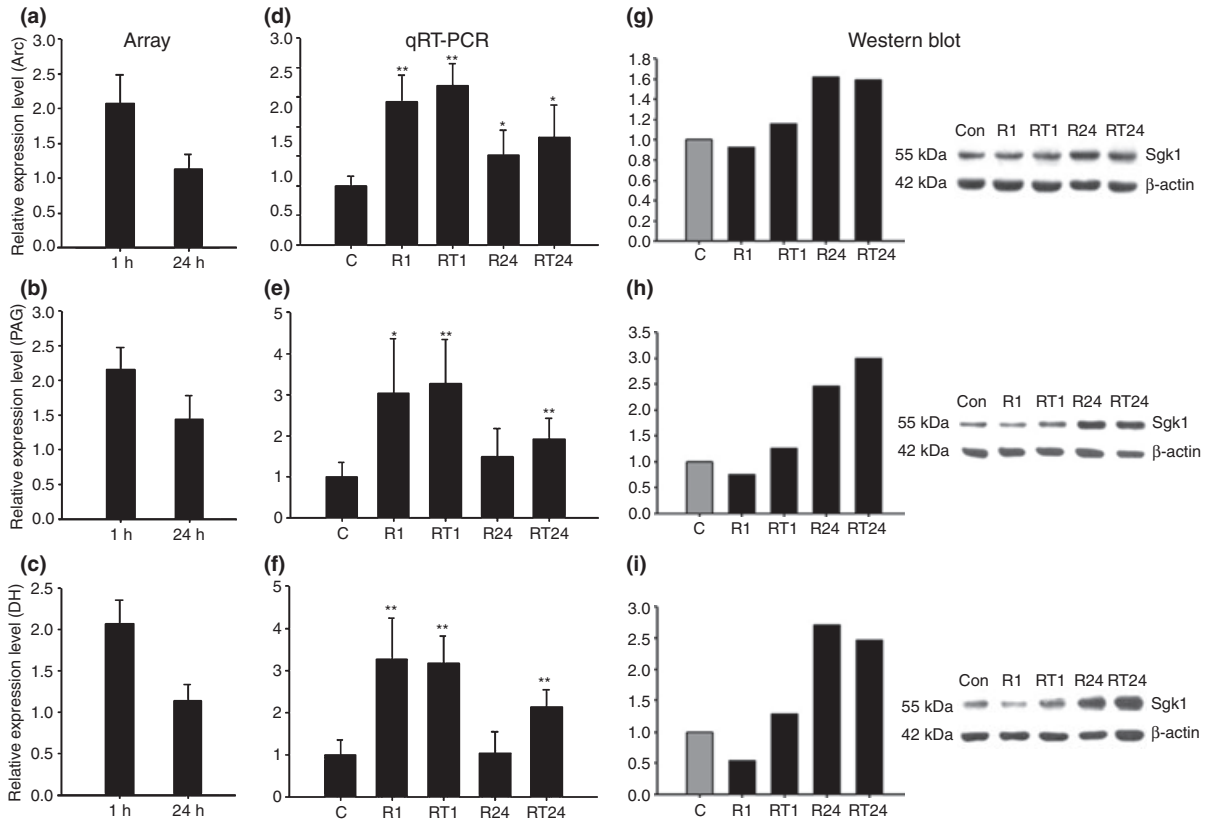

Fig. 4 Sgk1 gene expression and protein level in Arc, PAG and DH induced by temporary moderate restraint ($n = 5$ per group). (a), (b) and (c) showed that *Sgk1* relative fold change in microarray data with respect to control rats at Arc, PAG and DH at 1 h/24 h. qRT-PCR (d, e and f) and western blot (g, h and i) confirmed *Sgk1* up-regulation induced by temporary moderate restraint in Arc, PAG and DH. C: control group; R1: parallel with R1 group without TFL measurement; RT1: killed after restraint and nociceptive test and returned to home cages to recover 1 h; R24: parallel with R24 group without TFL measurement; RT24: killed after restraint and nociceptive test and return to home cages to recover 24 h. Data are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$ vs. control group.

regulations in the CNS regions resulted from the moderate restraint.

Regional- and time-dependent regulation of gene expression by moderate restraint

This study showed a spatiotemporal-dependent variation of global gene expression changes in the CNS regions after the temporary moderate restraint. The PCA showed that the gene expression of the Arc, PAG and DH were convergent and could not be distinguished from each other at 1-h time point (Fig. 1b). This convergence was not because of the same genes were simultaneously regulated at the Arc, PAG and DH. Furthermore, there were few genes in each region that were regulated at both 1-h and 24-h time points (Fig. 2a, d and g). These results suggested that the stress response induced by the temporary moderate restraint is a highly orchestrated biological process that involves the participation of many players at many different stages.

Combining the categorization by GO annotations and the key regulatory process identified by directed acyclic graph (DAG) analysis, we found that specific GO categories were uniquely enriched in a particular CNS region reflecting the interaction and integration of multiple neural circuits in response to external stimuli (Fig. 2; Tables S3 and S4).

Hypothalamus plays a central role in mediating stress responses, and Arc of the hypothalamus is the critical region (Herman *et al.* 2003; Kwon *et al.* 2006, 2008). The GO category 'response to stimulus' was significantly enriched with up-regulated genes at 1-h time point and these genes were almost down-regulated at 24-h time point (Fig. 2b and c; Tables S3 and S4). The regulated genes belonging to this GO category suggested a change in stress responses state at the Arc mainly at the early stage.

Periaqueductal gray is an important integrative portion of the neuroaxis controlling the motivational state of an animal and is proposed to mediate the defensive responses that involve extremely vigorous forms of active behavior, such as flight-or-fight reactions to proximal danger stimuli (Carobrez *et al.* 2001; Brandao *et al.* 2008). At 1-h time point, we found that the regulated genes related to the rhythmic process-categories were enriched; at 24-h time point, we

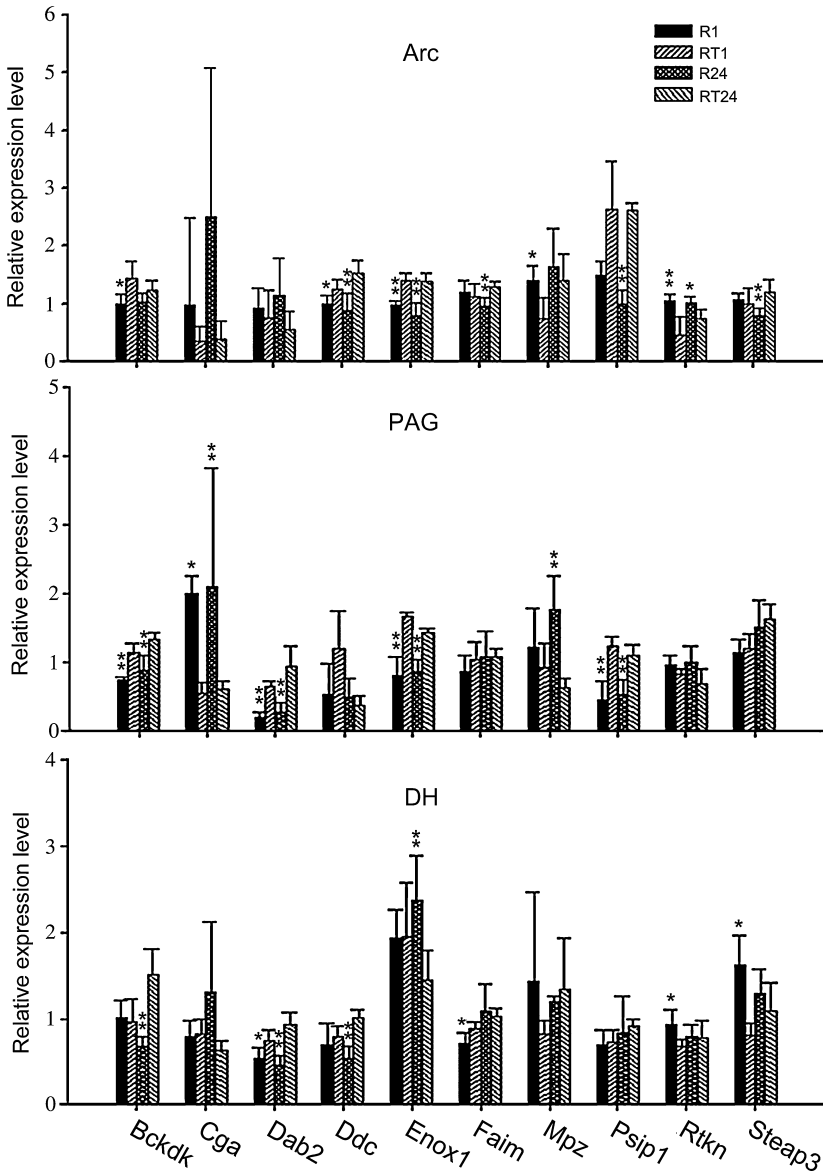

Fig. 5 Gene expression affected by TFL operation ($n = 5$ per group). Data (mean ± SEM) are normalized to control group. *p< 0.05, **p < 0.01, R1 group vs. RT1 group, R24 group vs. RT24 group. Abbreviations are the same as described in Fig. 4.

found that the genes belonging to the 'neurotransmitter uptake', 'cognition', 'fear response' and 'regulation of muscle contraction' were regulated. These genes are possibly participated in the regulation of defensive responses. For example, the expression of *Cry2* was down-regulated by the temporary moderate restraint (Tables S3 and S4). It has been shown that the sympathetic neural activity is elevated in *Cry2* knockout mice (Ikeda *et al.* 2007). This suggested that the temporary moderate restraint could elevate sympathetic neural activity to assure physiological needs in stress state by down-regulating the *Cry2* expression.

Dorsal horn, the dorsal section of the gray matter of the spinal cord, receives and regulates somatosensory information entering the spinal cord. *Ass1* and *Gad2*, which participate in neurotransmitter regulation, were found in the enrichment categories of 'glutamine family amino acid metabolism' and 'arginine metabolism' at 1-h time point by DAG (Table S4). Argininosuccinate synthetase (ASS) regulates nitric oxide production (Wiesinger 2001). Glutamic acid decarboxylase (GAD) is known as a crucial rate-limiting enzyme of GABA production (Wu *et al.* 2007). In this study, the expressions of *Ass1* and *Gad2* were decreased, which indicated the participation of nitric oxide and GABA signaling in response to restraint stress at the DH region. At 24-h time point, the regulated genes were enriched in the GO categories by DAG, such as 'neuron migration', 'microtubule organizing center organization and biogenesis' and 'anatomical structure development', etc. These genes are likely to influence the neuronal morphogenesis and then regulate the functions of the nervous system (Conde and Caceres 2009).

Besides the GO categories related to neural and behavioral effects, the GO categories related to immune function were

also of special interest. The GO categories of 'positive regulation of T cell proliferation', 'B cell receptor signaling pathway' and 'immune system process' were significantly enriched with regulated genes in the three CNS regions by DAG (Table S4). Previous studies suggested that moderate stress enhances the immune response (McEwen 2000). Our results found immune related genes were broadly regulated in the different CNS regions, suggesting that the moderate stress may activate immunoendocrine responses in broad CNS regions. Furthermore, the moderate stress may affect cardiovascular, immune and other systems via neural and immunoendocrine mechanisms (McEwen 2007).

Simultaneously regulated genes in different CNS regions

At 1-h time point, 14 genes were simultaneously regulated in the Arc, PAG and DH; at 24-h time point, there were 123 simultaneously regulated genes in response to the moderate restraint (Fig. 3; Tables S5 and S6). Furthermore, these genes were regulated towards the same directions (up/down) in the three CNS regions investigated, suggesting that these genes may play widespread roles in the CNS. *Sgk1* was one of the most highly up-regulated genes in response to the moderate stress in the three CNS regions. *Sgk1* belongs to the cAMP-dependent, cGMP-dependent, and protein kinase C subfamily of the serine/threonine protein kinases (Chen *et al.* 1999; Lang *et al.* 2006). Little is known about the role of Sgk1 in CNS. More recently, Sgk1 was found to play an important role in learning and memory formation and consolidation (Chao *et al.* 2007; Tyan *et al.* 2008), facilitation of the expression of long-term potentiation (Ma *et al.* 2006), neuronal plasticity (Chun *et al.* 2004; Yang *et al.* 2006), reduction of neuroexcitability (Bohmer *et al.* 2004), and act as a neuroprotective factor antagonizing neural cell death in neurodegeneration (Schoenebeck *et al.* 2005). Combining with our result, Sgk1 may be an important regulated gene in CNS in response to the temporary moderate stress. So these simultaneously regulated genes may play general and important roles in CNS in response to moderate stress.

The influence of TFL measurement

By comparing the gene expression between R1- and R24-groups with RT1- and RT24-groups, we found that thermal stimuli during TFL measurement influenced about 40–50% of the gene expression regulation compared with the restraint only (Fig. 5). This result suggested that experimental manipulation should be kept minimal in order not to affect the gene expression in the nervous system at a significant manner.

Conclusion

A systematic profile of gene expression in different CNS regions that respond to temporary moderate stress was shown in the present study. Critical genes and key biological processes involved in the moderate restraint stress were discussed, showing that temporary moderate external stimuli could regulate genes of specific GO categories in identified CNS regions related with specific neural function. Furthermore, immune-related genes were also regulated in broad CNS regions when exposed to temporary moderate stress, especially at the late stage. These results may help to elucidate the physiological processes involved in temporary moderate stress in the CNS.

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Conflict of interest statement

The authors declare no competing financial interests.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Moderate restraint stress model: the photo of rat when the received restraint.

Figure S2. There is no different in pain threshold change between before and after restraint stress.

Figure S3. Validation of genes with qRT-PCR.

Table S1. Experiment design.

Table S2. Sequences of primers for qRT-PCR.

Table S3. Regulated_gene_list_and_GO_annotation.xls.

Table S4. The gene list of enriched GO categories in biological process by GOTM.

Table S5. Co-regulated genes in all three CNS regions at 1-h time point.

Table S6. Co-regulated genes in all three CNS regions at 24-h time point.

Table S7. Co-regulated_genes_GO_categories.xls.

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