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Distinct gene expression profiles in hippocampus and amygdala after fear conditioning

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Abstract

It is well known that the hippocampus and amygdala are involved in the formations of fear conditioning memories, and both contextual and cued fear memory requires activation of the NMDA receptors. However, the global molecular responses in the hippocampus and amygdala have not been investigated. By applying high-density microarrays containing 11,000 genes and expressed sequence tags, we examined fear conditioning-induced gene expression profiles in these two brain regions at 0.5, 6, and 24 h. We found that 222 genes in the amygdala and 145 genes in the hippocampus showed dynamic changes in their expression levels. Surprisingly, the overall patterns of gene expression as well as the individual genes for the amygdala and hippocampus were drastically different and only small number of genes exhibited the similar regulation in both brain regions. Based on expression kinetics, the genes from the amygdala can be further grouped into eight unique clusters, whereas the genes from the hippocampus were placed into six clusters. Therefore, our study suggests that different genomic responses are initiated in the hippocampus and amygdala which are known to play distinct roles in fear memory formation.

Keywords: High-density microarrays; Fear memory; GluR1

1. Introduction

Completion of genome sequences marks an exciting new era for the study of gene function in biological processes. Understanding how the genome functions as a whole in the complex behavioral situation presents a great challenge. Knowing when and where genes are expressed and regulated can provide crucial clues as to the molecular and cellular mechanisms of a given behavior. On the other hand, the patterns of genes expressed in the brain can provide valuable information about the cognitive state. Recent progress in gene chip or DNA microarray technology makes it possible to monitor large-scale gene expression induced by behavioral learning paradigm. This approach has offered a view of molecular and genetic responses underlying many behavioral and cognitive changes including brain aging, effects of environment enrichment on brain function, and development of the hippocampus [10].

Emotional memories tend to be long lasting and play an important role in regulating the behavioral responses of animals. Most of the current knowledge of emotional memories comes from the study of classical fear conditioning. It is believed that contextual fear conditioning is dependent on the structural integrity of the hippocampus and amygdala, whereas cued fear conditioning is hippocampal-independent, but requires the amygdala [7,30]. Pharmacological and genetic experiments further suggest the formation of many associative memories including contextual and cued fear memory requires activation of the NMDA receptor [33,40,41]. In this study, we report the application of gene chip technique for the analysis of

Abbreviations: NMDA, *N*-methyl-D-aspartate; LTD, long-term depression; GABA, gamma-aminobutyric acid; AMPA, amino-3-hydroxy-5-methyl-4-isoxalone propionic acid; GluR1, glutamate receptor 1; CaMKII, calcium-calmodulin-kinase II

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the large-scale transcriptional responses that occur in the amygdala and hippocampus after fear conditioning.

2. Materials and methods

2.1. Animals and fear conditioning training

Animals used in this study were 5-month-old C57BL6/CBAF1 adult mice (Jackson lab, USA). Animals were housed in an environment of $23 \pm 0.5\,^{\circ}C$ with a relative humidity of $50 \pm 10\%$. Every cage had a complete exchange of air 15–18 times per hour and a 12-h light–dark cycle with no twilight. Water and food were continuously available.

To create contextual and cued fear memories, we used a single CS/US pairing paradigm [25,40]. Before training, animals were individually handled for 1 week, and then followed by adaptation to the chamber a day before the experiment for 5 min per session and three sessions total. For the fear conditioning training, the conditioned stimulus (CS) was an 85 dB sound at 2800 Hz and the unconditioned stimulus (US) was a continuous scrambled foot shock at 0.75 mA. The background white noise was a constant tape recording of radio noise (68 dB). During a single training session for both contextual and cued conditioning, mice were put individually into the chamber and allowed to explore the environment freely for 3 min, and then were exposed to the CS for 30 s. At the last 2 s of the CS, the US was delivered for 2 s. After the CS/US pairing, the mice were allowed to stay in the chamber for another 30 s and then returned to their home cages immediately. We individually placed 10 mice back into the same shock chamber 1 and 24 h after training for 5 min to first test contextual fear memory retention. We then tested the cued fear memory retention in the same animals using a different chamber. Memory is measured as percentage of freezing responses. In contextual fear memory retention, a significant difference in freezing response was found in both 1-h retention ($F(2, 24) = 5.062$, $p < 0.05$) and 24-h retention ($F(2, 25) = 5.223$, $p < 0.05$). A further post hoc analysis revealed the significant difference between untrained groups and conditioned animals ($p < 0.05$, respectively). In cued fear memory retention, mice exhibited robust freezing responses when tested at both 1 h ($F(2, 24) = 4.672$, $p < 0.05$) and 24 h ($F(2, 25) = 5.518$, $p < 0.01$) after training. Data are presented in mean ± S.D.

2.2. Tissue collection and RNA preparation

To establish comprehensive gene expression profiles underlying the contextual and cued fear conditioning, we chose three time points (0.5, 6, and 24 h). A total of about 50 mice were used for the gene chip experiments. For each time point, we trained, decapitated and dissected hippocampi and amygdalas from 10 to 14 trained mice, and separated them into two independent tissue pools (5–7 animals/pool) for the extraction of poly(A) mRNA and probe labeling. The RNA was extracted and stored as previously described [21].

2.3. Gene expression analysis

The high-density oligonucleotide microarray (gene chip) analysis was conducted as previously described [21,26]. Data were analyzed with the Affymetrix GeneChip expression analysis Software, version 3.1. Mu11KsubA and Mu11KsubB (GeneChip, Affymetrix, Santa Clara, CA) were used containing 13,069 probe sets corresponding to more than 11,000 genes and expressed sequence tags. To ensure the reliability of the data, we conducted hybridization experiments in duplicates consisting of two independent mRNAs and two sets of duplicate microarrays were used. In agreement with our previous experience with DNA microarrays [21,26], we found our procedures involving duplicate experiments provided consistent and reproducible hybridization signals. Indeed, several genes such as actin and cell adhesion molecule, L1, have been shown by independent methods to be similarly regulated by fear conditioning [20,39].

2.4. Real-time RT-PCR

Validation of selected genes was performed using real-time RT-PCR with the ABI 7700 Sequence Detection system. Total RNA was extracted and reverse transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies, Invitrogen, Carlsbad, CA). The house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. The sequences of primers and probes of the tested genes and GAPDH were listed in Table 1. The PCR reaction was performed using 50 μl of total reaction mixture volume and 2 μl of cDNA reaction products at 1 cycle of 55 °C for 1 min, 1 cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 30 s and 55 °C for 1 min. TaqMan probes for the genes were obtained from Perkin-Elmer. All the probes were labeled with the fluorescent dyes 6-carboxyfluorescein (FAM) and 6-carboxy-tetramethyl rhodamine (TAMRA). The TaqMan reaction buffer contained 5.5 mM $MgCl_2$; 200 nM each of dATP, dCTP, and dGTP; 400 nM dUTP, 0.5 U of uracyl DNA glycosylase, and 1.25 U of AmpliTaq gold. TaqMan probe concentrations were maintained at 100 nM, while PCR primer concentrations were systematically varied in all combinations. The fold change of the targets genes was calculated using the $2^{-\Delta\Delta Ct}$ methods.

2.5. GluR1 staining

For immunohistochemistry studies, mice were handled and trained using the same fear conditioning protocol as used for gene chip analysis. Six hours and twenty four hours after fear conditioning, mice were anesthetized with avertin (0.2 ml per 10 g body weight, i.p.) and perfused through heart as previously described [26]. Briefly, brains were removed

Table 1
Primers and probes used in the real-time RT-PCR experiments

Genes name	Forward primer	Reverse primer	TaqMan Probe
GAPDH	CTTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGAG	CCTGGCCAAGGTCATCCATGACAACTTT
NF1	CCCTTGTTCTCAGTGGGATGA	TGGTAGAGTAAATGCCGGG	TGGCTCGGGTTCTGGTCACTCTGTT
Vimentin	CAGATCGATGTGGACGTTTCC	ATACTGCTGGCGCGCACATCA	AGCCTGACCTCACTGCTGCCC
APP	CGTCCGTGCCGAACAGA	GGTCCACCATGCGCACAT	AGACAGACAGCACACCCTAAACCATTTTG
PKC-δ	CAAAGGCCGCTTCGAACTC	TGCAGTCCGCAGATGATCTC	ACCGGGCTACGTTTTATGCAGC
U2AF65	CATCACCCCAATGCAGTACAAG	GAAGGGCAGTGGCTGGAAT	CCATGCAAGCTGCGGGTCA
PLC-α	GGGTCATGATGGTGGCAAAG	GTTTTACGGCTAGCTACAGCAAAGT	AATTCCTTGATGCTGGACACAAACTC
CaMKII-	CTACGGTGGCATCCATGATG	CCGGGCGTTGAATTTGC	ATCGCCAAGAGACGGTAGAGTGCTT
14-3-3	GGTGGCCTACAAAAACGTGGTA	CTTCTGCTCAATGCTCGAGATC	CCGCAGTCCGCCTGGAGGG
Pantophysin	TGCATTGCTGCCCTTCTG	TGGGCAGTTTGCGACTATCC	TCTATGTTGGTTACACGAACCTCTACC
Synaptotagmin	CAAAAGTCCACCGAAAACC	CCGAGTATGGCACCTTGAAAG	TCAATCCAGTCTTCAATGAACAGTTT
MOBP	ACGAGCACTCAGGATAGGCTTT	GTAGCCCATTGGTGAGACAAATT	TGGATTGGATGAAGCTAGCCTGG
NCSP F3	TTATGCTCGACGCCAATGG	CCGGCCTGCGTGTTTC	AACTGCTCATCCGAAATGCGCAGC
CCT	ACTTGCTTGTGGTGGGATAGCT	CCCTGCATGTCCCAAACAG	TTCCTTTGATGACCTGAATCCTG
NAP-22	GGGAGGGAGGCGTTTGA	AATACTGCTTTCATAAAGATCTGATTGC	TCTGCCACAGGACCAGTGCCA
MAP4	CCAGAAGATGCAAGCTTTTGTTT	CATCACGGTGCTCGTTAAAGG	CAGCCTCAGCAAGTGTTAGATACTGACCAGG
Clathrin	TGACCATGCAAGACTGTCAATG	GGAGAGCGCTGTGATCCAAT	CGACAAAGACAACACTAATCAGCATATCCT
Enkephalin	AATTTCCTGGCGTGCACACT	CTTGCAGGTCTCCCAGATTTTG	AATGTGAAGGACAGCTGCCTTCTT
GABAR-AP	GCGCCCTTCCTGCTTGT	GTGGAGGCTCTGGCAGAGTAA	TCTCTTGTAAATAACTGGCTGTTCTCA
Calmodulin synthesis	TGCCATGAGCAGGTTTTCTTT	GGTACTCGGACACTATTTTTTTGTACTG	AGTGGCTAAACAAAGTTTAAAAGCAAGTAACA

and post-fixed in 4% paraformaldehyde in 0.1 M PBS for 3 h and then cryoprotected in 30% sucrose in 0.1 M PBS. Twenty five micrometres free-floating slices through amygdala were obtained using a Leica cryostat (Germany). After blocking in 0.1 M PBS containing 3% normal goat serum, slices were incubated at 4 °C overnight in 0.1 M PBS containing 3% goat serum, 0.25% Triton X-100, and 0.25 μg/ml rabbit anti-glutamate receptor 1 (CHEMCOM International Inc., Temecula, CA) followed by an incubation at room temperature for 1 h in 0.1 M PBS containing 1:200 biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA). Slices were then incubated with avidin/biotinylated enzyme complex for 5 min followed by incubation with nickel-3-3 diamino benzidine (nickel-DAB). Slices were mounted on gelatinized slides, stained with cresyl violet, and coverslipped. The staining intensities in each of the nuclei of amygdala were measured using ImagePro (Media Cybernetics, Silver Spring, MD). The faint signal in corpus collosum was used as internal background for subtraction as well as for standardization between cross-sections and cross animal comparisons.

3. Results

3.1. Distinct gene expression patterns in hippocampus and amygdala after fear conditioning

To examine the gene expression cascade in these brain regions induced by fear conditioning, we first trained a group of mice to produce robust contextual and cued fear memories as measured by both 1 h short-term and 1 day long-term retention tests (Fig. 1). The trained mice exhibited robust freezing responses when tested at both 1 and 24 h after training. For the gene profiling experiments, three time points

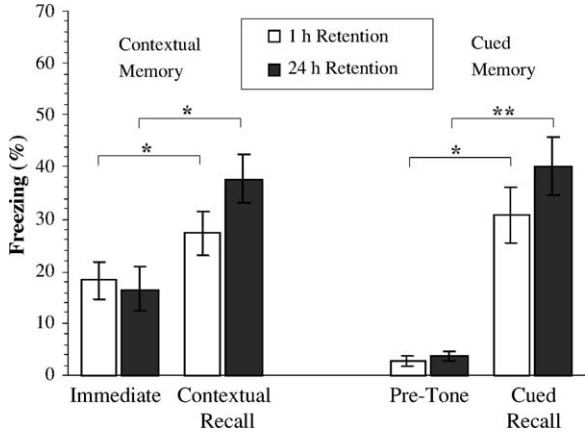

Fig. 1. Formations of short-term (1 h) and long-term (1 day) contextual and cued fear memories in mice. Memory is measured as percentage of freezing responses. In both contextual fear memory retention and cued fear memory retention, mice exhibited robust freezing responses when tested at both 1 and 24 h after training.

Table 2
Common set of genes regulated in both the amygdala and hippocampus

Accession number	Gene name	Fold change					
		Hippocampus (h)			Amygdala (h)		
		0.5	6	24	0.5	6	24
Genes showing the same regulation							
X51438	Vimentin		3			3	
M13366	Glycerophosphate dehydrogenase		2.55			2.55	
M27844	Calmodulin		−3.6			−3.6	
M73329	Phospholipase C-alpha		−3.3			−3.3	
AA655109	Eukaryotic translation initiation factor 3		−3			−3	
L04280	Ribosomal protein (Rpl12)	−2.0	−2.5		−3.4	−2.5	
Genes showing similar regulation, but with different time onset							
AA059550	Ectonucleotide pyrophosphatase/phosphodiesterase 2			2		3.1	
X79082	Kinase 1	2				4.35	
X59728	Gas5 growth arrest specific protein	2				3.55	
AA408185	Splicing factor, arginine/serine-rich 7	2				3.15	
AA655109	Ribosomal protein S3		−2		−2.2		
Genes showing opposite regulation							
U60150	Vesicle-associated membrane protein VAMP-2	−3.3	−2.7	−3.2	4.05		8.5
X61435	Kinesin heavy chain	−2.2	−2.5				3.2
U07950	GDP-dissociation inhibitor	−2.1	−2.9				2.5
X61434	cAMP-dependent protein kinase C β-subunit		−2.6		2.25		3.55
W57404	Carboxypeptidase H precursor		−2.4		4.05		5.65
L04961	Nuclear-localized inactive X-specific Transcript			−5			4.25
AA388848	Splicing factor, arginine/serine-rich 5		2		−3.15		
L13171	Myocyte-specific enhancer factor 2			−10.7	3.5		

(0.5, 6, and 24 h) were chosen to monitor the dynamical changes in gene expression over the first 24 h after training. The experiments revealed that expressions of 222 genes in the amygdala and 145 genes in the hippocampus were consistently and reproducibly changed for more than two-fold changes. A description of these genes and ESTs can be accessed on the web (http://sbg.ecnu.edu.cn/DATA.htm). Some of the representative genes are listed in Tables 2–4.

Surprisingly, the overall patterns of gene expression as well as the individual genes for the amygdala and hippocampus were drastically different (Fig. 2). Of them, only six genes (Table 2) showed the same up- or down-regulation at the same time point(s) in both regions. Interestingly, another five genes showed the similar trends in the changes of expression level (either increase or decrease), but differed in their time courses. In addition, we also observed that eight other genes were common to both brain regions but showed opposite regulation in their expression levels (Table 2).

In the hippocampus, among these 145 genes whose expressions have changed by fear conditioning paradigm, 33 genes (23%) increased expression while 112 genes (77%) were down regulated between the 0.5 to 24 h period (Fig. 2). Nearly all of these genes (96.5%) returned to basal levels by 24 h. In the amygdala, a total of 222 genes changed expression after fear conditioning. About 123 of these genes (55%) exhibited up-regulation, while 99 genes (45%) showed decreased expression (Fig. 2). Similar to the expression kinetics in the hippocampus, fear conditioning triggered expressional changes in most of the genes subsided to basal level at 24 h.

To further analyze these fear conditioning-triggered transcriptional responses, we grouped the genes in clusters based on their intrinsic expression kinetics over the four time points. Most of the 145 genes from the hippocampus were placed into six clusters (Fig. 3A and C), some of which were shown in Table 3. The majority of these genes belonged to clusters that

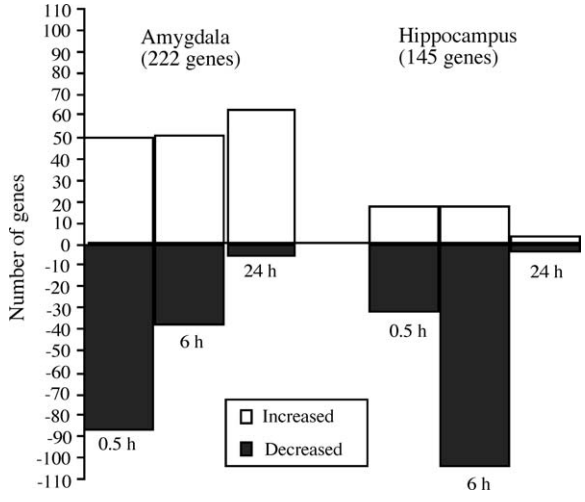

Fig. 2. Fear conditioning triggered expressional changes in 222 genes in the amygdala and 145 genes in the hippocampus at 0.5, 6, and 24 time points. Those changes are reflected in both directions, either up-regulated or down-regulated. A total of 11,000 genes and ESTs were screened in duplicates. The cut-off threshold is two-fold changes in the expression level above the zero point.

Table 3
Representative genes changed in the hippocampus after fear conditioning

Cluster	Accession number	Gene name	Fold change (h)		
			0.5	6	24
I	L25274	Glycoprotein (DM-GRASP)	2		
	ET63017	Cadherin-8	2.8		
	U56649	Phosphodiesterase (PDE1A2)	2.6		
II	D30730	GTPase activating-protein (NF1)		5.4	
	X51438	Vimentin		1.9	
	X60304	Protein kinase C-δ		6.2	
	X64587	Splicing factor U2AF65		4.8	
III	X56007	Adhesion molecule on glia (AMOG)	−2.7	−3.2	
	AA015415	Kinesin light chain 1 (KLC1)	−5.8	−9.7	
	AA066354	JAK1 protein tyrosine kinase	−6.8	−6.3	
	AA002629	Calcineurin B	−3	−4.4	
	AA108330	Astrocytic phosphoprotein PEA-15	−2.3	−3.2	
	AA000227	Diacylglycerol kinase	−19.6	−7.6	
IV	M73329	Phospholipase C-α (PLC-α)	2	2.3	
V	AA107895	Cathepsin D	−2.8		
	AA008502	Neuron specific gene family member 1	−11.9		
VI	AA066335	Amyloid precursor protein (APP)		−2.5	
	D21165	Visinin-like Ca2+-binding protein		−2.2	
	M27844	Calmodulin synthesis (CaM)		−2.2	
	W12204	Ca2+/calmodulin-dependent protein kinase II isom γ-b (CaMKII)		−3.2	
	X61434	Protein kinase C β subunit		−2.6	
	AA218341	Protein phosphatase type 1-α (PP1)			
	Z67745	Protein phosphatase 2A (PP2A)		−2	
	W46019	Protein kinase regulator 14-3-3		−2.8	
	M63436	$GABA_A$ receptor alpha-1 subunit		−2.2	
	D87898	ADP-ribosylation factor (ARF1)		−2.1	
	AA067362	Vesicle protein pantophysin		−3.8	
	*D*37792	SynaptotagminI/65		−3.2	
	U58886	Endophilin I		−3.3	
	AA118297	Neuronal protein 25 (NP25)		−2.6	
None	X16314	Glutamine synthetase			2.6
	U60150	Vesicle-associated membrane protein 2	−3.3	−2.7	−3

showed a decrease either at 6 h only (Cluster III: 78 genes) or at both 0.5 and 6 h (Cluster IV: 25 genes). In contrast, most of the 222 genes from the amygdala could be grouped into eight different clusters (Fig. 3B and D). Some important ones were listed in Table 4. The largest clusters contained genes that showed increased expression at 6 h (Cluster II: 43 genes) or decreased expression at 0.5 h (Cluster VII: 57 genes).

Distinct genomic responses in the amygdala and the hippocampus were further evident from distribution of genes based on classification of the cellular functions (Fig. 4). The hippocampus showed a higher percentage of signalling genes (34%) with increased expression compared to that of the amygdala (23%) (Fig. 4A and B). Of 222 genes from the amygdala, about 22% of the genes that were increased by fear conditioning encode structural/cytoskeleton protein genes, compared to that only 6% of 145 genes in the hippocampus are related with cellular structures (Fig. 4A and B). In addition, larger numbers of genes (33%) increased in the hippocampus were related with DNA/RNA regulation, whereas in the amygdala, only 5% of genes belonged to this category.

We also found that about 29% of genes whose expression levels were decreased in the hippocampus by fear conditioning consisted of signalling molecules (Fig. 4C). The second largest group of down-regulated genes belonged to transcriptional factors. Interestingly, the largest proportion of down-regulated genes in the amygdala was made of transcriptional factors (Fig. 4D). Thus, our analysis further illustrated that the molecular compositions of gene expressions in the hippocampus and amygdala were very different.

3.2. Validation of microarray-based expression profile by real-time RT-PCR

To further confirm the gene expression profiling data from our gene chip experiment, we have performed quantitative real-time PCR experiment. Total of 20 genes were chosen from hippocampus and amygdala. The results demonstrated that all 20 genes were shown similar expression changes in

Table 4
Representative genes changed in the amygdala after fear conditioning

Cluster	Accession number	Gene name	Fold change (h)		
			0.5	6	24
I	AF026124	Schwannoma-associated protein (SAM-9)	2.35		
	AB006361	Prostaglandin D synthetase	3.85		
	I31397	Dynamin	3.1		
	D38613	921-L presynaptic protein	3.05		
	AF026489	β-III spectrin	4.1		
II	R75491	Myelin-associated oligodendrocytic basic protein (MOBP)		2.85	
	X94310	L1		2.4	
	X14943	Neuronal cell surface protein F3		3.25	
	Z31557	Chaperonin containing TCP-1 (CCT)		2.6	
	X90875	FXR1		4.5	
	AA031158	NAP-22		2.3	
	M72414	Microtubule-associated protein 4 (MAP4)		3.3	
	X70398	P311		2.25	
III	AA139495	Clathrin, heavy polypeptide		−2.6	
	M73329	Phospholipase C-alpha		−3.35	
	M13227	Enkephalin		−5.15	
IV	AA239103	$GABA_A$ receptor-associated protein-like 2	−2.75	−2.9	
	AA409978	Calmodulin synthesis	−2.9	−2.15	
V	W76777	α-actinin	6.4	2.55	6.3
	W89940	A-X actin	41.9	10.6	25.15
	X57497	Glutamate receptor 1 (GluR1)	6.5	3.55	4.8
VI	D86177	Phosphatidylinositol 4-phosphate 5-kinase			3.05
	U20365	γ-actin			9.9
VII	U19582	Oligodendrocyte-specific protein (OSP)	−2.95		
	L02526	MEK1 protein kinase	−2.1		
	U60001	Protein kinase C inhibitor	−2.55		
	Z70023	Connexin-30	−2.15		
	X07215	Proteolipid protein (PLP)	−2.05		
VIII	AA111149	α-tubulin	3.95		5.35
	AA059763	β-tubulin	4.7		5.15
	AA122619	Protein phosphatase 2A inhibitor (PP2AI)	2.3		2.05
	*X*61434	Protein kinase C β-subunit	2.25		3.55
	U27106	Clathrin-associated AP-2, AP50 subunit	2.45		3
	U60150	Vesicle-associated membrane protein VAMP2	4.05		8.5
None	U06922	Signal transducer and activator of transcription (Stat3)	3.05	3.15	
	Z25524	Integrin associated protein (IAP)	−2.25		−2.05

both gene chip and real time PCR experiments (Table 5). It is well known that the real-time PCR method is more sensitive and reliable than microarray technology. Indeed, the fold changes of most of these genes were higher in our real-time PCR experiment compared to the gene chip data (Table 5).

3.3. Increase in GluR1 protein in amygdala after fear conditioning

To determine whether those observed gene expression changes were reflected at protein level, we focused on the AMPA receptor GluR1, which showed increased mRNA expression in amygdala at 0.5, 6, and 24 h after fear conditioning. We performed quantitative immunohistological analysis and found that expression of the GluR1 protein showed consistent increases both 6 and 24 h after training in the amygdala (Fig. 5). Therefore, this result extended the change of GluR1 mRNA to protein level. More importantly, it also provided a potential mechanistic explanation for the recently reported findings that fear conditioning enhances AMPA mediated transmission in the amygdala neurons [18,31].

While we only focused on GluR1 for detailed analysis here, interestingly, another identified gene, L1, a cell adhesion molecule, is recently shown to increase protein level after fear conditioning [20]. We hope that our establishment of the public gene profiling database (http://sbg.ecnu.edu.cn/DATA.htm) will facilitate further characterizations on the genes reported here and is a useful resource that should be shared with other researchers in the field.

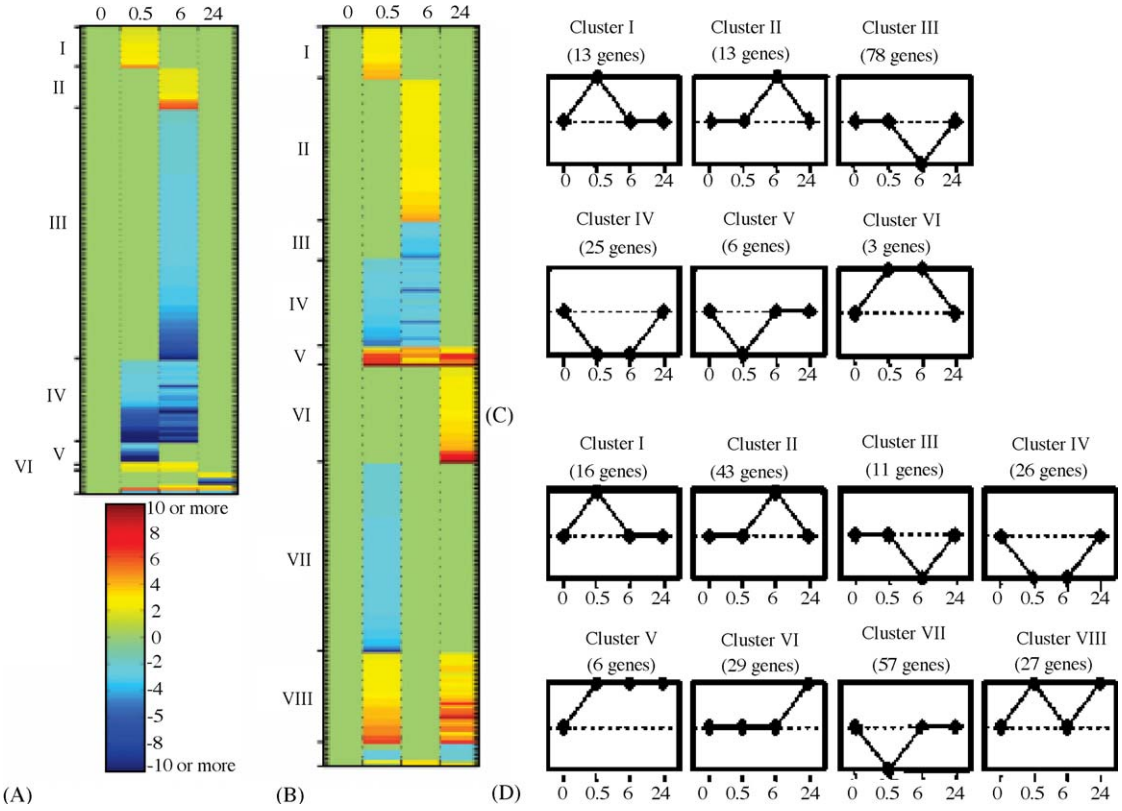

Fig. 3. Drastically different gene expression profiles triggered by fear conditioning in the hippocampus (A) and amygdala (B). Numbers in the *X*-axis represents time points in hours (0, 0.5, 6, and 24). The *Y*-axis represents the numbers of genes that show dynamic changes (coded in color). The Roman numbers (I–VI) along the *Y*-axis represent the gene clusters with similar expression kinetics. The color bar on the left corner represents the scale of changes in expression. (C) Most of the 145 genes identified in the hippocampus can be grouped into six clusters (I–VI). (D) Most of the 222 genes whose expression were changed in the amygdala could be placed into eight clusters (I–VIII). For illustration, changes of gene expression in each cluster (in C and D) are simply represented in a binary mode in the *Y*-axis. The *X*-axis shows the four time points (0, 0.5, 6, and 24 h after training). Numbers of genes in each particular cluster is shown at the top center of each cluster.

Table 5
Validation of microarray-based expression profile by real-time RT-PCR

Tissues	Accession number	Gene name	Fold change	
			Real-time RT-PCR	Microarray
Hippocampus	D30730	GTPase activating-protein (NF1)	9.95	5.4
	X51438	Vimentin	6.47	1.9
	AA066335	Amyloid precursor protein (APP)	−7.87	−2.5
	X60304	Protein kinase C-δ	6.16	6.2
	X64587	Splicing factor U2AF65	6.82	4.8
	M73329	Phospholipase C-α (PLC-α)	5.34	2.3
	*W*12204	Ca2+/calmodulin-dependent protein kinase II isom γ-b (CaMKII)	−5.47	−3.2
	W46019	Protein kinase regulator 14-3-3	−7	−2.8
	AA067362	Vesicle protein pantophysin	−7.97	−3.8
	D37792	SynaptotagminI/65	−3.69	−3.2
Amygdala	R75491	Myelin-associated oligodendrocytic basic protein (MOBP)	11.65	2.85
	X14943	Neuronal cell surface protein F3	9.95	3.25
	Z31557	Chaperonin containing TCP-1 (CCT)	6.69	2.6
	AA031158	NAP-22	8.52	2.3
	M72414	Microtubule-associated protein 4 (MAP4)	11.37	3.3
	AA139495	Clathrin, heavy polypeptide	−9.15	−2.6
	M73329	Phospholipase C-alpha	−6.26	−3.35
	M13227	Enkephalin	−9.3	−5.15
	AA239103	$GABA_A$ receptor-associated protein-like 2	−10.63	−2.9
	AA409978	Calmodulin synthesis	−8.53	−2.15

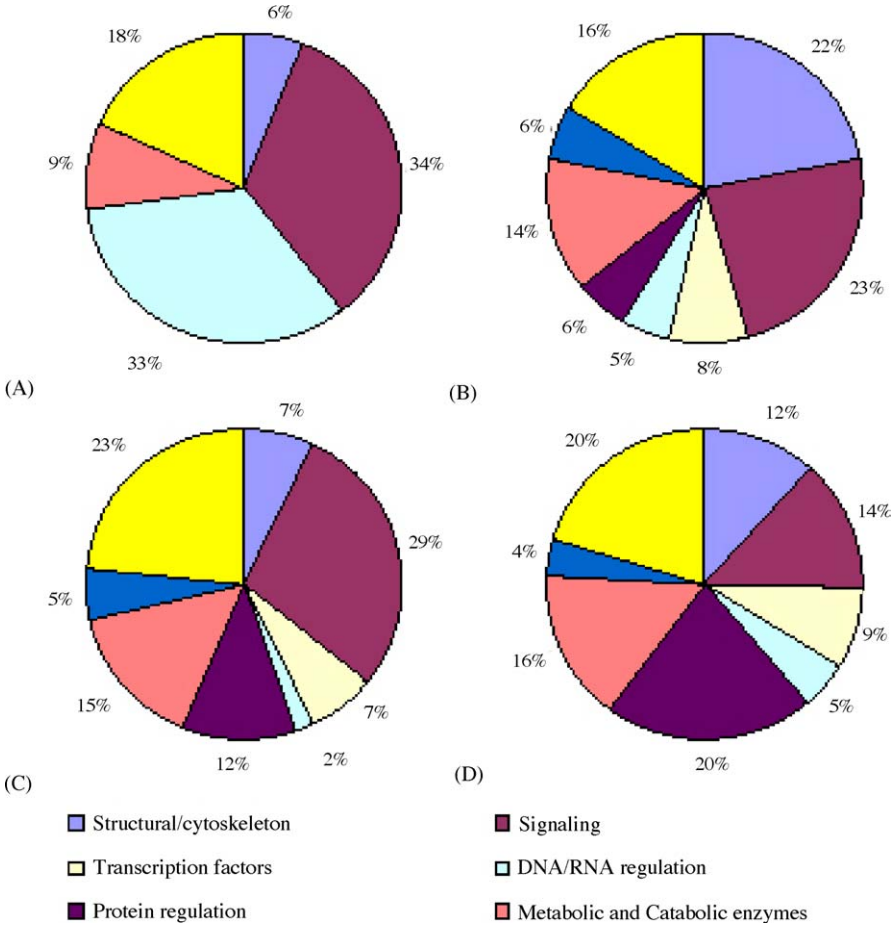

Fig. 4. Differences in the amygdala and hippocampus shown through distribution of all the identified genes based on classification of biological function. Illustrated in pie graphs are the percentages of genes in each functional class increased in the hippocampus (A) and amygdala (B), as well as those genes decreased their expression in the hippocampus (C) and amygdala (D).

4. Discussion

In an effort toward the understanding of the molecular genetic responses produced by fear conditioning training, we applied gene chip technology that allowed us to simultaneously examine large-scale gene expression changes in the amygdala and hippocampus after paired fear conditioning. Using Affymetrix GeneChip arrays, we screened over 11,000 mouse genes and ESTs, and identified 346 different genes that showed dynamic changes within the first 24 h after fear conditioning.

4.1. Fear conditioning-triggered gene expression profiles in the hippocampus

Twenty-seven of the 38 signalling molecules in the hippocampus showed decreased expression. Eighteen of these were down-regulated at 6 h, but not at 0.5 h or 24 h. For instance, the protein kinase regulator 14-3-3, which has previously been identified as a learning-related gene in *Drosophila* [38], shows a down-regulation 6 h after fear conditioning. In addition, 14-3-3 appears to interact with the C-terminus of $GABA_B$ receptors in tissue culture neurons [6]. We also found that $GABA_A$ receptor alpha-1 subunit was decreased at 6 h. This raises the interesting possibility that the formation of contextual fear memory involves modifications of GABA receptor-mediated inhibition of hippocampal circuits excitability. Interestingly, it has been reported that GABAA specific antagonist picrotoxin mediated currents that contributed to synaptic integration of US and CS in fear conditioning [32].

We also noticed that several proteins involved in synaptic vesicle trafficking and neurotransmitter release were down-regulated after fear conditioning, such as vesicle-associated membrane protein (VAMP) and synaptotagmin. Pantophysin [46], a vesicle protein related to synaptophysin and usually co-distributed with VAMP, was decreased at the 6 h time point. In addition, other protein involved in vesicle formation and assembly, for example, endophilin I [35] and ADP-ribosylation factor (ARF1) [8] were shown similar changes.

Modification of synaptic plasticity, such as depotentiation and long-term depression, is known to involve serine/threonine protein phosphatase 1 (PP1) and 2A (PP2A) [22]. These phosphatases interact closely with a variety of

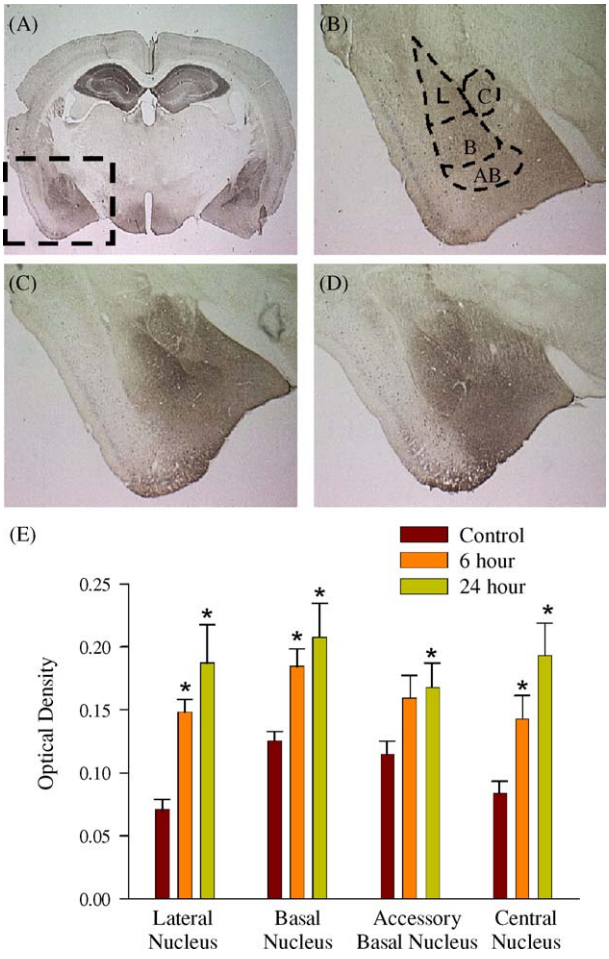

Fig. 5. Increase in GluR1 protein after fear conditioning. (A) A coronal section of mouse brain showing amygdala nucleus enclosed in the dot-lined box. (B) The area containing amygdala nucleus from control mouse is shown. Amygdala nucleus is divided into the lateral nucleus (L), basal nucleus (B), accessory basal nucleus (AB), and central nucleus (C). (C) Increased staining in the amygdala nucleus 6 h after fear conditioning. (D) Increased GluR1 staining in the amygdala 24 h after fear conditioning. (E) Quantitative measurement of GluR1 staining in various subnucleus of the amygdala. Asterisk (*) indicates the statistically significant increases in the staining intensities of GluR1 protein in the subregions of amygdala nucleus of the trained mice.

kinases including protein kinase C (PKC) and CaMKII. Interestingly, we found that PP1, PP2A, PKC, and CaMKII all showed a coordinated down-regulation 6 h after fear conditioning.

Several genes in the hippocampus showing learning related changes have previously been implicated in learning and memory disorders in humans. For example, Alzheimer's disease-related gene amyloid precursor protein (APP) was decreased in the hippocampus after fear conditioning. Moreover, two additional proteins, kinesin light chain 1 (KLC1) and the splicing factor, U2AF65, known to interact with APP, also changed expression level.

Another gene involved in learning disorders is the neurofibromatosis type 1 gene (NF1). Loss of NF1 function can lead to memory deficits in both mutant mice [37] and *Drosophila* [9]. We found that NF1 expression exhibited an increase at 6 h after fear conditioning, suggesting NF1 is tightly regulated during memory processes.

In addition to genes involved in direct regulation of neuronal function, we have also observed that a number of genes frequently expressed in non-neuronal cells showed altered expression in the hippocampus. For example, the glial enzyme glutamine synthetase showed an increase at the 24 h time point. Interestingly, it is reported that in Alzheimer's disease patients there was a decreased expression of glutamine synthetase in astrocytes [29]. Another gene expressed predominately in glia is vimentin which also showed an up-regulation after learning. This gene encodes an intermediate filament protein. Because intermediate filaments are a major component of the cytoskeleton in astrocytes [19], the increase in vimentin suggests that fear conditioning evokes structural changes in astrocytes as well.

Thirteen genes showing learning-related regulation in the hippocampus do not have a clearly assigned function or are completely novel. Only seven of these genes have been named, including neuronal protein 25 (NP25) [28] and neuron specific gene family member 1 (NSG1) [2]. While the functions of these genes are not clear, identification of these novel genes by our gene chip technique points to their potential importance in memory formation.

4.2. Fear conditioning-triggered gene expression profiles in the amygdala

From our simultaneous measurement of expression profiles of the same set of 11,000 genes and ESTs in the amygdala, 222 genes were responsive to fear conditioning learning in the amygdala, with 123 genes up-regulated and 99 genes down-regulated. Some of the representative genes are listed in the Table 4.

Perhaps the most noticeable feature for the amygdala gene profiles is that genes encoding structural and cell adhesion proteins occupy a major percentage (22%) of total genes involved. For example, many of them have been clearly linked with synaptic, dendritic and axonal structures such as actin, brain Spectrin, tubulin, and microtubule associated proteins [44]. β-III spectrin, a Golgi- and vesicle-associated protein, which has been shown to interact with the NMDA receptor, showed an increase at 0.5 h. Also, a microtubule associated protein (MAP4), β-tubulin, α-tubulin and cytosolic chaperonin CCT were up-regulated after training. CCT is a molecular chaperone required for the folding of α-actin and β-tubulin [15]. The up-regulation of CCT after learning provides further evidence for the requirement of functional α-actin and β-tubulin to undergo learning induced changes of cytoskeletal structure.

Several genes regulated in the amygdala are involved in the dynamic turnover and physical regulation of ionotropic receptors. The $GABA_A$ receptor associated protein (GABARAP), known to bind to $GABA_A$ receptors both in vitro and in vivo [45], decreased at 0.5 and 6 h after training. GABARAP can modulate channel kinetics by promoting

the clustering of $GABA_A$ receptors through microtubules [5]. The down-regulation of GABARAP after fear conditioning suggests that the inhibitory effects of the $GABA_A$ receptors is being decreased to allow for an increase in synaptic excitability.

The activity-dependent trafficking of AMPA receptors at synapses has emerged as one of the leading possible mechanisms for altering synaptic strength [3,16,44]. We found that the AMPA receptor subunit, GluR1, showed an increase of 6.5, 3.55, and 4.8-fold at 0.5, 6, and 24 h, respectively. Besides, we found several genes implicated in the insertion and removal of AMPA receptors. For example, AMPA receptors are specifically regulated by dynamin-dependent endocytosis [4]. Dynamin, a GTPase known to be involved in the fission step of vesicle formation [34], showed an increase at 0.5 h after training. In addition, it is reported that ligand-induced endocytosis of AMPA receptors is regulated by clathrin-coated pits [34]. Coincidently, the clathrin-associated AP-2 protein was also increased in expression after 0.5 and 24 h. Furthermore, it has been shown that CaMKII–actinin–actin complex provides an additional physical interacting site for anchoring AMPA receptors at synapses [3]. Indeed, both α-actinin and actin are drastically increased at all time points. All of these strongly suggested the involvement of AMPA regulation after fear conditioning. Furthermore, AMPA receptor antagonist CNQX has been shown to affect fear learning in rats [13].

We observed that the protein phosphatase 2A inhibitor showed an increase at both 0.5 and 24 h. It is interesting to note that protein phosphatase 2A (PP2A) can dephosphorylate AMPA receptors and CaMKII [14]. Up-regulation of PP2A inhibitor indicates the possible alteration in the signal transduction. We also found that SAM-9, which is a member of the phospholipase D superfamily [24], showed an increase at 0.5 h. In addition, phosphatidylinositol-4-phosphate-5-kinase (PIP5K), which is reduced by 50% in the frontal cortex of Alzheimer's patients [12], was increased after 24 h.

We also found that a number of genes known to be linked with human mental diseases have changed their expression levels. The fragile-X-related gene (FXR1) that encodes a ribosome-associated, RNA-binding protein [43], showed an increase 6 h after fear conditioning. It has been shown that FXR1 knockout mice displays significant deficit in fear conditioning [23]. Because FXR1 is located in spines and dendrites of neurons and thought to play a role in translational regulation of selective messenger RNA transcripts, the up-regulation of FXR1 expression by fear conditioning suggests that FXR1 is an important molecule in memory formation.

Several glia-enriched or glial-specific genes altered their expression in response to fear conditioning. This observation is consistent with a recent finding that glia can control synapse number in vitro and the possibility that glia may play a role in the changes underlying synaptic plasticity [42]. For example, astrocyte-specific connexin-30 (Cx30) [27] showed a decrease at 0.5 h after training. Also, several genes important in myelin formation or stabilization changed expression levels including myelin-associated oligodendrocytic basic protein (MOBP) [17], proteolipid protein (PLP) [47], and oligodendrocyte-specific protein (OSP) [1].

We found that among 222 genes showing dynamic expression changes in the amygdala, there are 41 genes with no apparent known function. Only 11 of these genes have been named, while the remaining 30 genes are completely novel. However, several of these genes appear to have very interesting features. Three previously identified genes, F3, 921-L, and P311 showed an up-regulation after fear conditioning. F3, suspected to be a neural adhesion molecule, increased three-fold at the 0.5 h time point. A significant decrease in F3 mRNA has been previously observed in 30-month old rats compared with younger rats [36]. This age-related decrease along with the increase observed in this study suggests F3 plays a role in memory formation.

One surprising aspect of this study is the large number of genes decreased after fear conditioning. In particular, there were 78 genes decreased in the hippocampus 6 h after training, indicating that a coordinated down-regulation in gene expression is an important part of the overall genomic program. This indicates that down-regulation of gene expression is an active and integral part of transcriptional processes in response to external stimuli. Further experiments will be required to elaborate on this finding.

Finally, by comparing the fear conditioning-induced genes identified in this study to the expression profiles of other cognitive processes that affect memory [10,11,26], we have noticed that a small set of genes induced by fear conditioning are also identified by our gene chip analysis from animals subjected to enriched environment (which improves learning and memory) or aging brains (which are typically associated with decline in learning and memory). For example, genes similarly regulated by environmental enrichment and fear memory include clathrin, synaptogamin I/65, VAMP-2, GluR1, and ryanodine receptor type 2 [26], whereas genes regulated in both aging and fear memory include dyanmin, clathrin-associated AP-2 protein, prostaglandin D synthetase, and protein phosphatase 2A (PP2A) [11]. Identification of these common genes, known to be critically involved in synaptic plasticity, demonstrates the usefulness of our genomic microarray approach. The transcriptional convergence of these genes in several memory–related behavioral paradigms points to their potential roles in the critical regulation of learning behaviors.

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