RESEARCH ARTICLE

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cDNA microarray analysis of gene expression in anxious PVG and SD rats after cat-freezing test

Abstract To identify genes involved in the development of anxiety or fear, we analyzed the gene expression profiles of the cortex of anxious hooded PVG and Sprague-Dawley (SD) rats after exposure to the cat-freezing test apparatus. These two rat strains showed a marked difference in the extent of anxious behavior on the cat-freezing test; the hooded PVG rats showed highly anxious behavior while a low anxiety state was observed in SD rats. A cDNA microarray consisting of 5,931 genes was employed to investigate the global mRNA expression profiles of anxiety-related genes. According to the assumption that an abundance ratio of ≥1.5 is indicative of a change in gene expression, we detected 16 upregulated and 38 downregulated genes in PVG hooded and SD rats. Some of these genes have not yet been associated with anxiety (e.g. FGF), while other genes were recently found to be expressed in an anxious state (e.g., rat nerve growth factor-induced gene, NGFI-A). Our study also focused on the expression of some neurotransmitter receptors that have already been proven to be relevant to anxiety or fear, e.g., γ-aminobutyric acid (GABA), cholecystokinin (CCK) and $5\text{-}HT_3$ receptors. To further confirm the microarray data, the mRNA expressions of three genes: rat activity-regulated cytoskeleton-associated gene (Arc), rat NGFI-A gene and rat $5\text{-}HT_3$ receptor ($5\text{-}HT_3R$) mRNA, were studied by reverse transcription-polymerase chain reaction (RT-PCR). The results of RT-PCR were basically consistent with those from cDNA microarray. Our study therefore demonstrated that the microarray technique is an efficient tool for analyzing global expression profiles of anxiety-related genes, which may also provide further insight into the molecular mechanisms underlying the states of anxiety and fear.

Keywords Anxiety and fear · cDNA microarray · RT-PCR · Gene expression · Cat freezing test

H. Wang and Y.Z. Zhu made equal contributions to this work

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Introduction

The significance of anxiety or fear in health and disease is well recognized today but its underlying molecular and neurological mechanisms are still poorly understood. Various conventional methods, e.g. northern blot (Lee et al. 1993; Hinks et al. 1996), in situ hybridization (Birzniece et al. 2002; Keck et al. 2002; Neumaier et al. 2002), and reverse transcription-polymerase chain reaction (RT-PCR; Farook et al. 2001; Muller et al. 2001), have been used to monitor differences in gene expression in various animal models of anxiety, but only a limited number of genes have been proved to contribute to anxiety/fear development. Although much evidence indicates the involvement of the γ-aminobutyric acid (GABA), serotonin (5-HT), norepinephrine, dopamine, and neuropeptide transmitter systems in the pathophysiology of anxiety (Graeff et al. 1996; Harold et al. 1996; Lang et al. 1998), little information is available concerning the function of other anxiety/fear-related genes, especially their expression profiles in anxious state.

The identification of all genes involved in anxiety or fear would be challenging with traditional techniques because they are highly focused, targeting only specific genes. The advent of DNA microarray technology ("DNA chips") has made possible the analysis of gene expression for tens of thousands of genes in parallel. So far, this technique has not been employed to examine gene expression changes in any animal models of anxiety. Very recently, Landgrebe et al. (2002) examined the effects of some antidepressants on gene expression in the

mouse brain with cDNA microarrays containing 3,624 expressed sequence tags (ESTs). In this study, the IncyteGenomics rat GEM cDNA microarray containing 5,931 annotated genes was used to compare the large-scale gene expression profiles between the cortex of PVG hooded and SD rats after cat-freezing test. The cat-freezing test (Blanchard et al. 1991, 1993) was designed to assess defensive behavior of rats in a situation where a predator is encountered. It focuses on risk assessment behavior, including freezing and locomotor activity. Two rat strains, PVG hooded and SD rats, were selected for this test because they showed marked difference in the extent of anxious behavior when exposure to a cat. The expression tendency of three genes obtained from microarray results was further confirmed by RT-PCR. The information on gene expression profile in anxious states may serve to enhance our understanding of the molecular mechanisms underlying anxiety and fear.

Methods

Cat-freezing test

Five male SD (Laboratory Animal Center, National University of Singapore) and PVG hooded rats (Animal Resources Center, Perth, Australia) weighing 200–250 g were housed two per cage in a temperature-controlled (22°C) colony room on a 12:12 h light:dark cycle, and with free access to food and water. The predator cat was a 4.3 kg male, which was selected for a total absence of aggressive behavior toward the rodents. All housing and behavioral procedures were approved by the animal ethics committee of National University of Singapore and conformed to the principles of laboratory animal care issued by the National Institutes of Health.

The test apparatus for cat exposure consisted of a completely enclosed black Plexiglas cat compartment (55×38×30 cm) with a wire mesh floor, and an open-top rat compartment (38×24×19 cm) made of clear Plexiglas to allow observation. The cat cage could be elevated 19 cm so that the rat cage could be slid underneath. At the beginning of the test, the cat was removed from the top cage, and the home cage containing the rat was placed under the empty cat chamber. Following a 10-min "pre-cat" period, the cat was introduced into the top chamber for 20 min. During the test period, the freezing behavior of rats, defined as the absence of all movements except those related to breathing, was monitored using a video camera mounted beside the clear Plexiglas rat cage. Freezing was expressed as the percentage of time the rat spent imobile during this test session. Locomotor activity was also monitored using an Opto-Varimex Mini (Columbus Instruments, Columbus, OH, USA), which measured activity by the number of interruptions of optical beams (15 in total) that were placed 2.5 cm apart.

RNA preparation

Rats were decapitated after exposure to the cat-freezing test. The brain was removed and cerebral cortex was isolated and immersed immediately in liquid nitrogen and stored at −70°C. For RNA isolation, brain tissue was briefly homogenized in a 10-fold volume (w/v) of ice-cold Trizol reagent (Gibco/BRL, Grand Island, NY, USA) with a Polytron homogenizer (Janke & Kunkel, Staufen, Germany). After phenol-chloroform extraction, total RNA was precipitated by adding isopropanol and centrifugation at 15,000 g for 12 min. The RNA pellets were suspended in diethylpyrocarbonate-treated water. Finally, the yield of total DNA was determined spectrophotometrically by measuring the absorbance at 260 nm.

cDNA microarray

The IncyteGenomics rat GEM cDNA microarray, which consisted of 5,931 cDNA clones, was used for this study. Preparation of rat microarrays, probe labeling, hybridization, microarray scanning, normalization and ratio determination were performed according to the protocols available from Incyte Pharmaceuticals, Inc. (Palo Alto, CA,USA). Briefly, poly(A)+ RNA of PVG hooded or SD rats was isolated from total RNA with Oligotex (Qiagen, Alameda, CA, USA) and reverse transcribed with 5'-Cy3- or Cy5-labeled random 9-mers (Operon Technologies, Inc., Alameda, CA, USA). The two fluorescent samples were simultaneously applied to a single microarray, where they competitively reacted with the arrayed cDNA molecules. After hybridization at 60°C for 6.5 h, slides were washed for 10 min in 1× standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), 1 mM DTT at 45°C, 3 min in 0.1×SSC, 0.1% SDS, 1 mM dithiothreitol (DTT) at 20°C, and for 1 min in reagent alcohol, and then dried. Microarrays were scanned in both Cy3 and Cy5 channels with Axon GenePix scanners (Foster City, CA, USA) with a 10-μm resolution. The signal was converted to 16-bits-per-pixel resolution, yielding a 65,536 count dynamic range. Incyte GEMTools software (Incyte Pharmaceuticals) was used for image analysis. The signal in each cell of the membrane was determined from the pixel density, and corrected for background signal. The intensity of the fluorescence at each array element was proportional to the expression level of that gene in the sample. The ratio of the balanced differential expression was finally used as a standard to evaluate the differences in expression of individual genes (Ono et al. 2000).

Reverse transcription-polymerase chain reaction

A sample of 5 μg total RNA from the tissue was incubated at 70°C for 10 min with oligo (dT)$_{12}$ primers and then at 48°C for 45 min with AMV reverse transcriptase (Promega, Madison, WI, USA) in the reaction buffer to synthesize single-stranded cDNA. The reaction buffer contained20 mM Tris-HCl, 2.5 mM MgCl$_2$, 0.5 mM dNTPs and 10 mM DTT. Double-stranded DNAs were synthesized and amplified in a 100 μl PCR reaction mixture containing 20 mM Tris-HCl, 1.25 mM MgCl$_2$, 0.2 mM dNTPs, 0.2 mM of each primer, and 1 unit of Taq DNA polymerase (Promega). Preliminary experiments demonstrated that the PCR reaction was in the exponential phase and each PCR reaction was repeated at least three times in a Perkin Elmer 2400 thermocycler. According to the GenBank accession numbers and sequences that corresponded to the individual probes in the microarray, we synthesized the PCR primers for Arc, NGFI-A and 5-HT$_3$R. The housekeeping gene β-actin was used as an internal standard in PCR analysis. This demonstrated that equal amounts of cDNA from the different tissues were being assayed for the individual mRNA expression. Forward and reverse primers for individual genes as well as the expected length of PCR product are shown in Table 1. The temperatures and time schedules were as follow: for Arc, 30 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 1 min; for NGFI-A, 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min; for 5-HT$_3$R, 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min.

Quantification of PCR products

For quantitative analysis of the mRNA, PCR products were separated by electrophoresis on 1% TAE agarose gel, which was stained with ethidium bromide (1 μg/ml) and photographed under ultraviolet light. Individual lanes on the exposed films were scanned using a computer-assisted laser scanner and the integrated density of the bands corresponding to different mRNAs was

Table 1 The primer sequence, expected length of PCR product and corresponding GenBank accession number of Arc, NGFI-A, 5-HT$_3$R and β-actin

Gene	Primer		Expected length (bp)	GenBank accession number
	Forrm	Sequence		
Arc	Sense	5'-ATACCAGTCTTGGGTGCCAG-3'	357	U19866
	Antisense	5'-AGTGTCTGGTACAGGTCCCG-3'		
NGFI-A	Sense	5'-GCAACAATTTGTGGCCTGAA-3'	512	M18416
	Antisense	5'-GAGTTGGGACTGGTAGGTGT-3'		
5-HT$_3$R	Sense	5'-TTATGTGTATGTGCACCATCAAGGTGAA-3'	956	U59672
	Antisense	5'-ACAGCAGCGTGTCCAGCACATATCCCACC-3'		
β-actin	Sense	5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3'	838	BC014401
	Antisense	5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'		

measured by NIH image-processing software. Ratios of the corresponding density (e.g., Arc/β-actin or 5-HT$_3$R/β-actin) were calculated for each sample and used for quantitative calculations and comparisons.

Results

Behavior of PVG hooded and SD rats on cat exposure test

Two strains of rats, PVG hooded and SD, showed marked difference in the extent of anxious behavior when exposed to a predator (Fig. 1). Freezing behavior was observed in both strains during cat exposure. PVG hooded rats demonstrated a remarkably high level of freezing behavior and a correspondingly low locomotor activity. In contrast, SD rats showed markedly less freezing behavior and a relatively high locomotor activity. This means that SD rats were in a low-anxiety state, while PVG hooded showed very high anxiety behavior on exposure to the same stimulus.

cDNA microarray

The IncyteGenomics rat cDNA microarray, which consisted of 5,931 cDNA clones, was employed to compare the large-scale gene expression between the cortex of PVG hooded and SD rats. A representative set of arrays is shown in Fig. 2. Based on the assumption that an abundance ratio of ≥1.5 is indicative of a definitive change in gene expression (Botta et al. 2000), we detected 16 upregulated and 38 downregulated genes in PVG hooded and SD rats, respectively, when the two strains were compared. Tables 2 and 3 shows these genes and their abundance ratios between PVG hooded and SD rats. The results contained some genes that have not yet been associated with anxiety, such as rat fibroblast growth factor (FGF), as well as some genes that were recently found to be expressed differentially in an anxious state, such as rat NGFI-A gene. Until now, the functions of most genes in anxiety are not clear, especially the relationship between the alterations of their expression and the anxiety-like behavior. However, some of them,

Freezing behaviour of PVG hooded and SD rats on cat exposure test
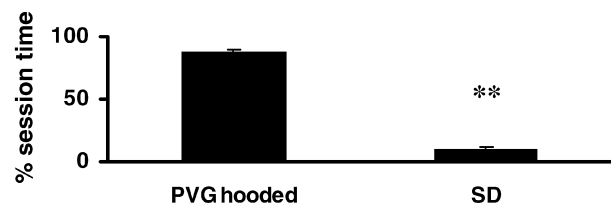
(a)

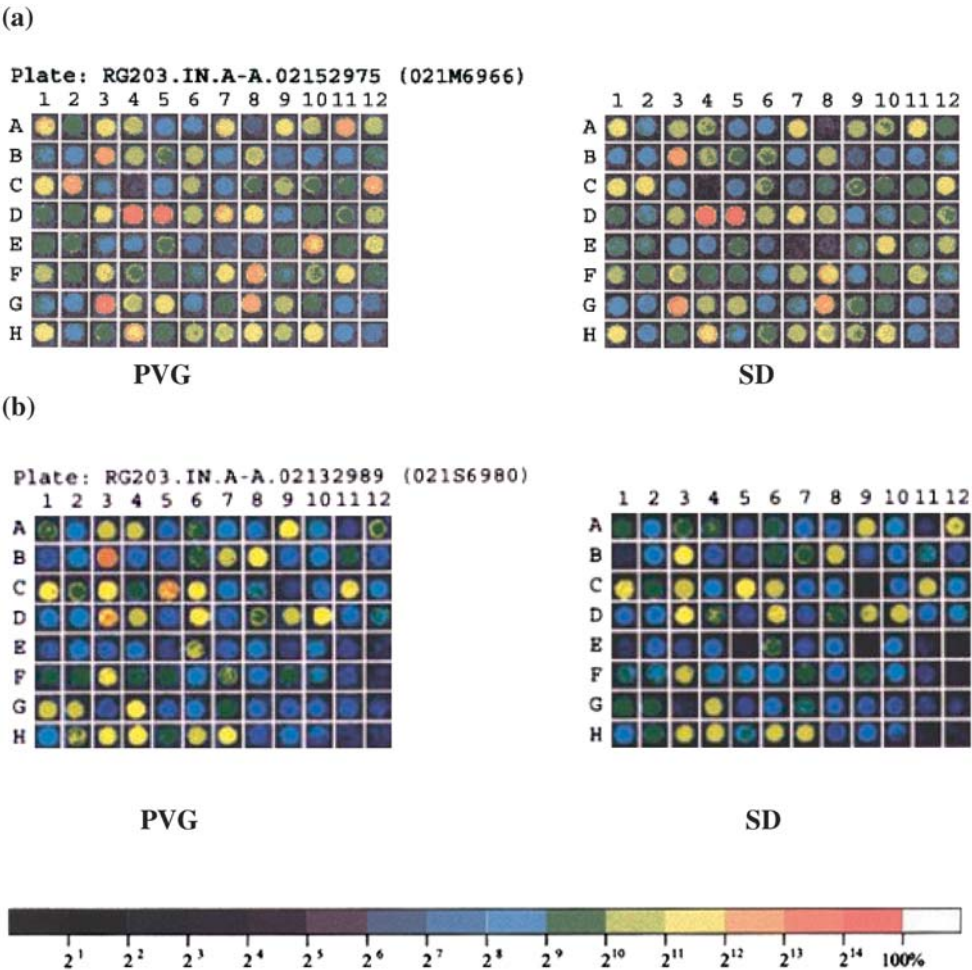**Locomotor activity of PVG hooded and SD rats on cat exposure test**
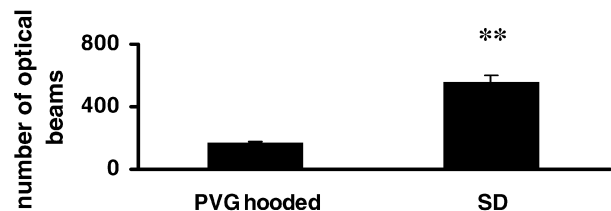
(b)

Fig. 1a, b Freezing behavior and locomotor activity of PVG hooded and SD rats on exposure to cat freezing test apparatus. **a** Freezing behavior, expressed as the percentage of time the rats spent on immobilization during the test session. PVG hooded rats 87±3%, SD rats 9±3%. **b** Locomotor activity, expressed as total number of beam interruptions during the entire period. PVG hooded rats 170±9, SD rats 548±51. Each experimental group comprised five animals. **$**P<0.005$, freezing behavior and locomotor activity were significantly different between the two strains of rats

e.g., microtubule-associated protein (MAP), might play a role in motor functions and other anxiety-related behavior.

Since it is well-acknowledged that norepinephrine, benzodiazepine, serotonin, and dopamine neuronal systems, as well as a considerable number of neurotransmitters and their receptors, are involved in anxiety/fear development, the differential expressions of GABA receptors, CCK receptors and 5-HT$_3$ receptors are shown

Fig. 2a, b Representative images of cDNA microarray results comparing PVG hooded and SD rats. The IncyteGenomics cDNA microarray was hybridized with fluorescent-labeled target cDNA prepared from the cortex of the two strains of rats. **a** Well C7 in plate 021M6966 contains the probe for rat fibroblast growth factor putative isoform 2. The signal ratio between PVG and SD is 2.1. **b** Well A12 in plate 02156980 contains probe of rat activity-regulated cytoskeleton-associated (Arc) gene mRNA. The ratio of SD rats over PVG hooded rats is 2

(a)

Plate: RG203.IN.A-A.02152975 (021M6966)

PVG   SD

(b)

Plate: RG203.IN.A-A.02132989 (021S6980)

PVG   SD

2^1 2^2 2^3 2^4 2^5 2^6 2^7 2^8 2^9 2^{10} 2^{11} 2^{12} 2^{13} 2^{14} 100%

Table 2 The 16 upregulated genes in PVG hooded rats compared to SD rats. *Ratio* is the balanced differential expression between two samples. If the ratio is positive, the gene in PVG channel is upregulated; a negative ratio means the gene in SD rat channel is upregulated (*cds* coding DNA sequence)

Ratio	Gene name
2.1	Rat fibroblast growth factor putative isoform 2 (FGF) mRNA, complete cds, alternatively spliced
2.0	Mouse FK506 binding protein 51 mRNA, complete cds
1.8	Messenger RNA for rat preproalbumin
1.7	Rat gastric intrinsic factor mRNA, complete cds
1.6	Rat MHC class I antigen (RT1.EC3) gene, complete cds
1.6	Mouse S3–12 mRNA, complete cds
1.6	Rat caveolin-3 mRNA, complete cds
1.5	Rat mRNA for pre-α-inhibitor, heavy chain 3
1.5	Rat synaptotagmin VI mRNA, complete cds
1.5	Mouse ERCC2 gene, genomic sequence
1.5	Mouse major histocompatibility locus class II region
1.5	Rat mRNA (rls2var1) for leuserpin-2
1.5	Rat Wistar transforming growth factor β3 mRNA, complete cds
1.5	Rat mRNA for fibronectin
1.5	Mouse mRNA for type II 57 kDa keratin
1.5	Mouse protein phosphatase 1 binding protein PTG mRNA, complete cds

in Table 4. GABA receptor subtypes tended to be downregulated in PVG compared with those in SD rats. Overexpression of 5-HT$_3$R was observed in PVG rats. For the CCK receptor family, it was found that CCK receptors are upregulated in PVG in comparison with SD, while CCK-A receptor is downregulated. Although the abundance ratio of most of these genes did not reach the normal standard (1.5), the subtle changes showed that these genes could be differentially expressed between these two rat strains.

Table 3 The 38 upregulated genes in SD rats compared to PVG hooded rats. *Ratio* is the balanced differential expression between two samples. If the ratio is positive, the gene in PVG channel is upregulated; a negative ratio means the gene in SD rat channel is upregulated (*cds* coding DNA sequence)

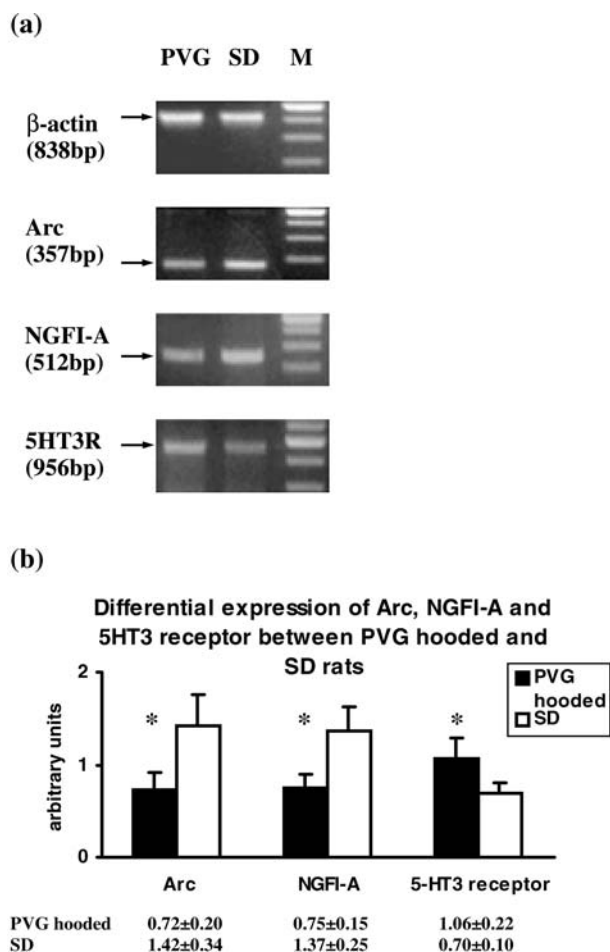| Ratio	Gene name
−2.0	Rat activity-regulated cytoskeleton-associated gene (Arc) mRNA, complete cds
−1.9	Rat carbonic anhydrase III (CA3) mRNA, complete cds
−1.8	Mouse (Mus musculus domesticus) transcription factor IID (Tbp) mRNA, complete cds
−1.8	Rat partial mRNA for MHC class Ia A2 h antigen (RT1-A2 h gene)
−1.7	Mouse myelin proteolipid protein mRNA, complete cds
−1.7	Rat SC1 protein mRNA, complete cds
−1.7	Rat prealbumin (transthyretin) mRNA, complete cds
−1.7	Rat nerve growth factor-induced (NGFI-A) gene, complete cds
−1.7	Rat prostaglandin $F_{2\alpha}$ receptor mRNA, complete cds
−1.6	rat myelin basic protein (mbp) gene mRNA
−1.6	Rat mRNA for neurodegeneration associated protein 1, complete cds
−1.6	Rat (clone pCNPII) 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPII) mRNA, complete cds
−1.6	Rat microtubule-associated protein 1A MAP1A (Mtap-1) mRNA, complete cds
−1.6	Rat mRNA for microtubule associated protein IB
−1.6	Rat partial GR gene for glucocorticoid receptor, promoter region
−1.6	63 kDa Calmodulin-stimulated phosphodiesterase
−1.6	Rat mRNA for cytoplasmic dynein 74 kDa intermediate chain
−1.6	Rat neural membrane protein 35 mRNA, complete cds
−1.6	Rat non-processed neurexin I-β mRNA, complete cds
−1.6	microtubule-associated protein, MAP-115
−1.6	Rat rab GDI α mRNA
−1.6	Rat Ca^{2+}-dependent activator protein (CAPS) mRNA, complete cds
−1.5	Mouse epidermal keratin type I intermediate filament gene, exon 1
−1.5	Rat mRNA for calcineurin A
−1.5	Rat Myo5a mRNA for myosin-Va, complete cds
−1.5	Mouse mRNA for neuroserpin
−1.5	Mouse $GABA_A$ receptor γ-2 subunit mRNA, complete cds
−1.5	Rat kinesin-like protein KIF1B (KIF1B) mRNA, partial cds
−1.5	Mouse zinc-finger protein (Peg3) mRNA, complete cds
−1.5	Mouse mRNA for SmN protein
−1.5	Rat $GABA_A$ receptor α-1 subunit gene, complete cds
−1.5	Mouse ubiquitinating enzyme E2–20 K mRNA, complete cds
−1.5	Rat mRNA for SNERG-1 protein
−1.5	Rat mRNA for cathepsin L (EC 3.4.22.15)
−1.5	Rat GluT and GluT-R glutamate transporter mRNA, complete cds
−1.5	Rat γ-adducin mRNA, complete cds
−1.5	Rat D-binding protein mRNA, complete cds
−1.5	Rat metabotropic glutamate receptor 2 mRNA, primary transcript

Table 4 Receptors of some neurotransmitters that were differentially expressed and their abundance ratios between PVG hooded and SD rats. Ratio is the balanced differential expression between two samples. If the ratio is positive, the gene in PVG rat channel is upregulated; a negative ratio means the gene in SD rat channel is upregulated (*cds* coding DNA sequence)

Ratio	Gene type	Gene name
−1.5	GABA receptor	Rat $GABA_A$ receptor α-1 subunit gene, complete cds
−1.3		Rat mRNA for $GABA_BR1b$ receptor
−1.3		Rat $GABA_{B1}$ receptor ($GABA_BR1$) gene, exons 1–9
−1.3		Rat $GABA_A$ receptor α-6 subunit gene, complete cds
−1.2		Rat $GABA_A$ receptor α-4 subunit gene, complete cds
−1.2		Rat $GABA_A$ receptor δ-subunit gene, complete cds
1.2		Rat mRNA for $GABA_A$ receptor γ-1 subunit
−1.2		Rat $GABA_A$ receptor α-5 subunit gene, complete cds
−1.2		Rat $GABA_A$ receptor α-6 subunit cDNA
1.3	CCK receptor	Rat brain cholecystokinin receptor mRNA, complete cds
−1.2		Rat gene for cholecystokinin type-A receptor (CCKAR), complete cds
1.2	5-HT$_3$ receptor	Rat 5HT$_3$ receptor mRNA, complete cds

RT-PCR

To confirm further the microarray data, we selected three genes of interest and examined their mRNA expression using semi-quantitative RT-PCR. After amplification, we obtained 357-, 512- and 956-bp length PCR products, which were consistent with Arc, NGFI-A and 5-HT$_3$R. The gel image of electrophoresis is shown in Fig. 3a. The density of the individual bands was quantified by NIH imaging system (Fig. 3b). It was observed that the mRNA level of Arc was increased about 1.9-fold in SD rats compared with that in PVG hooded rats. The expression of NGFI-A showed the same tendency as Arc, with an increase of 1.8-fold in SD rats. By contrast, SD rats had a decreased expression of 5HT$_3$ receptors in comparison with PVG hooded rats. The expression ratio of 5HT$_3$ receptor in PVG hooded to SD is 1.5. RT-PCR experiments on the expression of these three individual genes

(a)

(b)

Differential expression of Arc, NGFI-A and 5HT3 receptor between PVG hooded and SD rats

	Arc	NGFI-A	5-HT3 receptor
PVG hooded	0.72±0.20	0.75±0.15	1.06±0.22
SD	1.42±0.34	1.37±0.25	0.70±0.10

Fig. 3a, b Expression of mRNA for Arc, NGFI-A, 5-HT$_3$ receptor (5HT3R) and β-actin in PVG hooded and SD rats by reverse transcription-polymerase chain reaction (RT-PCR). **a** Gel image of electrophoresis of Arc, NGFI-A, 5HT3R and β-actin. PCR products were separated by 1% TAE agarose gel, which was stained with ethidium bromide (1 μg/ml) and photographed under UV light. A 200-bp DNA step ladder (*M*) was used to monitor the size of individual bands. The length of Arc, NGFI-A and 5HT$_3$R is 357-, 512- and 956-bp, respectively. **b** Quantitative result of RT-PCR products of Arc, NGFI-A and 5HT3R. The integrated density of the bands corresponding to different mRNAs was measured by NIH image-processing software. Ratios of the corresponding density (e.g., Arc/β-actin or 5-HT3R/β-actin) were calculated for each sample and used for quantitative calculations and comparisons. *$P<0.05$, significant difference between the two strains of rats

indicated the same tendency as the data from the microarray study.

Discussion

The major issue in the post-genome sequencing era is determination of gene expression patterns in various biological systems. Many conventional techniques have been successfully used to detect and quantify gene expression at mRNA levels, including northern blot, ribonuclease protection, RNA blot and RT-PCR, but these molecular biology and cell biology experiments have been limited to the analysis of individual genes, or, at best, small groups of genes. Clearly, to relate changes in gene expression to changes in function, it would be desirable to examine differential expression of groups of genes and, ideally, to examine the entire repertoire of genes expressed in a special biological sample, i.e., the "gene profile" or "transcriptome" for that sample (Lomax et al. 2000; Yoshikawa et al. 2000).

Recently, new methods to define gene expression patterns, including differential display, suppression subtractive hybridization, cDNA microarray and serial analysis of gene expression (SAGE), have been developed. Microarray is one of the latest breakthroughs in experimental molecular biology, which allows us to investigate simultaneously the expression profiles of large numbers of genes, from hundreds to thousands to whole genomes (Blohm and Guiseppi-Elie 2001). As a result, the scope of applications for microarrays has broadened rapidly, from drug discovery and classification of cancers to analysis of splice variants as well as downstream targets of transcription factors. One of the most popular experimental platforms is used for comparing mRNA abundance in two different samples (or a sample and a control).

This study was designed to compare large-scale gene expression profiles in two rat strains, PVG hooded and SD, after the cat-freezing test. The IncyteGenomics cDNA microarray used in the current study contains 5,931 annotated genes, which are separated into three categories, namely enzyme hierarchy, function hierarchy and pathway hierarchy. After appropriate normalization of data from samples, we obtained a considerable number of genes that are differentially expressed between the two strains of rats. Typically, it is assumed that abundance ratios of above 1.5 are indicative of a change in gene expression. Based upon this criterion, we detected a total of 16 upregulated and 38 downregulated genes in PVG rats compared with SD rats.

The list of 16 up-expressed genes in PVG hooded rats is shown in Table 2. Most of them, such as rat FGF, have not previously been associated with anxiety. FGF is synthesized by a variety of cell types during the process of embryonic development and in adult tissues and shows a biological profile that includes mitogenic and angiogenic activity, with a consequent crucial role in cell differentiation and development (Botta et al. 2000; Powers et al. 2000). Our result showed that FGF mRNA is upregulated in PVG hooded rats compared with SD rats. As no previous report documenting the relationship between anxiety and FGF has been published, further investigations are needed to determine exactly what role, if any, these genes may play in anxiety/fear-related behavior.

Among the list of downregulated genes in PVG hooded rats, two genes: rat Arc gene and rat NGFI-A gene, draw our attention. They all belong to immediate-early genes (IEGs), whose expression is believed to be one of the first events that occur sequentially when a cell is stimulated. The IEGs can be classified into two groups. The first group includes genes that encode transcription

factors which regulate the expression of target genes by means of binding to a specific sequence of DNA. The second group of IEGs includes genes that encode proteins which are spread throughout the cytoplasm into the peridendritic region of cells and may directly modify cellular function.

Arc, one of the second group IEGs, is a cytoskeleton-associated protein and is selectively localized in neural dendrites. Some reports have shown that the action of psychostimulants leads to increased expression of Arc, whereas antipsychotics prevent its expression. Fosnaugh et al. (1995) have reported that the acute systemic administration of cocaine elicits a robust and transient rise in Arc mRNA levels in the striatum. Kodama et al. (1998) found that levels of Arc mRNAs in brain regions increased significantly from 0.5–1 h after acute and chronic administration of methamphetamine. Phencyclidine (PCP) has potent psychotomimetic effects in humans and induces psychosis. Administration of PCP increased Arc mRNA levels in the prefrontal cortex, nucleus accumbens and posterior cingulated cortex. Pretreatment with some atypical antipsychotics prevents this expression (Nakahara et al. 1999). These findings in combination with our evidence that Arc expression is upregulated in low-anxiety SD rats demonstrated that Arc is associated with anxiety development.

So far, the literature regarding the expression alterations of NGFI-A during the anxiety state is still very controversial. The common concept is that NGFI-A is related to contextual fear conditioning and NGFI-A mRNA expression is activated during the acquisition or consolidation of fear learning. Malkani and Rosen (2000) therefore hypothesized that the anxiolytic diazepam would block both contextual fear and the concomitant increase in NGFI-A mRNA expression. Unfortunately, the data they obtained is contradictory. Diazepam significantly increased EGR-1 mRNA expression in the central nucleus of the amygdala in a dose-dependent manner. Bjartmar et al. (2000) also proved that NGFI-A mRNA expression increased profoundly in the hippocampus formation and the cerebral cortex after chronic treatment with different antidepressants. Withdrawal following intravenous cocaine administration produces ultrasonic vocalization in rats, indicative of anxiety-like behavior, and the reduction of NGFI-A messenger RNA expression (Mutschler et al. 2000). Although our experiment differs from other studies in that the presence of the cat as an unconditioned stimulus was tested on PVG hooded and SD rats that were not treated with any drug, the previous studies may indirectly support our finding that NGFI-A is downregulated in high-anxiety PVG hooded rats. The exact molecular mechanism underlying the role of NGFI-A expression on conditioned and unconditioned fear development and their relationship still needs further investigation.

The alterations of other genes in the list have not previously been associated with anxiety-related behavior. Nevertheless, some genes, such as MAP, might be connected with motor functions. MAP, together with tubulins, forms the microtubules, which are major cytoskeletal filaments in eukaryotic cells. Alterations in hippocampal MAP-2 have been proved to be highly correlated with contextual memory. The decreased expression of MAP-1A, MAP-1B and MAP-115 in PVG hooded rats was detected in our study. MAP-1A, MAP-1B and MAP-2 are the major structural MAPs in the nervous system with the function of modulation of microtubule dynamics. Moreover, NGFI-A overexpression was found to be accompanied by an increase in the amount of phosphorylated MAP-1B (Pignatelli et al. 1999). Hoogenraad et al. (2002) demonstrated that targeted mutation of MAP-115 affected dynein motor regulation. All the above evidence proved that MAPs could affect the cytoskeletal structure, and alterations of their expression appeared to be connected with freezing behavior and locomotor activity.

Microarray-based gene expression measurements are still far from giving estimates of mRNA counts per cell in the sample. The signal in each cell of the membrane can be assessed not only by the absolute intensity, but also by many other factors, such as uniformity of the individual pixel intensities, or the shape of the spot. Unfortunately, there is currently no standard way of assessing the spot measurement reliability. The measurements are relative by nature: essentially we can compare the expression level either of the same gene in different samples, or of different genes in the same sample (Cheung et al. 1999; Getz et al. 2000). Expression data analysis methods are only in their infancy. Novel analytical tools have been constructed to address every component of the microarray experiment to optimize performance (Ramakrishnan et al. 2002). However, ideal systems with maximized sensitivity and data reproducibility have not been achieved.

Since it is well-accepted that norepinephrine, benzodiazepine, serotonin and dopamine neuronal systems, as well as a lot of neurotransmitters, are responsible for the anxiety/fear development (Bloom and Morales 1998; Dauge and Lena 1998), we also pay much attention to the expression of these neurotransmitters and their receptors. The changes in gene expression of GABA receptors, CCK receptors, 5-HT$_3$ receptors were listed in Table 4; however, most of the abundance ratios did not reach the normal standard. Currently, for most microarray technology platforms, only the ratio of the background-subtracted signals of the given sample and the control is meaningful (Brazma and Vilo 2000), but such typical inference that abundance ratios of 1.5–2 are indicative of a change in gene expression is very crude. The key issue is the determination of whether a ratio is significantly high or low in order to conclude whether the gene is upregulated or downregulated. A refined hypothesis is proposed that the measured intensities forming the ratio are assumed to be combinations of signal and background. The new method, which involves a signal-to-noise ratio, could improve the accuracy of microarray data when the normalization was carried out (Chen et al. 2002). The value of microarray-based gene expression measurements would be considerably higher if reliability

and limitations of particular microarray platforms for particular kinds of measurements, as well as cross-platform comparison and normalization, were studied.

In order to validate the data of our microarray, we employed the traditional semi-quantitative RT-PCR method to compare the mRNA expression level of three genes that were included in microarray probes. It was found that the gene expressions of Arc and NGFI-A were individually increased about 1.9- and 1.8- fold, respectively, in SD rats when compared with PVG hooded rats. This tendency is consistent with the microarray results, which indicated that Arc and NGFI-A were markedly overexpressed by 2.0- and 1.7-fold in the cortex of SD rats in comparison with those in PVG hooded rats. Meanwhile, the fact that the $5HT_3R$ had a decreased expression in SD rats compared with PVG hooded was detected by both RT-PCR and microarray. The consistent results between RT-PCR and microarray demonstrated that the data from microarray analysis reliably reflected the proportions of mRNA levels between the two strains of rats. cDNA microarray reports large-scale expression changes that are highly reproducible and can be validated by other techniques.

In conclusion, our study demonstrated that microarray is an efficient tool for analyzing the expression profiles of anxiety-related genes. It further confirmed that some neurotransmitters and their receptors are related to anxiety development. Meanwhile, we also detected some other genes, which have not yet been connected with anxiety. These data have been in part proved by RT-PCR and will be helpful in further investigating the molecular mechanisms of anxiety and fear. Like genome sequencing, the systematic gene expression profile is not an end in itself. It is a long way from having detailed gene expression profiles to real understanding of underlying cellular processes.

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