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Research

Differential transcriptional response to nonassociative and associative components of classical fear conditioning in the amygdala and hippocampus

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Classical fear conditioning requires the recognition of conditioned stimuli (CS) and the association of the CS with an aversive stimulus. We used Affymetrix oligonucleotide microarrays to characterize changes in gene expression compared to naive mice in both the amygdala and the hippocampus 30 min after classical fear conditioning and 30 min after exposure to the CS in the absence of an aversive stimulus. We found that in the hippocampus, levels of gene regulation induced by classical fear conditioning were not significantly greater than those induced by CS alone, whereas in the amygdala, classical fear conditioning did induce significantly greater levels of gene regulation compared to the CS. Computational studies suggest that transcriptional changes in the hippocampus and amygdala are mediated by large and overlapping but distinct combinations of molecular events. Our results demonstrate that an increase in gene regulation in the amygdala was partially correlated to associative learning and partially correlated to nonassociative components of the task, while gene regulation in the hippocampus was correlated to nonassociative components of classical fear conditioning, including configural learning.

Although post-translational modification of existing molecules may be sufficient for the storage of short-term memory for conditioned fear, long-term memory is mediated by changes in gene expression induced by the activation of intracellular signaling pathways (Abel and Lattal 2001), and these occur at precise times after training (Bernabeu et al. 1997; Bourchouladze et al. 1998). Characterizing transcriptional regulation during memory formation is therefore a key challenge for understanding the mechanism supporting long-term memory storage. Recent studies have revealed that learning induces a complex reprogramming of gene expression involving the regulation of many genes. For example, hippocampal gene expression has been examined following training for eye-blink conditioning (Cavallaro et al. 2001; Donahue et al. 2002), spatial navigation (Cavallaro et al. 2002; Leil et al. 2002, 2003), swim-escape (Irwin 2001), spatial discrimination (Robles et al. 2003), passive avoidance (Robles et al. 2003), and classical fear conditioning (Levenson et al. 2004). There was little agreement in the lists of genes identified after these various forms of training, possibly because of the variation in the type of training that occurred and the range of time points examined.

Although microarrays have not previously been used to examine gene expression in the amygdala following behavioral manipulation, several genes are known to be differentially regulated in the amygdala during memory storage. For example, *Fos* is induced in the rat amygdala following both conditioned and unconditioned fear (Campeau et al. 1991), and *Fos* and *Egr-1* are up-regulated following contextual fear conditioning (Campeau et al. 1991; Rosen et al. 1998). When genes known to be up-

regulated by seizure were examined in the amygdala following cued fear conditioning, *Fos*, *EGR-1*, *Jun*, *NF1*, *Gphn*, *Nrgn*, *Nr4a1*, *Actn1*, *16c8*, and *Cdh2* were all found to be up-regulated (Ressler et al. 2002). In another study, subtractive hybridization was used to identify 12 genes regulated in the amygdala by classical fear conditioning (Stork et al. 2001). Thus, the amygdala has been demonstrated to undergo complex changes in gene expression following behavior that requires further characterization.

The present study focuses on gene regulation in both the amygdala and hippocampus following classical fear conditioning. Classical fear conditioning provides a means to correlate the degree of gene regulation in a particular brain region with the proposed role of that brain region in learning and memory. The amygdala has been proposed to function in the associative component of classical fear conditioning, while the hippocampus has been proposed to support the configural component of the task (Maren 2001). Circuitry in the amygdala may be modified during classical fear conditioning such that a subsequent appropriate sensory input would cause activity patterns in amygdala via the thalamus that activate areas of the brainstem responsible for innate fear responses (Pare et al. 2004). In the hippocampus, modification of the synaptic circuitry has been shown to play a role in behavior, such as the modification of place cell fields (Sharp et al. 1985; Jeffery and Hayman 2004). In the processing of contextual fear conditioning, the hippocampus is likely to store a conjunctive representation of the context, as demonstrated by experiments combining pre-exposure to the training context and protein synthesis inhibitors (Barrientos et al. 2002).

Like neurons that act in networks to relay information across brain regions, genes are likely to act coordinately in functional networks to support cellular processes underlying long-term memory storage. The goal of this experiment was therefore to examine these two brain regions and generate hypotheses regarding the similarities and differences in gene regulation re-

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sponsible for these similarities and differences in the regional response to behavioral manipulation. To accomplish this, we used Affymetrix mouse genome U74v2A microarrays to examine gene expression patterns, comparing mice that were naive, exposed to conditioned stimuli (CS), or conditioned to fear those CS. We used statistical methods to identify regulated genes and computational methods to predict underlying regulatory mechanisms.

Results

We examined the transcriptional changes in the amygdala and the hippocampus 30 min after classical fear conditioning. Fear-conditioned (FC) mice were conditioned to fear a novel context plus tone by a single presentation of these CS paired with a mild footshock. CS-exposed (CS) mice received exposure to the CS without shock. Naive (N) mice received no behavioral manipulation on the day of training (Fig. 1A). Training consisted of a 3-min exposure to the conditioning chamber, a 30-sec exposure to tone, and (for FC training only) a 2-sec, 1.5-mA footshock. Classical conditioning produced robust fear upon exposure to the CS 24 h after training. FC-trained mice demonstrated robust freezing behavior ($58.4\% \pm 3.4\%$) compared to CS mice ($4.5\% \pm 1.2\%$) in response to the CS presented 24 h later (Fig. 1B). Thus classical fear conditioning produced evidence of robust learning.

Microarray analysis reveals different expression patterns in amygdala and hippocampus

The groups of mice used for microarray analysis were trained in parallel with those tested behaviorally. The expression profiles in the amygdala and hippocampus were examined 30 min after training, corresponding to the timing of immediate-early gene induction. Samples produced from either four amygdalae from two mice or two hippocampi from one mouse were measured on individual Affymetrix MGU74v2A microarrays (amygdala, N: $n = 10$ arrays, 20 mice; FC: $n = 6$ arrays, 12 mice; CS: $n = 6$ arrays, 12 mice; hippocampus, N: $n = 10$ arrays, 10 mice; FC: $n = 8$ arrays, 8 mice; CS: $n = 8$ arrays, 8 mice). Intensity scores were determined by Robust Multiarray Average, and MvsA plots were examined to ensure there was no bias due to signal intensity (data not shown).

Volcano plots were produced to gain perspective on global trends in our data (Fig. 2). In these pairwise group comparisons,

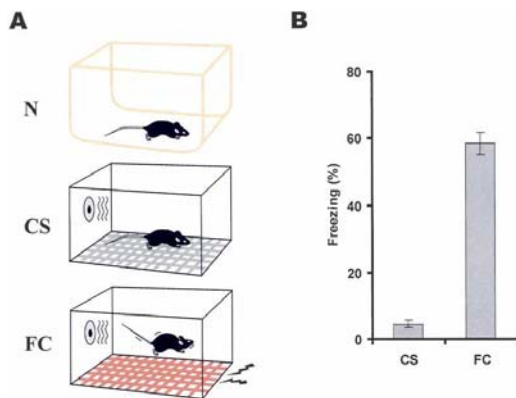


Figure 1. Classical fear conditioning. (A) Mice were left in their home cage (N), exposed to the conditioned stimulus (CS), or conditioned to fear the CS by coadministration of footshock (FC) and were then dissected 30 min later or tested for conditioned fear at 24 h. (B) Mice that were FC-trained demonstrated robust associative learning by exhibiting freezing behavior during ~60% of the re-exposure to the training environment at 24 h. CS-trained mice demonstrated only baseline levels of freezing behavior.

contrast P -values from mixed model ANOVA are plotted against the fold change, expressed as the base two log. The volcano plots demonstrate that the most significantly up-regulated genes were more statistically significant compared to down-regulated genes because each scatterplot demonstrates more data points in the upper right portion of the plot than in the upper left. The most significant genes in the hippocampus had similar patterns of significance in both FC versus N (Fig. 2A) and CS versus N (Fig. 2B), whereas the FC versus N comparison in the amygdala (Fig. 2C) shows many more data points with P -values $< 1E - 6$ compared to the CS versus N comparison (Fig. 2D). The 50 most significantly regulated probe sets in FC mice compared to N mice according to P -value are presented for the hippocampus (Table 1) and amygdala (Table 2).

CS and FC training produced similar gene regulation in the hippocampus, but in the amygdala, FC training produced significantly greater levels of gene regulation compared to CS training

Expression levels in CS mice were highly similar to FC mice in the hippocampus (Fig. 3). This was not the case in the amygdala, where average FC versus N regulation in the amygdala was significantly greater than CS versus N (Fig. 3). Because most of the 50 most significant genes followed this expression pattern, the difference in FC regulation in the 50 most significantly regulated amygdala genes compared to the 50 most significantly regulated hippocampus genes was very highly significant ($P < 10^{-5}$, paired t -tests). We validated that our microarray results were accurate by testing expression levels for *Fos*, *Dusp1*, *Nr4a1*, *Egr1*, *Junb*, *Gadd45b*, *Gadd45g*, and *Btg2* using quantitative real-time PCR (qPCR). For qPCR, tissue samples taken from independent training sessions were used ($n = 6$ for each group), and cDNA samples from both hippocampus and amygdala were prepared from single animals. For all genes tested, qPCR confirmed the up-regulation following FC observed in our microarray data. Because most fold-change values were small in this experiment (see Tables 1 and 2; qPCR data are given in parentheses), we determined the geometric average of fold change and used paired t -tests on log scale data to determine the significance of group differences for the set of genes examined. According to qPCR, the hippocampal genes we examined were up-regulated ~96% in CS and 85% in FC groups with no significant difference between these groups, whereas the genes we examined in the amygdala demonstrated 67% increase in expression in CS mice, while FC mice demonstrated a significantly greater 107% increase ($P < 0.05$).

Transcription factor-binding site analysis identified promoter models that predict gene regulation with some regional specificity

To account for the observed expression patterns, we generated promoter models based on the hypothesis that multiple transcription factors act coordinately to regulate many of the most significantly regulated genes. Transcription factor-binding sites were predicted for each of the 50 most significantly regulated genes in each brain region using previously described methods (Hannenhalli and Levy 2003). Twenty of the 50 most significantly regulated genes in the hippocampus were linked to all nine transcription factor-binding sites in the hippocampus model: *CREB*, *E2F1*, *Pax4*, *Sp1*, *GATA1*, *AP2*, *ZF5*, *AP-1*, and *Nrf-1*. Ten of the 50 most significantly regulated amygdala genes were linked to all 11 transcription factor-binding sites in the amygdala model: *CREB*, *E2F1*, *Pax4*, *Sp1*, *GATA1*, *AP2*, *ZF5*, *Ets1*, *Elk1*, *Myc*, *Max*, and *USF*. To examine the accuracy of our transcription

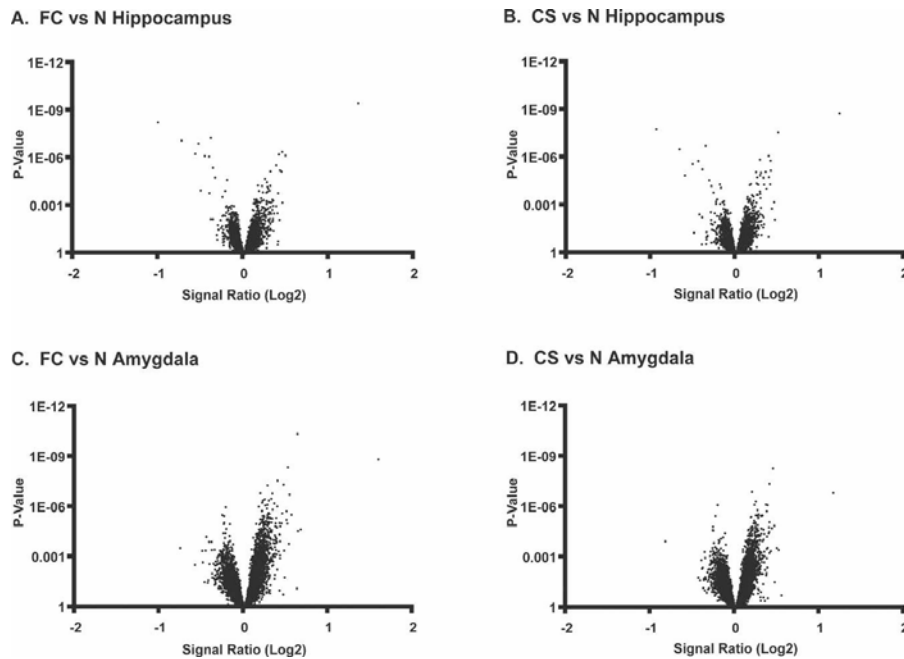


Figure 2. Volcano plots demonstrate the relationship between significance and Affymetrix signal ratios for gene regulation in the hippocampus and amygdala. Contrast P -values from a mixed model ANOVA are plotted in negative log scale on the y -axis against the base 2 log of the Affymetrix signal ratio for each probe set on the x -axis.

factor-binding site predictions, we examined six transcription factor-binding sites that were previously identified in the *Fos* promoter using Dnase1 protection assays (Shaw 1992; Lavrovsky et al. 1994; Schild-Poulter et al. 1996). We found that our annotation correctly identified the cAMP response element, the serum response element, and three out of four AP2-binding sites (data not shown). We then tested whether our transcription factor-binding site models could identify genes from the entire genome that were not among the genes used to produce the models, but were likely to be regulated in our data at lower levels of significance. High scoring matches for all nine binding sites identified in the hippocampal data were located upstream of 65 genes, and the expression of 32 of these genes had been measured by microarray. These 32 genes were significantly up-regulated in the hippocampus ($P = 0.001$) but regulation in the amygdala was not significant ($P = 0.22$) (Fig. 4). The 11 transcription factors identified for the amygdala were located upstream of 59 genes, of which we had expression data for 29 genes. These 29 genes were significantly induced in the amygdala following classical fear conditioning ($P = 0.003$), but regulation was not significant in the hippocampus ($P = 0.11$) (Fig. 4).

Discussion

Our study is the first to characterize gene regulation in the amygdala following classical conditioning using microarrays and to compare these changes to those observed in the hippocampus. Interestingly, we found that in the amygdala, changes in expression following FC versus N were greater than changes following CS versus N, whereas in the hippocampus, exposure to the CS or FC induced equivalent levels of gene regulation with respect to N mice. Our results are therefore consistent with proposed functions of the hippocampus and amygdala (Maren 2001), in which the hippocampus is involved in the configural component of the task, while the amygdala is involved in the associative component.

The similar transcriptional response to CS and FC training in the hippocampus indicates that nonassociative components

of the task alone are sufficient to drive the most significant regulatory changes observed in fear-conditioned animals, with associative components of the task having no significant additional effect. This result is consistent with the proposed role of the hippocampus in the configural component of fear conditioning (Rudy and Wright-Hardesty 2005), but the changes in expression could also be correlated to other nonassociative components of the task, such as arousal. Similarly, nonassociative components of fear conditioning may occlude otherwise significant effects of associative learning on hippocampal gene regulation in our data. Additional experiments using immediate shock or pre-exposure to induce memory deficits would be required to determine the more precise behavioral correlates for each gene. For example, more precise experiments have been previously examined in the case of Fos (Radulovic et al. 1998). In those experiments, Fos expression increased in the hippocampus following CS exposure, but this regulation was dramatically inhibited by pre-exposure of the training stimuli, demonstrating that arousal associated with training was not correlated to Fos regulation. Similarly, cued associative learning (including shock) was not correlated to Fos induction in the hippocampus when contextual training was blocked by pre-exposure (Radulovic et al. 1998).

The graded levels of regulation in the amygdala may indicate that some cell populations of the amygdala are involved in the processing of CS and US, and that additional activity in these populations occurs during associative processes (Maren and Quirk 2004). Alternatively, the graded response may be due to gene regulation in additional nuclei of the amygdala when the CS is paired with the unconditioned stimulus. Although additional experiments would be required to determine which of these two possibilities is occurring for each gene in our data, the relevant experiment has been conducted for Fos, and in that experiment, Fos expression in the central nucleus was partially correlated with associative learning, but Fos expression in other nuclei correlated with other components of the task such as exposure to novelty (Radulovic et al. 1998).

We focused our studies on genes regulated by FC versus N because attempts to identify “associative-learning genes” in our data using FC versus CS comparisons were not successful. In the hippocampus, only the most significantly regulated gene, uridine monophosphate kinase, had a P -value $<10^{-3}$; however, that corresponded to a fold change of only 6%. In the amygdala, JunB, FOSB, and nucleolin demonstrated significance of P -value $<10^{-3}$. The significance of JunB was accounted for in our examination of FC versus N genes. The differential regulation of FosB in FC versus N was validated, but the 11% FC versus CS fold change could not be validated by qPCR. Nucleolin demonstrated a change of only 9%. Overall, the changes in expression demonstrated in FC versus CS in the amygdala included several of the regulatory changes identified in FC versus N studies and were more significant than FC versus CS changes in the hippocampus. For consistency with previous studies, we included a tone in our training protocol. Previous results indicate that this auditory cue may be processed by the hippocampus along with other elements

Table 1. The 50 most significantly regulated probe sets in the hippocampus

P-value (FC vs. N)	Probe set	Symbol	Molecular role	N (log ₂)	CS vs. N	FC vs. N
Up-regulated genes						
3.2E-10	160901_at	Fos	DNA-binding transcription factor	5.4	134 (352)%	153 (303)%
3.7E-07	104598_at	Dusp1	Phosphatase	7.4	33 (37)%	36 (61)%
5.2E-07	103501_at	Pura	DNA-binding transcription factor	7.7	41%	33%
6.1E-07	102371_at	Nr4a1	DNA-binding transcription factor	7.2	33 (104)%	40 (58)%
2.6E-06	98579_at	Egr1	DNA-binding transcription factor	8.4	27 (55)%	30 (40)%
5.5E-06	104510_at	Cacna2d1	Voltage-dependent calcium channel	6.7	31%	34%
6.1E-06	102363_r_at	Junb	DNA-binding transcription factor	7.6	20 (50)%	24 (52)%
6.3E-06	103460_at	Ddit4	Unknown	7.1	28%	36%
3.1E-05	98782_at	Cplx2	Binds syntaxin	8.2	23%	20%
4.8E-05	94274_at	Ube2s	Ubiquitin carrier	7.2	12%	12%
5.0E-05	102362_i_at	Junb	DNA-binding transcription factor	7.6	22%	24%
5.2E-05	99597_at	Gnai2	GTP binding	8.4	22%	16%
5.9E-05	160617_at	Klf13	DNA-binding transcription factor	7.9	31%	21%
7.8E-05	102342_at	Nsf	Binds AMPA GluR2	10.3	25%	22%
1.0E-04	96497_s_at	Myt1l	DNA-binding transcription factor	5.5	22%	23%
1.1E-04	94405_at	Slc6a6	Taurine transporter	5.1	8%	13%
1.2E-04	93896_at	Ptprd	Protein tyrosine phosphatase	6.3	25%	21%
1.3E-04	104663_at	Pip5k1b	Phosphatidylinositol 4-phosphate 5-kinase	8.1	18%	17%
1.4E-04	93985_at	Tiparp	Amino acid ADP-ribosylation	6.3	14%	20%
1.5E-04	103393_at	Pspc1	RNA binding	5.7	22%	32%
3.1E-04	92982_at	Bmp8a	Secreted signaling molecule	5.2	15%	15%
3.7E-04	160925_at	Nras	GTP binding	6.5	13%	11%
3.8E-04	101930_at	Nfix	DNA-binding transcription factor	9.2	33%	26%
4.1E-04	99865_at	Bmpr2	Receptor transmembrane serine/threonine kinase	5.9	13%	17%
4.2E-04	160603_at	Pparbp	Transcriptional cofactor	6.2	19%	27%
4.3E-04	103243_at	Emp2	Associates with $\beta(1)$ integrin	7.5	9%	11%
4.5E-04	99109_at	Ier2	Unknown	5.1	20%	27%
4.7E-04	92195_at	Cebpg	DNA-binding transcription factor	4.8	15%	20%
4.7E-04	104743_at	Cdh13	Cell adhesion molecule	5.8	22%	15%
4.9E-04	100446_r_at	Sprr1b	Structural	10.2	8%	10%
5.0E-04	102972_s_at	Dab1	Interacts with protein kinase pathways	7.1	18%	12%
5.1E-04	98927_at	Rab6	GTP binding	10.1	26%	20%
5.4E-04	92703_at	Pb1	Kinetochores-associated proteins	6.3	19%	23%
5.6E-04	92899_at	Gad2	γ -Aminobutyric acid (GABA) synthesis	5.3	12%	25%
5.9E-04	100405_at	Cbx3	Binds heterochromatin	6.6	37%	37%
6.0E-04	93740_at	Nsep1	DNA-binding transcription factor	10.8	10%	9%
6.2E-04	98914_at	Asf1a	Nucleosome component	7.5	5%	8%
6.8E-04	99878_at	Ddx10	RNA helicases	4.1	8%	9%
7.2E-04	92362_at	Dusp8	Phosphatase	5.7	22%	20%
8.7E-04	97682_r_at	Gstm3	Transport/detoxification; major GST in brain	7.9	10%	11%
9.4E-04	103288_at	Nrip1	Transcriptional activity of the estrogen receptor	6.8	14%	27%
Down-regulated genes						
5.0E-08	161053_at	Ssty1	Unknown	3.9	-22%	-24%
1.1E-07	103847_at	Ssty2	Unknown	4.8	-27%	-31%
4.9E-07	94027_at	Cd84	Cell adhesion	7.6	-30%	-33%
2.2E-05	104581_at	Zdhhc6	Metal binding	3.7	-12%	-13%
9.8E-05	95705_s_at	Actb	Cytoskeletal	8.7	-34%	-30%
1.1E-04	97524_f_at	Amy2	Hydrolyze 1,4- α -glucoside bonds	4.1	-7%	-14%
1.4E-04	102818_at	Xmr	Unknown	4.1	-19%	-25%
2.5E-04	94028_f_at	Cd84	Cell adhesion	5.1	-17%	-16%
9.1E-04	M12481_M_st	Actb	Cytoskeletal	4.9	-14%	-10%

Genes were ranked by contrast *P*-values from FC versus N group comparisons using mixed model ANOVA. Affymetrix probe set identifiers were matched to gene symbols, and molecular roles are reported. Affymetrix signal levels calculated by RMA are reported for naive (N) mice along with percent change for FC versus N and CS versus N comparisons. qPCR values are given in parentheses.

of the context and may also be processed directly by the amygdala through an alternate route that does not pass through the hippocampus (Maren 2001). The cue was therefore not expected to have any effect on hippocampal expression, other than to serve as a salient element of the novel environment. In the amygdala, the parallel processing of the cue could have induced parallel gene regulatory events in the amygdala. However, the cognitive correlates of these molecular events could still be classified as associative or nonassociative regardless of whether they are associated with cued or contextual conditioning.

Of the 50 most significantly regulated genes in our FC versus N data, 12 genes in the hippocampus and 11 genes in the amygdala had been previously described as being induced by some

form of neural activity. Only two genes that we found to be up-regulated after fear conditioning (*Fos* and *Egr1*) were also identified in a previous study that examined gene regulation in two subregions of the hippocampus 1 h after classical fear conditioning (Levenson et al. 2004). The lack of overlap between these results may be due to the time points selected or the method used to obtain the tissue samples and prepare cDNA. Of the 50 most significant genes in each brain region from our data, six genes are common between these lists: *Fos*, *Dusp1*, *Nr4a1*, *Egr1*, *Junb*, and *Ier2*. Six genes shared between two top 50 lists would be highly unlikely based on chance (χ^2 , $P < 0.001$), suggesting that a cohort of genes is coordinately regulated in both brain regions. However, the divergence of these lists suggests that

Table 2. The 50 most significantly regulated probe sets in the amygdala (FC vs. N)

P-value (FC vs. N)	Probe set	Symbol	Molecular role	N (log ₂)	CS vs. N	FC vs. N
Up-regulated genes						
3.8E-11	102362_l_at	Junb	DNA-binding transcription factor	7.11	32 (7)%	55 (63)%
1.3E-09	160901_at	Fos	DNA-binding transcription factor	5.26	123 (205)%	200 (512)%
3.8E-09	102371_at	Nr4a1	DNA-binding transcription factor	6.44	28 (86)%	43 (86)%
4.2E-08	99109_at	ler2	Unknown	3.97	30%	38%
4.7E-08	96318_at	ly6e	Unknown	6.47	17%	21%
1.4E-07	98456_at	Stk19	Serine/threonine kinase	6.61	14%	14%
1.5E-07	101979_at	Gadd45g	Upstream activator of p38 and JNK MAPKs	6.18	18 (53)%	26 (40)%
1.7E-07	98579_at	Egr1	DNA-binding transcription factor	7.98	32 (66)%	45 (44)%
3.0E-07	98878_r_at	Aaas	Nuclear pore/adaptor	5.42	21%	25%
3.1E-07	95726_at	Mlf2	Unknown	9.54	13%	18%
3.7E-07	96554_r_at	Taf15	DNA-binding transcription factor	3.72	11%	22%
3.7E-07	96872_at	Sgta	Unknown	8.89	14%	16%
5.0E-07	104684_at	Grin1	Ligand gated ion channel	9.74	13%	16%
5.7E-07	103367_at	Galgt1	Biosynthesis of glycosphingolipids	6.98	24%	28%
6.6E-07	103277_s_at	Tnrc11	Unknown	6.23	18%	21%
7.2E-07	101487_f_at	Ly6e	Unknown	10.03	12%	19%
8.6E-07	99872_s_at	Ftl1	Iron binding	8.70	29%	31%
8.8E-07	98429_at	Lypla2	Lysophospholipase	8.87	8%	12%
1.1E-06	92618_at	Serf2	Unknown	4.13	11%	20%
1.6E-06	92375_at	Ascc1	DNA-binding transcription factor	5.95	6%	15%
1.6E-06	104598_at	Dusp1	Phosphatase	6.87	29 (55)%	41 (95)%
2.1E-06	102092_s_at	Ovgp1	Unknown	4.37	25%	36%
2.6E-06	161806_r_at	Atp6ap1	Proton pump	4.86	29%	47%
2.7E-06	104123_at	Fcho1	Unknown	6.77	18%	21%
3.1E-06	93126_at	Ckb	Creatine kinase	11.20	16%	17%
3.1E-06	160503_at	Fbl	RNA binding	7.56	12%	16%
3.6E-06	99014_at	Apbb1	Unknown	9.76	14%	18%
3.8E-06	96008_at	Dad1	Unknown	9.57	19%	23%
4.3E-06	92987_at	Slc4a3	Chloride/bicarbonate exchanger	8.02	22%	30%
4.4E-06	160242_at	Flcn	Unknown	6.33	14%	21%
4.4E-06	92714_at	Icam5	Intercellular adhesion	8.62	20%	20%
4.5E-06	100492_at	Ap2a2	Tropomyosin-binding	8.78	24%	23%
4.7E-06	160215_at	Aes	Repressor of expression	9.41	19%	26%
5.0E-06	94918_at	Aars	tRNA synthase	7.39	11%	17%
5.2E-06	101094_at	Hig1	Unknown	4.87	9%	15%
5.2E-06	93733_r_at	Rgs19ip1	RGS protein degradation	6.19	9%	14%
6.6E-06	101583_at	Btg2	Regulates transcription	8.07	12 (87)%	20 (204)%
6.6E-06	93618_at	Spnb3	Binds Arp1	8.64	12%	19%
7.1E-06	94780_at	Zbtb20	DNA-binding transcription factor	5.21	20%	21%
7.1E-06	92622_at	Spin1	Transporter	7.82	15%	16%
7.2E-06	97319_at	Rrad	G-protein	5.75	9%	18%
7.3E-06	93738_at	Slc2a1	Glucose transporter	6.59	13%	16%
7.4E-06	101007_at	Mknk2	Serine/threonine kinase	6.21	13%	23%
7.7E-06	102835_at	Ap2a2	Lipid binding clathrin	7.01	19%	25%
8.1E-06	99486_at	Cenpb	Centromere binding	6.74	23%	21%
8.2E-06	161666_f_at	Gadd45b	Upstream activator of p38 and JNK MAPKs	4.92	31 (21)%	33 (52)%
Down-regulated genes						
9.5E-07	92665_f_at	Xlr	Unknown	3.02	-6%	-14%
2.8E-06	161499_f_at	Rpl7l1	Unknown	4.77	-13%	-17%
3.1E-06	103925_at	Mllt3	DNA-binding transcription factor	7.02	-15%	-15%
8.2E-06	94074_at	Fcgr3	IgG binding	4.97	-10%	-15%

Genes were ranked by contrast *P*-values from FC versus N group comparisons using mixed model ANOVA. Affymetrix probe set identifiers were matched to gene symbols, and molecular roles are reported. Affymetrix signal levels calculated by RMA are reported for naive (N) mice along with percent change for FC versus N and CS versus N comparisons. qPCR values are given in parentheses.

additional cohorts of genes are being regulated in a brain-region-specific manner. We used EASE analysis (Hosack et al. 2003), Ingenuity Pathway Analysis (<http://www.ingenuity.com>), and literature survey to identify gene functions for our lists of significantly regulated genes. Many of the genes identified in both the amygdala and hippocampus are categorized as DNA-binding transcription factors, immediate early proteins, or cell signaling genes (Tables 1, 2).

We identified groups of transcription factor-binding sites shared among the differentially expressed genes in the 5 kp upstream of each gene (Hannenhalli and Levy 2003). The promoter models we thus generated successfully predicted regulation and also demonstrated tissue specificity. Because we were interested

in building transcriptional models that include many transcription factor-binding sites that regulate cohorts of related genes (Harbison et al. 2004; Chesler et al. 2005), we used graph theoretical analysis to search for the largest group of transcription factor-binding sites that demonstrated complete linkage to the largest number of genes among each of our top 50 gene lists. In the hippocampus, our transcription factor-binding site model was used to predict 65 potentially regulated transcripts. In the amygdala, our model was used to predict 59 potentially regulated transcripts. Because our models rely on the assumption that coordinated binding of many transcription factors is occurring at each promoter, electromobility shift assays would not be appropriate to validate these results. Here we used a strategy in which

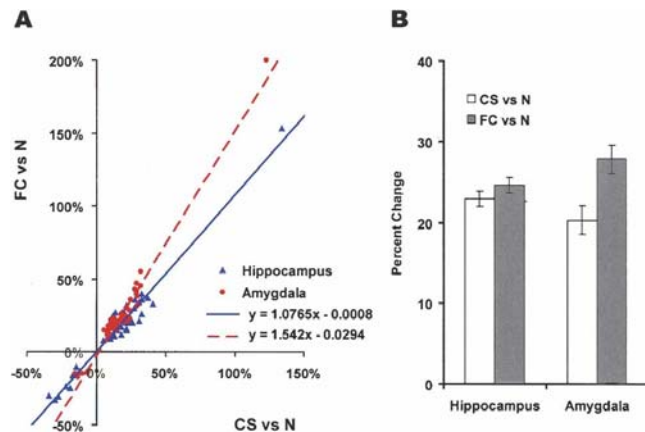


Figure 3. Gene regulation was equivalent following associative learning and CS exposure in the hippocampus, whereas gene regulation in the amygdala was greater following associative learning than nonassociative learning. (A) FC versus N regulation is plotted against CS versus N regulation for each of the top 50 genes in the hippocampus (blue triangles) and amygdala (red circles). Hippocampal genes were located near the identity function, as demonstrated by the slope of the regression line (blue solid line, slope = 1.08, $R^2 = 0.96$). Amygdala genes were located along a line of greater slope (red dashed line, slope = 1.54, $R^2 = 0.96$). (B) Group statistics based on geometric averages across each set of 50 genes demonstrate that FC training in the hippocampus does not produce significantly greater regulation than CS training. In the amygdala, FC training does produce significantly greater regulation. Error bars represent SEM of pairwise changes.

changes in the expression of genes predicted by our models were examined in our existing microarray data. Although not all predicted genes were present on the array, the average expression values from 32 genes in the hippocampus and 29 genes in the amygdala demonstrated the expected pattern of up-regulation following classical fear conditioning, with some degree of regional specificity (Fig. 4). The overlap between regulatory modules generated for these two brain regions, including *CREB*, *E2F1*, *Pax4*, *Sp1*, *GATA1*, *AP2*, and *ZF5*, again suggests that common mechanisms are at work in both the hippocampus and amygdala, while our ability to predict gene regulation from these lists with some regional specificity supports the idea that additional brain-region-specific mechanisms exist. Brain-region-specific transcrip-

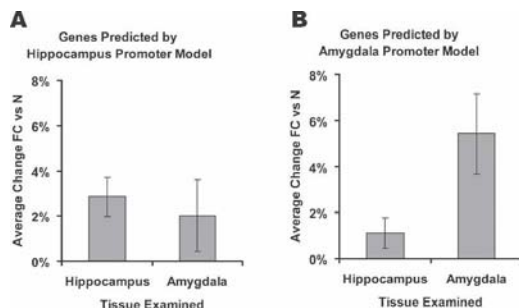


Figure 4. Transcription factor-binding site models predict regulation of additional genes for both amygdala and hippocampus with some specificity. (A) A set of transcription factor-binding sites identified from the 50 most significantly regulated hippocampal genes was detected in 32 additional genes, which showed significant up-regulation in the hippocampus ($P < 0.001$), but up-regulation in the amygdala was not significant ($P > 0.05$). (B) A set of transcription factor-binding sites identified from the 50 most significantly regulated amygdala genes was detected in 27 additional genes, which showed significant up-regulation in the amygdala ($P < 0.005$), whereas up-regulation in the hippocampus was not significant ($P > 0.05$).

tion factor-binding sites that together confer the specificity of the models include Ets1, Elk1, Myc/Max, and USF binding sites in the amygdala and Nrf1 and AP-1 in the hippocampus. It will be interesting to examine the role of these transcription factors in regionally specific gene regulation during memory storage. Chromatin immunoprecipitation (ChIP) from neural tissue following pharmacological manipulation has recently been accomplished (Kumar et al. 2005). By expanding these techniques to examine combinatorial binding of transcription factors using ChIP following behavioral manipulation, future experiments could address the hypotheses generated here. In future experiments, it will also be important to develop genetic and genomic approaches to identify the direct target genes of transcriptional regulators in vivo, as indirect effects are almost always possible with current molecular techniques.

To identify additional *trans*-acting genetic regulators, we used WebQTL (Wang et al. 2003; <http://www.genenetwork.org>) to search for gene cliques within our top 50 gene lists that were coordinately linked to genetic loci in an independent data set (Chesler et al. 2005). We found only one clique that was linked to two loci and contained genes that were regulated in both brain regions: *Fos*, *Dusp1*, *Nr4a1*, *Ier2*, *Egr1*, *Junb*, *Gadd45b*, and *Btg2*. One linkage locus on chromosome 12 contained 65 transcripts that included *Myt1l*, which was one of the 50 most significantly regulated genes in the hippocampus (Table 1) and was also regulated in the amygdala ($P < 0.001$). Another linkage locus on chromosome 18 contained ~54 transcripts that included *Camk2a*, a known regulator of immediate-early genes in response to neural activity (Colbran and Brown 2004). Future experiments would be required to determine how genetic variations in genes at these linkage sites, such as *Myt1l* and *Camk2a*, act to regulate the behaviorally responsive gene clique identified here.

Based on our results, we hypothesize that classical fear conditioning induces changes in the expression of a large battery of genes in both brain regions within 30 min. Some of these genes may be regulated in both brain regions as a cohort that responds to a set of shared transcription factor-binding sites, while additional genes may be regulated by regionally specific involvement of particular transcription factor-binding sites, as has been observed during development (Davidson et al. 2002) and exposure to environmental stimuli (Harbison et al. 2004). Our main findings are that during classical fear conditioning, exposure to CS drives gene regulation in the hippocampus, while in the amygdala, genes are regulated partially by CS but are more greatly regulated by FC. Associative learning therefore correlated with gene regulation in the amygdala, while nonassociative components of classical fear conditioning correlated with gene regulation in both the amygdala and the hippocampus.

Materials and Methods

Animals

Six- to 8-wk-old male C57BL/6J mice were obtained from Jackson Labs (Bar Harbor, ME). Mice were housed under a 12-h light/12-h dark cycle (lights on at 7 a.m.) and allowed access to food and water ad libitum. All experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and performed in accordance with all National Institutes of Health guidelines. All mice were between 8 and 14 wk old at the time of behavioral training.

Behavior

Animals were individually housed and handled for 6 d prior to behavioral training. Handling consisted of removing mice from their home cage for 3 min while in the training room. Animals were always handled, trained, and tested at the same time of day (10:30 a.m. to 11:30 a.m.) to control for circadian effects. Experi-

ments were performed in parallel. Naive (N) mice were dissected between 11 a.m. and 12 p.m. on day 7. CS-exposed (CS) mice were exposed to the conditioning chamber for 3 min. From 2:00 to 2:30 the mice were exposed to a tone (2800 Hz, 85 dB). CS mice were dissected 30 min after context/cue exposure. Fear-conditioned (FC) mice were exposed to the same CS as CS mice, but additionally received a scrambled 2-sec 1.5-mA footshock from 2:28 to 2:30. FC mice were likewise dissected 30 min after training. Additional animals were given the same behavioral treatment in parallel, but were tested on day 8. Testing consisted of re-exposure to the CS for 3 min. Freezing behavior was scored by sampling at 5-sec intervals to determine the percentage of time that the mice spent in a stereotypical frozen posture. Groups of mice used for microarray analysis were trained in parallel with those tested behaviorally.

Dissection

Brains were rapidly dissected, placed in ice cold PBS for 10 sec, and then transferred to a mouse brain matrix (ASI Instruments). The matrix was occluded so that the brain rested at a consistent angle of 45°, and a 10- μ m blade was inserted at the posterior boundary of the circle of Willis. By following the 45° angle of the brain matrix, we were able to expose the amygdalae without damaging the hippocampi. The amygdalae were dissected by cutting along the line of the external capsule following the angle of the lateral cortex from the surface of the amygdala to the bifurcation of the external capsule and then teasing the amygdala away from the optic tract, internal capsule, striatum, and other dorsal/medial structures. The resulting sample therefore included the cortical amygdaloid, basolateral, basomedial, lateral, central, and medial nuclei, but not the posterior portion of the amygdala. Hippocampi were dissected by teasing apart the posterior cerebral hemispheres and the lateral ventricles to unroll the posterior cerebrum. Hippocampi were carefully separated from the cortex, fimbria, and choroid plexus.

RNA extraction, cDNA synthesis, and hybridization

RNA extraction, cDNA synthesis, and hybridization were based on Affymetrix GeneChip Sample Cleanup Module (Mat. No. 1,020,407, 03/2002; Affymetrix) with some modifications. Briefly, RNA extraction was performed by homogenizing two hippocampi from one mouse or four amygdalae from two mice in 1 mL of Trizol reagent (Invitrogen) using a dounce homogenizer. Samples were extracted with 300 μ L of chloroform using spin-lock tubes (Eppendorf). Total RNA was precipitated with 1 μ L of glycogen (10 mg/mL), 1/10 vol of 3 M NaOAc, and 2 vol of ethanol, then desalted with cold 80% ethanol, dried, and resuspended in sterile distilled water. RNA cleanup was performed using the RNeasy kit (Qiagen) according to instructions, followed by quantification by spectrophotometry. Synthesis of cDNA from purified total RNA was performed using SuperScript II (Invitrogen) according to instructions. Briefly, 5 μ g of total RNA was reverse-transcribed in 20- μ L reactions using 200 U RT (SuperScript II). Temperature adjustment and first-strand synthesis were performed at 42°C. Second-strand synthesis was performed using DNA ligase, Polymerase I, and RNase H from *Escherichia coli* (SuperScript II) according to instructions, followed by T4 DNA Polymerase to finish second-strand synthesis. Cleanup of double-stranded cDNA was performed using the GeneChip Sample Cleanup Module (Affymetrix) according to instructions. Synthesis and cleanup of biotin-labeled cRNA was performed by T7 polymerase in vitro transcription using the BioArray HighYield RNA Transcript Labeling Kit (Affymetrix). Ten microliters of cDNA was used for labeling reactions for each sample. Quantification of cRNA was performed by spectrophotometry, subtracting carryover cDNA from total concentration to obtain the concentration of labeled cRNA. Initial samples were analyzed by agarose gel electrophoresis stained with ethidium bromide. Fragmentation of 20 μ g of cRNA was performed by metal-induced hydrolysis using 5 \times fragmentation buffer supplied with the GeneChip Sample Cleanup Module (Affymetrix). The cRNA products were fragmented to 200 nt or less, heated at 99°C for 5

min, and hybridized for 16 h at 45°C to Affymetrix mouse genome U74v2A microarrays by the Penn Microarray Facility. The microarrays were washed at low (6 \times SSPE) and high (100 mM MES, 0.1 M NaCl) stringency and stained with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycoerythrin stain. A confocal scanner was used to collect fluorescence signal at 3 μ m resolution after excitation at 570 nm. The average signal from two sequential scans was calculated for each microarray feature.

Expression analysis

Robust Multiarray Average (Bolstad et al. 2003) was used to compute intensity scores from image (.cel) files. Mixed model ANOVA was performed for each brain region using group (n, cs, fc) fixed variables and batch (1–4) random variables, and *P*-values were calculated from pairwise contrast calculations using Partek Pro software (Partek). Microarray data are available through GEO accession number GSE3963.

Quantitative real-time PCR

Quantitative PCR was performed according to the TIGR protocol for two-step RT PCR (Hegde et al. 2000) with some modifications. RNA extraction was performed by homogenizing two hippocampi from one mouse in 1 mL or two amygdalae from one mouse in 300 μ L of Trizol reagent (Invitrogen), using a dounce homogenizer. Samples were extracted with 1/3 volume chloroform using spin-lock tubes (Eppendorf). Total RNA was precipitated with 1 μ L of glycogen (10 mg/mL), 0.1 vol of 3 M NaOAc, and 2 vol of ethanol, then desalted with cold 80% ethanol, dried, and resuspended in sterile distilled water. RNA cleanup was performed using the RNeasy kit (Qiagen) according to instructions. DNase treatment and removal were performed with DNA-free (Ambion) according to instructions and resuspended in 100 μ L of sterile water. RNA precipitation was performed using 1 μ L of glycogen, 50 μ L of 7.5 M ammonium hydroxide, and 250 μ L of ethanol, then desalted with cold 80% ethanol, dried, and resuspended in sterile distilled water and quantified by spectrophotometry at 260 nm. To produce cDNA, 2 μ g of RNA from each sample was reverse-transcribed in 100- μ L reactions using Taqman RT reagents (ABI), according to instructions. Additional 2- μ g RNA samples were prepared in parallel but without reverse transcriptase to act as a control for genomic contamination in quantitative PCR. Reverse-transcription conditions were 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C to heat-kill the enzyme. For each quantitative PCR reaction, 100- μ L RT reactions were diluted to 1500 μ L with sterile water, and 14 μ L of each sample was used for each qPCR reaction. qPCR was performed using 15 μ L of Quantitect SYBR master mix (Ambion) plus 0.5 μ L of each 10 μ M primer, pre-mixed and aliquoted for consistency. The efficiency of each primer set was determined for each of the genes selected (*Fos*, *Junb*, *Nr4a1*, *Egr1*, *Dusp1*, *Gadd45b*, *Gadd45g*, *Btg2*) using a serial dilution of pooled cDNA. All qPCR was performed in triplicate. *Fos*, *Junb*, *Nr4a1*, *Egr1*, *Dusp1*, and *Gadd45b* qPCR products were each cloned and sequenced, and all six cloned products contained the appropriate sequence. Primer sequences are available upon request. Threshold cycle (C_t) values were adjusted for efficiency and then normalized to two internal control genes: *HPRT* and *Actin* γ . Median values from triplicate experiments were then used to generate group statistics. *P*-values were determined based on paired *t*-tests using log-scale values. Geometric averages were also calculated for each group.

Transcription factor-binding site analysis

TRANSFAC (Wingender et al. 1996) was used as a primary source of known binding profiles to annotate regions 5 kb upstream of all known RefSeq transcripts. This region was selected based on the fact that transcription factor-binding sites in several validated targets are localized in that region (Euskirchen et al. 2004). The annotation threshold for each position weight matrix was set so that the frequency of each PWM would be 1/50,000 bp in

nonconserved sequence and 1/5000 bp in conserved sequence, using human–mouse genome alignment from the UCSC database to determine conservation. The application of this method to the human genome has been previously described (Levy and Hannenhalli 2002). The upstream regions of the 50 most significantly regulated genes from each brain region were examined for each transcription factor-binding site, and the results were represented as a bipartite graph. All transcription factors from the largest completely connected subgraphs were combined to produce one promoter model for each brain region. For each model, genes that contain every site in the model were selected from genomic data.

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