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Identification of plasticity-associated genes regulated by Pavlovian fear conditioning in the lateral amygdala

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Abstract

Most recent studies aimed at defining the cellular and molecular mechanisms of Pavlovian fear conditioning have focused on protein kinase signaling pathways and the transcription factor cAMP-response element binding protein (CREB) that promote fear memory consolidation in the lateral nucleus of the amygdala (LA). Despite this progress, there still remains a paucity of information regarding the genes downstream of CREB that are required for long-term fear memory formation in the LA. We have adopted a strategy of using microarray technology to initially identify genes induced within the dentate gyrus following *in vivo* long-term potentiation (LTP) followed by analysis of whether these same genes are also regulated by fear conditioning within the LA. In the present study, we first identified 34 plasticity-associated genes that are induced within 30 min following LTP induction utilizing a combination of DNA microarray, qRT-PCR, and *in situ* hybridization. To determine whether these genes are also induced in the LA following Pavlovian fear conditioning, we next exposed rats to an auditory fear conditioning protocol or to control conditions that do not support fear learning followed by qRT-PCR on mRNA from microdissected LA samples. Finally, we asked whether identified genes induced by fear learning in the LA are downstream of the extracellular-regulated kinase/mitogen-activated protein kinase signaling cascade. Collectively, our findings reveal a comprehensive list of genes that represent the first wave of transcription following both LTP induction and fear conditioning that largely belong to a class of genes referred to as 'neuronal activity dependent genes' that are likely calcium, extracellular-regulated kinase/mitogen-activated protein kinase, and CREB-dependent.

Keywords: amygdala, dentate gyrus, fear conditioning, LTP, microarray, plasticity.

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Classical or Pavlovian fear conditioning has emerged as one of the best characterized learning paradigms in behavioral neuroscience (Davis 1992; Fanselow and LeDoux 1999; LeDoux 2000). In this associative learning paradigm, an animal learns to associate a benign stimulus (conditioned stimulus, CS), such as a tone, with a noxious stimulus (unconditioned stimulus, US), such as a foot shock. Because this learning paradigm is rapidly acquired, enduring, and quantifiable, much progress has been made in identifying the neurobiological mechanisms underlying fear learning at the behavioral, neurophysiological and molecular levels.

Most recent studies aimed at studying the cellular mechanisms of fear conditioning have supported the notion that fear acquisition and memory formation involves transmission and integration of CS and US information in the lateral nucleus of the amygdala (LA), where NMDA receptor (NMDAR)-driven alterations in protein kinase signaling pathways are thought to promote alterations in synaptic transmission, in part, by engaging cAMP-response element binding protein (CREB)-dependent transcription in the nucleus (Blair *et al.* 2001a; Josselyn *et al.* 2001; Schafe *et al.* 2001; Maren *et al.* 2003; Rodrigues *et al.* 2004). Indeed, long-term memory (LTM) formation of fear conditioning has been shown to require *de novo* mRNA transcription and protein synthesis in the LA. Intra-LA injection of inhibitors of either mRNA transcription or protein synthesis impairs memory consolidation of fear conditioning; that is, short-term memories are intact, while LTM is impaired (Bailey *et al.* 1999; Schafe and LeDoux 2000; Maren *et al.* 2003; Duvarci *et al.* 2008). Conversely, over-expression of CREB in the LA enhances fear memory

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Abbreviations used: CREB, cAMP-response element binding protein; CS, conditioned stimulus; ERK, extracellular-regulated kinase; HFS, high-frequency stimulation; LA, lateral nucleus of the amygdala; LFS, low-frequency stimulation; MAPK, mitogen-activated protein kinase.

consolidation (Josselyn *et al.* 2001). Collectively, these findings have contributed to the view that neural plasticity in the LA encodes key aspects of fear learning and memory storage (Fanselow and LeDoux 1999; Blair *et al.* 2001b; Schafe *et al.* 2001; Maren and Quirk 2004).

While the signaling mechanisms underlying fear memory formation in the LA have been extensively studied, relatively little progress has been made in identifying genes downstream of CREB that are regulated by fear learning in LA neurons. This lack of progress is likely due, in part, to the relatively small size of the LA, the low cell body density of the LA, and to the relatively small changes in gene expression induced by fear conditioning within the LA. Collectively, this leads to difficulties in unambiguously identifying gene expression changes following fear learning using existing methodologies that rely on a high signal-to-noise ratio. Indeed, the few studies that have attempted to examine the downstream genes that are regulated by fear memory formation in the LA have either identified only a few genes (Stork *et al.* 2001; Ressler *et al.* 2002) or have generated a list of genes from a microarray screen (Keeley *et al.* 2006).

While global gene expression profiling utilizing DNA microarrays offers an opportunity to identify genes that may be regulated during a particular stimulus, in practice, it can be very difficult to identify biologically meaningful changes (signal) versus the many false positives (noise) that inundate these types of screens. Typically, statistics are used to provide confidence that the genes identified are valid, but all too often these same genes are unable to be verified via other means, bringing into question the reliability of the screen (Tan *et al.* 2003; Miklos and Maleszka 2004; Frantz 2005). There are several reasons a screen may result in a low signal-to-noise ratio. For example, the stimulus may not induce strong gene expression, or the time point chosen to examine gene expression changes may not be optimal. Further, the noise may be increased due to contamination of either surrounding structures or cell types that do not undergo the same gene expression changes. These latter issues are particularly problematic for the brain as it is a heterogeneous structure composed of many cells types and distinct subregions.

In the present study, we attempted to circumvent many of these common pitfalls by choosing to first identify genes that are induced during *in vivo* long-term potentiation (LTP) at dentate gyrus synapses in urethane-anesthetized rats followed by global gene expression analysis of microdissected dentate gyrus at a time point (30 min) that is likely to capture the first wave of transcription induced following LTP. The use of the *in vivo* dentate gyrus LTP method to initially screen for genes regulated by synaptic plasticity offers several important advantages. First and foremost, it utilizes a pattern of stimulation that induces highly reliable and robust alterations in synaptic plasticity that shares many of the essential pharmacological and biochemical features of learning and memory (Bliss and Lomo 1973; Malenka and Nicoll 1999; Schafe *et al.* 2001; Rodrigues *et al.* 2004). Second, the dentate gyrus is a clearly identifiable region of high neuronal cell body density. This allows for highly accurate microdissection, limiting contamination from surrounding structures and virtually eliminating gene dilution/negation effects. Further, the use of the *in vivo* anesthetized LTP preparation has the additional advantage of avoiding gene-dilution effects which may be inherent in either awake-behaving models, due to variability in the baseline expression of activity-dependent genes (Cirelli *et al.* 2004), or *in vitro* slice methods, in which cutting the brain slice alone may result in significant changes in gene expression (Taubenfeld *et al.* 2002).

In our next series of experiments, we examined whether a portion of the genes that we found to be regulated by LTP in the dentate gyrus are also regulated in the LA following Pavlovian fear conditioning via qRT-PCR. We reasoned that hippocampal dentate gyrus neurons induced to undergo LTP and LA neurons undergoing emotional learning would likely undergo similar patterns of gene expression changes considering that both of these processes share many pharmacological and biochemical similarities and that both of these brain structures have been documented to be important for learning and memory (Schafe *et al.* 2001; McHugh *et al.* 2007).

Using this approach, we report one of the most comprehensive lists to date of plasticity-associated genes that represent the first wave of transcription following fear conditioning in the LA.

Materials and methods

Subjects

Adult male Sprague-Dawley rats (Harlan Laboratories, Somerville, NY, USA) were housed individually in plastic cages and maintained on a 12 h light/dark cycle. Food and water were provided *ad libitum* throughout the experiment. Animal use procedures were in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Yale University Animal Care and Use Committee.

Electrical stimulation experiments

For LTP stimulation experiments, rats (300–350 g) were anesthetized with urethane (2 i.p. injections at 10 min intervals; total of 1.6 mg/kg) and placed in a stereotaxic frame. The skull was exposed and the rats were implanted with a concentric bipolar stimulating electrode (model #NEX-100 from David Kopf Instruments, Tujunga, CA, USA), into the angular bundle of the perforant path (−7.8 AP, 4 ML, −3.4 DV). One-half hour following implantation of the stimulating electrode, rats were given LTP-inducing high-frequency stimulation (HFS) which consisted of six trains of pulses (400 Hz, 20 ms), delivered at a 10 s interval and repeated six times at an interval of 2 min. This protocol has been widely used in the perforant-dentate pathway, and results in reliable and robust

potentiation of dentate gyrus synapses (Davis *et al.* 2000). Low-frequency stimulation (LFS) controls received pseudotetanic stimulation consisting of six pulses, delivered at a 10 s interval, repeated six times with an interval of 2 min, to match the tetanus without inducing LTP. In all stimulation experiments, current was applied such that it moved from the tip to the tube of the bipolar stimulation electrode. The rats were killed 30 min following HFS or LFS and the brain was dissected and immediately frozen on powdered dry ice and stored at −80°C until further processing.

Dentate gyrus microdissection

Fresh frozen rat brains were mounted posterior side down on a sliding freezing microtome and tissue was removed to ∼ −2.5 Bregma. The coronal plane was brushed with RNA Later (Ambion, Austin, TX, USA) to prevent RNA degradation and to provide rigidity to the tissue slice during dissection. A 480 μm-thick section was taken between ∼ −2.5 to ∼ −3.0 Bregma and placed in a glass dish with RNA Later. A small portion of one blade of the dentate gyrus was selectively microdissected using a 1 mm punch tool. The microdissected samples were immediately placed in ice-cold microfuge tubes, frozen on dry ice, and placed at −80°C until RNA isolation. After the dentate gyrus was removed by dissection, the tissue was immersed in Nissl stain for 5–10 min and then visualized under a bright field microscope, to ensure the dentate gyrus was accurately dissected.

Microarray analysis

The ipsilateral dentate gyrus (side of stimulation) was microdissected from rats that received either HFS or LFS ($n = 4$ each). The total RNA from microdissected samples was isolated using RNA Aqueous Micro (Ambion, Austin, TX, USA). Samples were purified via precipitation using Pellet Paint NF (Novagen). Total RNA was amplified via a single round of amplification according to the manufacturer's recommendations using Amino allyl MessageAmp II (Ambion). All amplification reactions were incubated at 37°C for 14 h. The labeling of the amplified RNA with Cy5/Cy3 and microarray hybridizations were performed at the DUKE microarray facility using dual channel Rat OpArrays containing 27 044 gene transcripts. A total of four microarrays were performed in which one HFS (Cy5) sample and one LFS (Cy3) sample were hybridized to the same microarray slide. Resulting Genepix files (Molecular Devices, Sunnyvale, CA, USA) were imported into Genespring GX 7.3 (Silicon Genetics, Redwood City, CA, USA) for data analysis. Per chip intensity dependent Lowess normalization was performed. Gene lists were created based on the relatively stringent criteria that the gene must be at least 1.9-fold up or down-regulated in 4 of 4 arrays performed. This list resulted in 34 up-regulated genes and no down-regulated genes. All genes in this list exhibited a control or raw average signal value that was well above background and also had a *t*-test *p*-value of $p < 0.05$. Most of the genes were confirmed by a combination of *in situ* hybridization and/or qRT-PCR in separate cohorts of rats and therefore did not require the use of a multiple testing correction. Importantly, the MAQC Consortium has reported that this approach can be successful in identifying reproducible gene lists (Shi *et al.* 2006).

Fear conditioning

All rats were removed from the rat colony in their home cage and transported to our behavioral suite for habituation to handling and transporting each day for a total of 4 days prior to behavioral training. On the day of training, rats were transferred to the behavioral suite in the morning and allowed to remain in their home cages for approximately 3–5 h before training to minimize the effects of transfer from the colony room on basal levels of mRNA. Conditioned 'Paired' rats received three conditioning trials consisting of a 20 s, 5 kHz, 75 dB tone that co-terminated with a 1 s, 0.5 mA foot shock. The intertrial interval (ITI) was, on average, 120 s, and the total training time lasted 9 min. 'Immediate Shock' control rats were placed in the conditioning chamber and immediately given three 0.5 mA foot shocks and removed from the training chamber, a procedure that does not support fear learning (Fanselow 1980). 'Tone Only' rats were placed in the conditioning chamber and exposed to three tones [20 s, 5 kHz, 75 dB] without receiving shocks. 'Box only' rats were placed in the conditioning chamber for 9 min with no further stimulation. 'Naïve' control rats were handled and killed without exposure to further stimulation. For experiments depicted in Fig. 5, rats were also habituated to the training chamber for 15 min each day for 4 days. At the appropriate time point for each experiment, rats were anesthetized with CO_2 and decapitated. Brains were removed and immediately frozen on powered dry ice and stored at −80°C until further processing.

Behavioral testing

Testing for conditioned fear responses (*freezing*) was performed 24 h after behavioral training (LTM test). For the tone test, rats were placed in a distinct environment as compared with the training context. Rats were exposed to three CS tones (5 kHz, 75 dB, 30). Total seconds freezing during the CS presentations was scored for each rat, and this number was expressed as a percentage of the total CS presentation time. For analysis, freezing across each trial was averaged into a single score for each memory test. For context memory testing, rats were placed in the original training context. Total seconds freezing during the context exposure was scored for each rat, and this number was expressed as a percentage of the total context presentation. All data were analyzed with ANOVA and Duncan's *post hoc t*-tests. Differences were considered significant if $p < 0.05$.

LA microdissection

The LA microdissection was performed in a similar fashion as described above with some important modifications. The fresh frozen rat brains were mounted anterior face down on a sliding freezing microtome and tissue was removed to ∼ −3.6 Bregma. A 480 μm-thick section was taken between ∼ −3.6 to ∼ −3.1 Bregma and the LA was selectively microdissected using an X-ACTO knife. The dissection was performed on top of a light box to illuminate the amygdala and surrounding structures allowing for accurate microdissection of the LA.

RNA purification and cDNA synthesis

All tissue samples for qRT-PCR analysis were homogenized in a microfuge tube with 800 μL of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) with a plastic pestle. RNA isolation was performed according to the manufacturer's instructions. To enhance precipitation, 10 μg of Rnase-free glycogen (Invitrogen) and 1 μL of Pellet Paint NF were added. The isolated total RNA was DNase treated in a 100 μL reaction with 6.8 Krunitz Units of DNase I

(Qiagen, Valencia, CA, USA) for 10 min at 25°C followed by total RNA purification using the RNeasy MinElute Kit (Qiagen). RNA purification was performed according to the manufacturer's instructions. RNA quantities were measured using a NanoDrop (NanoDrop Products, Wilmington, DE, USA). Approximately 100 ng of total RNA was converted to cDNA for each sample in a 20 μL reaction using 250 nM of random hexamer primers (Invitrogen), 1 μL reverse transcriptase (Genisphere, Hatfield, PA, USA), and 1 μL SUPERase•In (Ambion). The reactions were incubated for 2 h at 42°C, and then inactivated by the addition of 3.5 μL 50 mM EDTA/5 M NaOH for 10 min at 65°C. The pH was normalized to physiological pH by the addition of 5 μL of 1 M Tris pH 7.4. The samples were precipitated using Pellet Paint NF, and re-suspended in 100 μL Tris–EDTA buffer and stored at −20°C. 1 μL of prepared cDNA was used per 16 μL qRT-PCR reaction.

Quantitative real-time PCR

qRT-PCR was performed using the ΔΔCt method as described previously (Ploski *et al.* 2006) using Quantitect SYBR Green (Qiagen) and a ABI 7900 instrument (Applied Biosystems, Foster City, CA, USA) with either custom designed primers (see Fig S2) at a concentration of 300 nM or QuantiTect PCR primers purchased from Qiagen (see Fig S3), at the concentration recommended by the manufacturer in 16 μL reaction volumes. For qRT-PCR performed with custom primers, the default settings of the ABI 7900 instrument (Applied Biosystems) were used, with the following modifications: cycling parameters, where for 40 cycles were 94°C for 2 s, 60°C for 30 s, 72°C for 30 s. For qRT-PCR performed with QuantiTect PCR primers the default settings of the ABI 7900 instrument were used, with the following modifications as recommended by Qiagen: cycling parameters for 40 cycles were 94°C for 15 s, 56°C for 30 s, 76°C for 30 s with data collection only during the annealing phase. For all qRT-PCR experiments, all samples were run in triplicate and relative gene concentrations were normalized against GAPDH levels. Custom gene specific primers were designed using Primer3 Software and primers used are listed in the Supporting information. Where appropriate, the data were analyzed using analysis of variance (ANOVA) and the Duncan's *post-hoc* test or a two-tailed *t*-test. Differences were considered significant if $p < 0.05$. For the most accurate representation of qRT-PCR experimental data variance, all qRT-PCR data are represented as the average threshold cycle (Ct) difference values for each group after normalization to GAPDH, with the error bars representing the standard error of the mean for each group. Corresponding data tables include the average fold change values which result from the transformation of the raw qRT-PCR data using the equation: $2^{(\text{average Ct difference value})}$ = average fold change.

In situ hybridization

In situ hybridization was performed as previously described (Newton *et al.* 2003; Ploski *et al.* 2008). Briefly, slide mounted cryostat cut frozen sections (15 μm) were hybridized with ^{35}S-radiolabeled antisense probes at 55°C for 14–18 h. Slide mounted sections were then washed, air-dried, and slides were exposed to Biomax MR autoradiographic film (PerkinElmer, Waltham, MA, USA) for 2–14 days. Relative gene expression changes were determined using ImageJ (US National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). Gene expression intensity was normalized against film background for each section, and averages were determined for at least two sections per slide per rat. Data were analyzed using a two-tailed *t*-test. Differences were considered significant if $p < 0.05$.

The DNA templates for transcription of RNA radioactive probes were generated by PCR amplification using gene-specific primers (see Fig S4). The reverse primer includes a T7 template sequence. Rat hippocampal cDNA was used as the template for PCR, which was performed in a MJ-Mini Gradient Thermal Cycler (BioRad, Hercules, CA, USA) using the Quantitect Sybr Green PCR kit (Qiagen). The PCR product was purified by ethanol precipitation and was resuspended in TE buffer. One microgram of the 300 bp PCR product was used to produce radiolabeled probe using a T7-based *in vitro* transcription kit (Megashortscript; Ambion) using [^{35}S]CTP (1.5 μCi). Removal of unincorporated nucleotides after the *in vitro* transcription reaction was performed using sepharose spin columns (Roche, Indianapolis, IN, USA).

Drug

SL327 (Tocris Bioscience, Ellisville, MO, USA) was solubilized in dimethylsulfoxide and injected intraperitoneally at a dose of 40 mg/kg 1 h before behavioral training. Injection volumes were 0.5 mL.

Results

Identification of genes induced during hippocampal LTP

In out first series of experiments, rats were anesthetized and implanted with a bipolar stimulation electrode into the angular bundle of the perforant path. Thirty minutes after implantation, rats were given HFS. Relative to LFS controls, we observed that HFS of the perforant path resulted in robust gene expression in the dentate gyrus. In total, we identified 34 genes that met criteria for inclusion in the data analysis (see Materials and methods) (Table 1). We collectively refer to these genes as 'plasticity-associated genes'. Approximately one-third of these genes are DNA-binding transcription factors, one-third enzymes, and one-third are genes that are involved in cellular signaling or have other miscellaneous functions. Importantly, we not only identified a number of genes that have been previously identified to be induced following LTP induction, such as Fos, Egr1, Jun, Nr4a1, Nr4a2, Arc/Arg3.1, Gadd45b, which underscores the validity of our screen, but we also identified numerous genes that have not been previously indentified to be induced following *in vivo* LTP, including Npas4, Atf3, Per1, Ar4l, Tiparp, Trib1, and many others (Table 1).

Considering that microarray screens are notorious for containing false positives, we placed a premium on secondary confirmation utilizing a combination of qRT-PCR and *in situ* hybridization. We found most of the genes to be significantly regulated in the dentate gyrus following HFS of the perforant path as compared with LFS of the perforant path, as determined by qRT-PCR using gene specific primers (two-tailed *t*-test, $p < 0.05$) (Table 1). Although in general the qRT-PCR data and DNA microarray data reflect similar average fold changes between HFS and LFS samples, there are differences. This is likely due to

Table 1 Identification of genes induced during dentate gyrus LTP

	Microarray			qRT-PCR		
Official gene symbol	Average fold change	SEM	*t*-Test *p*-value	Average fold change	Official full name	GenBank Accession #
Transcription factors						
Egr2	61.7	5.6	2.3E-05	170.5	NM_053633	Early growth response 2
Npas4	28.2	3.9	1.4E-04	76.2	NM_153626	Neuronal PAS domain protein 4
Fos	17.6	1.9	1.0E-04	40.8	NM_022197	FBJ osteosarcoma oncogene
Egr4	12.8	1.2	9.3E-05	20.0	NM_019137	Early growth response 4
Nr4a1	11.0	1.7	4.9E-04	8.0	NM_024388	Nuclear receptor subfamily 4, group A, member 1
Atf3	10.0	4.2	8.2E-03	9.3	NM_012912	Activating transcription factor 3
Egr1	9.9	0.7	2.8E-05	11.9	NM_012551	Early growth response 1
Junb	6.6	1.1	1.1E-03	11.9	NM_021836	Jun B proto-oncogene
Nr4a2	4.3	1.5	1.6E-02	n.d.	NM_019328	Nuclear receptor subfamily 4, group A, member 2
Jun	3.7	0.3	6.2E-05	7.8	NM_021835	Jun oncogene
Per1	3.6	0.8	5.7E-03	3.8	NM_001034125	Period homolog 1 (Drosophila)
Ddit3	3.3	0.4	7.6E-04	3.0	NM_001109986	DNA-damage inducible transcript 3
Structural/trafficking						
Arc	10.1	0.7	2.7E-05	14.9	NM_019361	Activity-regulated cytoskeleton-associated protein
Enzymes						
Dusp5	6.6	1.1	1.2E-03	19.4	NMJ.33578	Dual specificity phosphatase 5
Rffl	5.7	0.9	9.6E-04	n.d.	NM_001004068	Ring finger and FYVE like domain containing protein
Arf4l	5.7	0.3	2.3E-06	9.0	NM_001107052	ADP-ribosylation factor 4-like
Rnf39	5.2	1.3	5.9E-03	6.8	NM_134374	Ring finger protein 39
Rasllla	4.5	0.7	1.7E-03	9.5	NM_001002829	RAS-like family 11 member A
Tiparp	4.2	0.9	4.9E-03	4.5	NM_001107679	TCDD-inducible poly(ADP-ribose) polymerase
Ppplrl5a	3.6	0.3	1.6E-05	3.8	NM_133546	Protein phosphatase 1, regulatory (inhibitor) subunit 15A
Ptgs2	3.2	1.2	4.0E-02	n.d.	NM_017232	Prostaglandin-endoperoxide synthase 2
Sat1	3.2	0.3	3.5E-05	2.6	NM_001007667	Spermidine/spermine Nl-acetyl transferase 1
Mat2a	3.0	0.3	2.5E-04	3.0	NM_134351	Methionine adenosyltransferase II, alpha
Peli1	2.9	0.4	2.4E-03	n.d.	NM_001100565	Pellino 1
Signaling/misc						
Irs4	7.6	1.2	7.7E-04	n.d.	XM_235721	Insulin receptor substrate 4
RGD1564664	6.7	0.3	2.1E-06	14.8	NM_001110055	Similar to LOC387763 protein
Btg2	6.4	0.9	6.9E-04	7.9	NM_017259	B-cell translocation gene 2, anti-proliferative
Gadd45b	5.6	1.8	8.6E-03	10.4	NM_001008321	Growth arrest and DNA-damage-inducible, beta
Ier2	5.4	1.3	4.3E-03	7.6	NM_001009541	Immediate early response 2
Gadd45g	4.6	1.3	9.0E-03	6.5	NM_001077640	Growth arrest and DNA-damage-inducible, gamma
Trib1	3.4	1.2	3.0E-02	11.2	NM_023985	Tribbles homolog 1 (Drosophila)
Ier5	2.4	0.2	1.4E-04	n.d.	NM_001025137	Immediate early response 5
Trim35	2.4	0.3	8.1E-04	n.d.	NM_001025142	Tripartite motif-containing 35
Faml20b	2.2	0.1	2.5E-04	n.d.	NM_001107466	Family with sequence similarity 120B

Urethane anesthetized rats were implanted with a bipolar stimulation electrode into the perforant path. Thirty minutes after implantation, rats were given high frequency stimulation (HFS), which results in potentiation of dentate gyrus synapses, or low frequency stimulation (LFS) to match the tetanus without inducing LTP. Thirty minutes following stimulation, rats were killed and the dentate gyrus was microdissected from 480 μm coronal sections. RNA was purified, amplified and hybridized to Rat Operon oligo microarrays ($n = 4$), representing ~ 27 000 transcripts. Identified genes up-regulated upon HFS versus LFS are listed by official gene symbol, into categories: transcription factors, structural/trafficking, enzymes, and signaling/miscellaneous. The average fold change, standard error of the mean (SEM), and *t*-test *p*-value for the microarray results are listed next to each gene (see Materials and methods for inclusion criteria). The average fold change for each gene as determined by qRT-PCR secondary confirmation is listed to the right and was found to be significant ($p < 0.05$), data not shown (see Fig S1). n.d = not determined. Official full name for each gene is listed. See Fig S1 for additional information.

the fact that the RNA samples for the microarray were amplified (see Materials and methods) which can cause minor distortions in RNA levels and thus explain why the qRT-PCR results are not identical. In addition, different samples were utilized for these different methodologies which can also introduce variability.

In addition to qRT-PCR, we performed *in situ* hybridization for 14 randomly chosen plasticity-associated genes on coronal rat brain slices 30 min following HFS of the perforant path to emphasize the convincing change in gene expression (Fig. 1). In all cases, we observed an unambiguous induction of gene expression within the dentate gyrus on the side of the brain that received HFS (ipsilateral). In some experiments, we compared expression of a select number of genes following HFS to that of LFS (Fig. 1a–c). All genes showed statistically significant gene expression differences between the HFS and LFS groups as measured by the ratio of gene expression between ipsilateral dentate gyrus compared with the contralateral dentate gyrus of the HFS brains versus the ratio of ipsilateral dentate gyrus compared with the contralateral dentate gyrus of the LFS brains. Error bars represent SEM, $n = 3$, $p < 0.05$. Notably, rats that received LFS did not exhibit gene expression differences between the ipsilateral or contralateral sides (Fig. 1b). In other experiments, rats received HFS of the perforant path followed by gene expression analysis of the side of the brain ipsilateral to the stimulation to that on the contralateral side (Fig. 1d–f). As before, we observed an unambiguous induction of gene expression within the dentate gyrus on the side of the brain that received HFS (ipsilateral) relative to the contralateral side of the brain. Error bars represent SEM, $n = 3$, $p < 0.05$.

Identification of genes induced in the LA following fear conditioning

In our next series of experiments, we performed extensive gene expression profiling for the plasticity-associated genes identified in our first experiments on LA tissue following auditory fear conditioning. Although it is not feasible to test every plasticity-associated gene in every experiment, we have attempted to test a representative set of the most

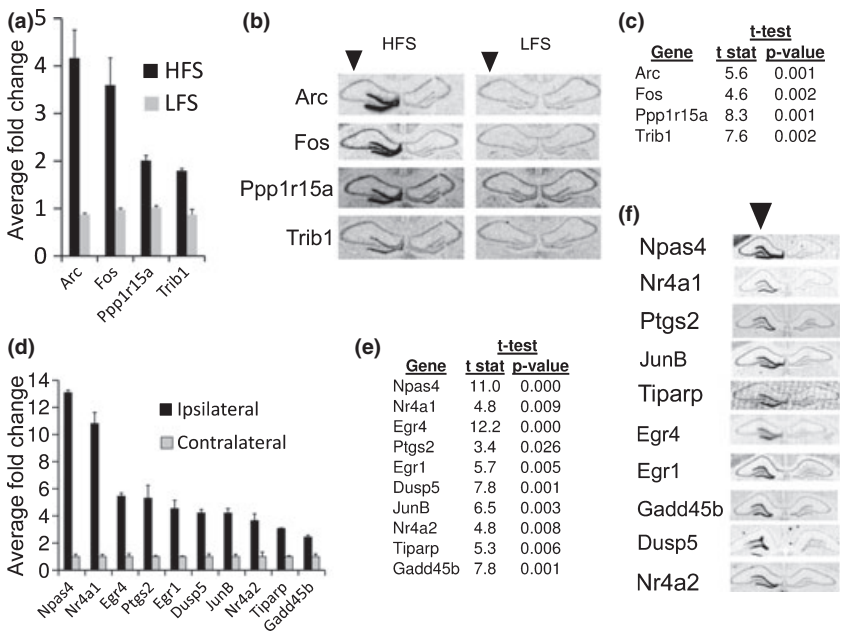
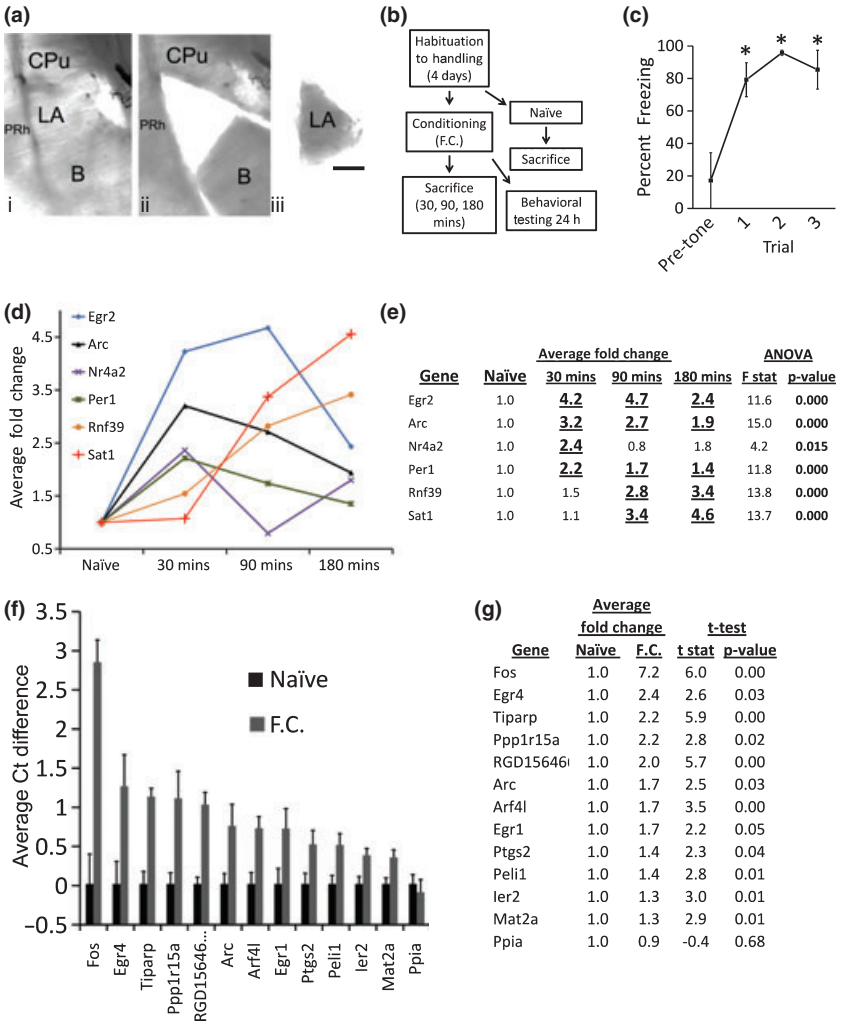


Fig. 1 Analysis of genes induced during dentate gyrus LTP via *in situ* hybridization. Urethane anesthetized rats were implanted with a bipolar stimulation electrode into the perforant path. Thirty minutes after implantation, rats were given HFS ($n = 3$) or LFS ($n = 3$). Thirty minutes following stimulation, rats were killed and cryostat-cut coronal thin sections containing the dentate gyrus were probed with radioactive oligonucleotide gene-specific probes. Probes were allowed to hybridize for 14–18 h and were exposed to film for 1–2 weeks. Gene expression in the dentate gyrus was quantified using Image J software. (a) Bar graph represents gene expression differences as determined by *in situ* hybridization for four genes (Arc, Fos, Ppp1r15a, Trib1), comparing the gene expression between the ratio of ipsilateral dentate gyrus compared to the contralateral dentate gyrus of the HFS brains versus the ratio of ipsilateral dentate gyrus compared with the contralateral dentate gyrus of the LFS brains. Error bars represent SEM. (b) Representative *in situ* hybridization images of the HFS induced gene induction (left panel) versus LFS induced gene induction (right panel) for selected genes represented in (a). Arrow designates the ipsilateral side. Unambiguous gene expression induction appears on the HFS, ipsilateral side. (c) All genes exhibited statistically significant gene expression differences between the HFS and LFS groups. $n = 3$, $p < 0.05$. (d) Bar graph depicts gene expression differences as determined by *in situ* hybridization between ipsilateral dentate gyrus versus the contralateral dentate gyrus for 10 additional genes. (e) All genes represented in (d) exhibited statistically significant gene expression differences between the ipsilateral and contralateral sides, error bars represent SEM, $n = 3$, $p < 0.05$. (f) Representative *in situ* hybridization images of HFS induced gene induction in the ipsilateral dentate gyrus versus the contralateral dentate gyrus for the 10 genes represented in (d). Arrow designates the ipsilateral side. Unambiguous induction of gene expression appears within the ipsilateral dentate gyrus.

robustly regulated genes. To maximize the chance for success, we ensured that all LA tissue samples were meticulously microdissected to limit gene dilution/negation effects (Fig. 2a and Methods). Further, the highly sensitive technique of qRT-PCR was utilized to examine gene expression changes in our samples.

Time course of gene expression changes in the LA following fear learning

In our first set of fear conditioning experiments, we sought to determine the time course of gene expression induction for a subset of plasticity-associated genes. Following conditioning, rats were removed from the conditioning chamber,



Gene	Naïve	Average fold change 30 mins	90 mins	180 mins	ANOVA F stat	p-value
Egr2	1.0	**4.2**	**4.7**	**2.4**	11.6	**0.000**
Arc	1.0	**3.2**	**2.7**	**1.9**	15.0	**0.000**
Nr4a2	1.0	**2.4**	0.8	1.8	4.2	**0.015**
Per1	1.0	**2.2**	**1.7**	**1.4**	11.8	**0.000**
Rnf39	1.0	1.5	**2.8**	**3.4**	13.8	**0.000**
Sat1	1.0	1.1	**3.4**	**4.6**	13.7	**0.000**

Gene	Average fold change Naïve	F.C.	t-test t stat	p-value
Fos	1.0	7.2	6.0	0.00
Egr4	1.0	2.4	2.6	0.03
Tiparp	1.0	2.2	5.9	0.00
Ppp1r15a	1.0	2.2	2.8	0.02
RGD15646	1.0	2.0	5.7	0.00
Arc	1.0	1.7	2.5	0.03
Arf4l	1.0	1.7	3.5	0.00
Egr1	1.0	1.7	2.2	0.05
Ptgs2	1.0	1.4	2.3	0.04
Peli1	1.0	1.4	2.8	0.01
Ier2	1.0	1.3	3.0	0.01
Mat2a	1.0	1.3	2.9	0.01
Ppia	1.0	0.9	-0.4	0.68

Fig. 2 Gene expression induced within the LA following Pavlovian fear conditioning (a) Depiction of microdissected LA from a 480 μm coronal section. i. tissue slice before dissection, ii. tissue slice after dissection, iii. LA tissue dissected, scale bar = 500 μm. B = Basal nucleus of the amygdala, PRh = perirhinal cortex, CPu = Caudate/Putamen. (b) Schematic of the behavioral protocol. Rats were habituated to handling for 4 days, fear conditioned (FC) with three tone-shock pairings and were killed either 30, 90, or 180 min following conditioning. A naïve group was included that was not conditioned. An additional group of rats was conditioned and tested for long term fear memory 24 h later. (c) Rats exhibit little freezing behavior before the presentation of the tone, but exhibit significant freezing during each tone presentation as compared to pre-tone freezing ($n = 4$; $p < 0.05$; Duncan's test). Error bars represent SEM. (d) Time course analysis of mRNA expression in the LA, 30, 90, or 180 min following fear conditioning utilizing qRT-PCR. All six plasticity-associated genes exhibited significant increases in gene expression relative to the naïve group following conditioning as determined by ANOVA ($n = 8$). Error bars omitted for clarity. (e) Data table for experiments depicted in (d), where the time points that are represented in bold/underline are significant ($p < 0.05$; Duncan's test). The ANOVA p-value is given for each gene. (f) qRT-PCR average Ct differences for an additional 12 plasticity-associated genes regulated by F.C. relative to a naïve group (naïve group normalized to zero). The housekeeping gene Ppia, which is not a plasticity-associated gene, is not regulated. Error bars represent SEM. (g) Associated data table for experiment depicted in (f) with average fold changes between fear conditioning and naïve groups. All 12 plasticity-associated genes exhibited significant gene expression changes following fear conditioning as compared to a naïve control group ($n = 8$; $p < 0.05$; two tailed t-test).

returned to their home cages and killed at either 30, 90, or 180 min following conditioning (Fig. 2b). A naïve group was also included that was not conditioned. An additional group of rats was conditioned and tested for long-term fear memory 24 h later. Notably, rats in this group exhibited little freezing behavior before the presentation of the tone, but exhibited significant freezing during each of the tone presentations as compared with pre-tone freezing ($n = 4$; $p < 0.05$; Duncan's test) (Fig. 2c). Rats do not freeze to the tone if they had not been previously conditioned (data not shown). Gene expression analysis via qRT-PCR revealed a robust increase in LA gene expression 30 min following fear conditioning for most of the genes tested. Specifically, Egr2, Arc/Arg3.1, Nr4a2, and Per1, exhibited a significant increase in gene expression at 30 min which typically slowly tapered off by 180 min ($n = 8$; $p < 0.05$; Duncan's test). Interestingly, Rnf39 and Sat1 exhibited a slow rate of gene expression induction that peaked at 180 min post fear conditioning ($n = 8$; $p < 0.05$; Duncan's test) (Fig. 2d and e).

Considering that most of the genes we tested in Fig. 2d were observed to exhibit significant induction of gene expression 30 min following conditioning, we went on to examine whether fear conditioning promoted significant increases in 12 additional genes in the LA at this time point (Fig. 2f and g), including Fos, Egr4, Tiparp, Ppp1r15a, RGD1564664, Arf4I, Egr1, Ptgs2, Peli1, Ier2, Mat2a, and the housekeeping gene Ppia. Relative to naïve controls, all genes examined, with the exception of Ppia, exhibited a significant increase in expression (Fig. 2f and g).

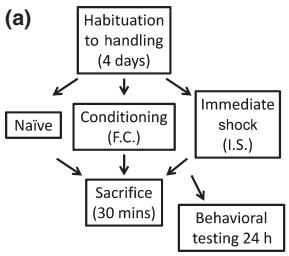
Training-specific regulation of gene expression changes in the LA following fear learning

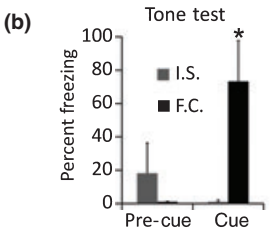
In our next series of experiments, we asked whether the observed training-induced changes in identified genes were specific to tone-shock pairing. Rats were habituated to handling for 4 days, followed by fear conditioning with three tone-shock pairings. As an additional control, a separate group of rats were presented with three immediate foot shocks. These 'immediate shock' control rats receive the same number and intensity of foot shocks as do rats in the traditional conditioning paradigm, but the shocks are given immediately when the rat is placed in the conditioning chamber. Importantly, this type of protocol does not support associative learning (Fanselow 1980), and is therefore useful for determining whether a gene is regulated by associative learning or by exposure to shock alone. A third group was not trained and served as a naïve control. A subset of these rats were killed 30 min post-conditioning for gene expression analysis and a subset were used for behavioral analysis for long-term fear memory tested 24 h later (Fig. 3a). Notably, rats that received the immediate shock treatment exhibited little to no freezing upon presentation of the auditory cue as compared with fear conditioned rats ($n = 4$; $p < 0.05$; Duncan's test) (Fig. 3b). Further, rats that received the immediate shock treatment exhibited significantly less freezing to the training context as compared with the rats that were fear conditioned ($n = 4$; $p < 0.05$; two-tailed t-test) (Fig. 3c), indicating that the rats that received the immediate shocks did not learn to associate the context with the foot shock.

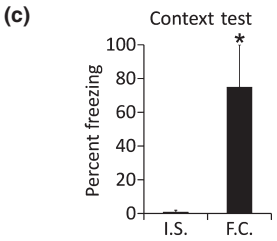
Gene expression analysis via qRT-PCR comparing fear conditioned, immediate shock or naïve rats revealed a robust increase in LA gene expression 30 min following fear conditioning for 16 plasticity-associated genes (Fig. 3d and e). The ANOVA revealed a significant effect for group ($n = 8$, $p < 0.05$) for all 16 plasticity-associated genes tested, with the fear conditioned group being significantly different from the naïve controls ($p < 0.05$; Duncan's test). The expression of at least seven plasticity-associated genes in fear conditioned groups was also observed to be significantly different from immediate shock controls ($p < 0.05$; Duncan's test), including Fos, Atf3, Egr2, Arc/Arg3.1, Gadd45g, Nr4a2, and JunB (Fig. 3d and e). Another three genes showed a clear trend toward significance ($p < 0.10$), including Nr4a1, Btg2, and Egr4. These findings are consistent with the hypothesis that Pavlovian fear conditioning regulates many of these genes, and the degree of regulation is not simply a result of exposure to foot shock alone.

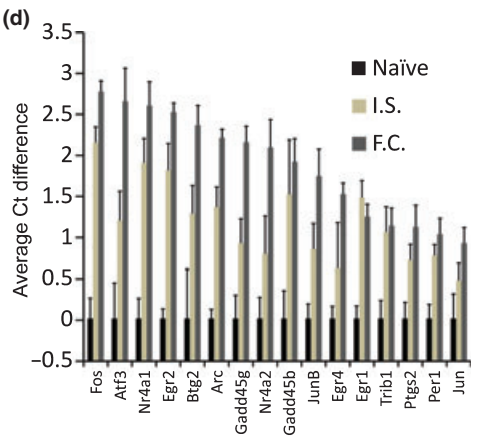
In our initial experiments, we compared gene expression in rats that received paired presentations of tone and shock to that in those receiving either immediate shock or to no stimulation. In this next experiment, we asked whether exposure to tone alone might regulate gene expression in the LA. Further, given that 'paired' rats in our initial experiments were not pre-exposed to the conditioning chambers prior to fear learning, the possibility remains that many of the genes that we observed to be regulated in the LA following fear learning may instead have been driven by the novelty of the conditioning chamber. Indeed, in a separate experiment (Fig S5), we found several genes to be regulated following exposure to the novel conditioning chamber alone. In that experiment, rats were either habituated to handling for 4 days prior to fear conditioning or habituated to both handling and to the conditioning chamber for 4 days prior to fear conditioning. Relative to a group of naïve rats, rats exposed to the novel conditioning chamber exhibited significant increases in the expression of Fos, Egr2, and Arc, an effect which was reduced, but not completely eliminated, by pre-exposure to the chamber. Importantly, paired rats exhibited significantly higher expression of all genes relative to box controls whether they were pre-exposed to the conditioning chamber or not (Fig. S5).

To address the remaining question of whether tone stimulation alone might regulate gene expression in the LA, rats in the present experiments were habituated to handling and to the training chamber for 4 days prior to conditioning. On the training day, one group of rats was conditioned with three tone-shock pairings, one group was presented









(e)

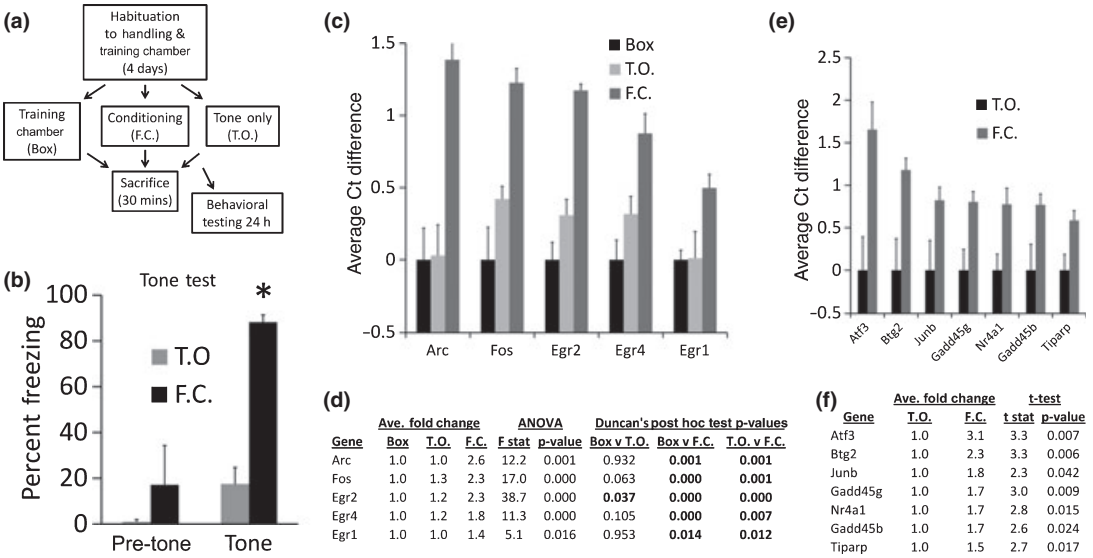
	Ave. fold change			ANOVA		Duncan's post hoc test p-value		
Gene	Naïve	I.S.	F.C.	F stat	p-value	N. vs I.S.	N. vs F.C.	I.S. vs F.C.
Fos	1.0	4.5	6.9	57.0	0.000	**0.00**	**0.00**	**0.04**
Atf3	1.0	2.3	6.3	10.8	0.001	**0.04**	**0.00**	**0.02**
Nr4a1	1.0	3.8	6.1	23.7	0.000	**0.00**	**0.00**	0.09
Egr2	1.0	3.5	5.8	38.5	0.000	**0.00**	**0.00**	**0.03**
Btg2	1.0	2.4	5.2	7.9	0.003	**0.04**	**0.00**	0.09
Arc	1.0	2.6	4.6	39.9	0.000	**0.00**	**0.00**	**0.00**
Gadd45g	1.0	1.9	4.5	18.2	0.000	**0.02**	**0.00**	**0.00**
Nr4a2	1.0	1.7	4.3	7.7	0.004	0.15	**0.00**	**0.02**
Gadd45b	1.0	2.9	3.8	6.0	0.010	**0.02**	**0.01**	0.52
JunB	1.0	1.8	3.4	10.3	0.001	**0.04**	**0.00**	**0.04**
Egr4	1.0	1.5	2.9	5.1	0.025	0.22	**0.01**	0.09
Egr1	1.0	2.8	2.4	19.7	0.000	**0.00**	**0.00**	0.37
Trib1	1.0	2.1	2.2	6.9	0.005	**0.01**	**0.01**	0.83
Ptgs2	1.0	1.7	2.2	6.9	0.012	**0.05**	**0.01**	0.23
Per1	1.0	1.7	2.1	10.4	0.001	**0.00**	**0.00**	0.29
Jun	1.0	1.4	1.9	3.6	0.050	0.18	**0.02**	0.21

Fig. 3 Analysis of gene expression induced within the LA following Pavlovian fear conditioning and foot shock. (a) Schematic of the behavioral protocol. Rats were habituated to handling for 4 days followed by fear conditioning or three immediate foot shocks (I.S.) and were killed 30 min later. All groups were compared against a separate group of naïve rats. Additional groups of rats that received either I.S. or F.C. were tested for long term fear memory 24hrs later. (b) Rats that received I.S. exhibit significantly less freezing upon presentation of the auditory cue as compared to the F.C. group ($n = 4$; $^*p < 0.05$; Duncan's test). Error bars represent SEM. (c) Rats that receive I.S. freeze significantly less to the training context as compared to the rats that were fear conditioned ($n = 4$; $^*p < 0.05$; two-tailed *t*-test). Error bars represent SEM. (d) Gene expression analysis via qRT-PCR comparing F.C. ($n = 8$), I.S. ($n = 8$) or naïve rats ($n = 8$) revealed a robust increase in LA gene expression 30 min following fear conditioning for 16 plasticity-associated genes. Graph of qRT-PCR average Ct differences between F.C., I.S. and Naïve groups (naïve group normalized to zero). Error bars represent SEM. (e) Associated data table for experiment depicted in (d) with average fold changes between F.C., I.S. and naïve groups. All genes tested exhibited statistically significant changes as revealed by ANOVA ($n = 8$, $p < 0.05$). Gene expression in the F.C. group was significantly different from the naïve group for all 16 genes tested ($p < 0.05$; Duncan's test). Numerous plasticity-associated genes also were significantly different between the I.S. and F.C. groups ($p < 0.05$; Duncan's test).

with three tones ('Tone only'; T.O.). A third group was simply placed into the training chamber, but received no stimulation ('Box'). A subset of these rats was killed 30 min post-training, and gene expression analysis was performed on LA tissue. A second subset was used for behavioral testing for long-term fear memory 24 h later (Fig. 4a). Notably, the 'tone only' rats were not observed to freeze to the presentation of the tone when tested for LTM. In contrast, rats that were conditioned with three tone-shock pairings exhibited a robust freezing response during tone presentation on the testing day ($n = 4$; $p < 0.05$; Duncan's test) (Fig. 4b).

Gene expression analysis via qRT-PCR comparing fear conditioned, tone only or box-exposed rats revealed a robust increase in LA gene expression 30 min following fear conditioning for five plasticity-associated genes (Fig. 4c and d). The ANOVA revealed a significant effect for group ($n = 8$, $p < 0.05$) for all five plasticity-associated genes tested, with the fear conditioned group exhibiting significantly more gene expression than the tone only or box control groups ($p < 0.05$; Duncan's test) (Fig. 4c and d). An additional analysis of seven genes showed significant increases in gene expression in fear conditioned rats relative to those receiving tone alone [Fig. 4e and f ($n = 8$, $p < 0.05$)].

Collectively, our findings are consistent with the hypothesis that Pavlovian fear conditioning, rather than exposure to tone alone, immediate shock alone, or to the conditioning chamber, regulates many of these plasticity-associated genes.



(d)

Gene	Ave. fold change Box	T.O.	F.C.	ANOVA F stat	p-value	Duncan's post hoc test p-values Box v T.O.	Box v F.C.	T.O. v F.C.
Arc	1.0	1.0	2.6	12.2	0.001	0.932	**0.001**	**0.001**
Fos	1.0	1.3	2.3	17.0	0.000	0.063	**0.000**	**0.001**
Egr2	1.0	1.2	2.3	38.7	0.000	**0.037**	**0.000**	**0.000**
Egr4	1.0	1.2	1.8	11.3	0.000	0.105	**0.000**	**0.007**
Egr1	1.0	1.0	1.4	5.1	0.016	0.953	**0.014**	**0.012**

(f)

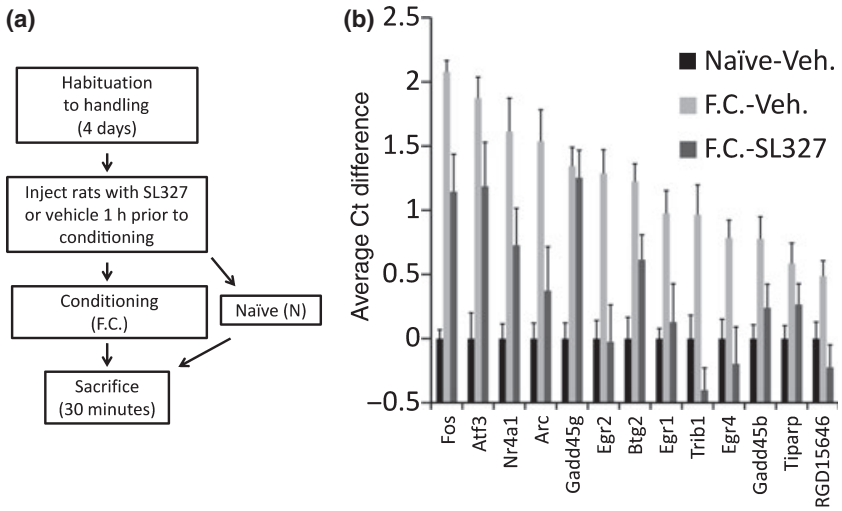
Gene	Ave. fold change T.O.	F.C.	t-test t stat	p-value
Atf3	1.0	3.1	3.3	0.007
Btg2	1.0	2.3	3.3	0.006
Junb	1.0	1.8	2.3	0.042
Gadd45g	1.0	1.7	3.0	0.009
Nr4a1	1.0	1.7	2.8	0.015
Gadd45b	1.0	1.7	2.6	0.024
Tiparp	1.0	1.5	2.7	0.017

Fig. 4 Analysis of gene expression induced within the LA following Pavlovian fear conditioning and tone presentation. (a) Schematic of the behavioral protocol. Rats were habituated to handling and to the training chamber, 'Box' for 4 days. On the training day, one group of rats received F.C. ($n = 8$), one group was presented with three tones (Tone Only, 'T.O.'; $n = 8$) and one group was simply placed into the training chamber ('Box'; $n = 8$). A subset of these rats was killed 30 min post-training and subset was used for behavioral testing 24 h later. (b) T.O. rats do not freeze significantly before or after the presentation of the tone when tested for LTM. In contrast, rats that received F.C. exhibit a robust freezing response during tone presentation, but not before ($n = 4$; $^*p < 0.05$; Duncan's test). (c) Gene expression analysis via qRT-PCR comparing fear conditioned, Tone only and Box only rats revealed a robust increase in LA gene expression 30 min following fear conditioning for 5 plasticity-associated genes tested. Graph of qRT-PCR average Ct differences between Box, T.O. and F.C. groups (naïve group normalized to zero). Error bars represent SEM. (d) Associated data table for experiment depicted in (c) with average fold changes among Box, T.O. and F.C. groups. ANOVA revealed a significant effect for group ($n = 8$, $p < 0.05$) for all five plasticity-associated genes tested, with the conditioned group exhibiting significantly more gene expression than the Tone only or Box only control groups ($p < 0.05$; Duncan's test). (e) Graph of qRT-PCR average Ct differences between T.O. and F.C. groups for an additional set of genes (T.O. group normalized to zero). Error bars represent SEM. (f) Associated data table for experiment depicted in (e) with average fold changes between T.O. and F.C. groups. All seven plasticity-associated genes exhibited significant gene expression changes following fear conditioning as compared to a T.O. control group ($n = 8$; $p < 0.05$; two tailed *t*-test).

ERK/MAPK regulation of conditioning-induced gene expression in the LA

Previous reports have implicated the extracellular-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling cascade in the consolidation of fear memories within the LA (Schafe *et al.* 2000, 2005). The requirement for the ERK/MAPK pathway for plasticity and memory consolidation is due at least in part to its role in providing a functional link between the post-synaptic membrane and nuclear gene expression changes mediated by CREB, which are thought to be critical for inducing the structural and functional changes that underlie LTP and LTM (Impey *et al.* 1998; Dolmetsch *et al.* 2001). Considering the ERK/MAPK pathway is essential for CREB mediated gene expression in neurons and both ERK/MAPK and CREB are essential in the LA for fear memory consolidation (Josselyn *et al.* 2001; Han *et al.* 2007, 2009), we examined whether the plasticity-associated genes we identified to be regulated by fear conditioning within the LA are downstream of ERK/MAPK signaling. Rats were habituated to handling for 4 days. On the day of training, rats received an intraperitoneal injection of either vehicle or the ERK/MAPK inhibitor SL327 at a dose that has previously shown to inhibit ERK/MAPK activation and long-term fear memory formation (Atkins *et al.* 1998). One hour after injection, rats were conditioned with three tone-shock pairings followed by killing 30 min later. A third group of rats received intraperitoneal injection of vehicle and were killed 1.5 h later to serve as a naïve control group (Fig. 5a).

Gene expression analysis via qRT-PCR for 13 plasticity-associated genes was performed on LA tissue comparing fear conditioned-SL327 rats, fear conditioned-vehicle rats, and naïve-vehicle rats (Fig. 5b and c). The ANOVA revealed a significant effect for group ($n = 8$, $p < 0.05$) for all 13 plasticity-associated genes tested, with the SL327-fear conditioned rats being significantly different from both the vehicle-fear conditioned rats and vehicle-naïve rats ($p < 0.05$; Duncan's test). Ten plasticity-associated genes



(c)

	Average fold change			ANOVA		Duncan's post hoc test p-values		
	Naïve	Fear conditioned				Naïve-Veh v.	Naïve-Veh v.	F.C.-Veh v.
Gene	Vehicle	Vehicle	SL327	F stat	p-value	F.C.-Veh	F.C.-SL327	F.C.-SL327
Fos	1.0	4.2	2.2	33.2	0.000	**0.000**	**0.000**	**0.002**
Atf3	1.0	3.7	2.6	15.2	0.000	**0.000**	**0.003**	0.065
Nr4a1	1.0	3.1	1.7	12.0	0.000	**0.000**	**0.039**	**0.014**
Arc	1.0	2.9	1.3	10.0	0.001	**0.001**	0.310	**0.004**
Gadd45g	1.0	2.5	2.4	20.6	0.000	**0.000**	**0.000**	0.704
Egr2	1.0	2.4	1.0	12.3	0.000	**0.000**	0.933	**0.000**
Btg2	1.0	2.3	1.5	13.3	0.000	**0.000**	**0.017**	**0.018**
Egr1	1.0	2.0	1.1	6.6	0.006	**0.004**	0.663	**0.009**
Trib	1.0	1.9	0.8	12.6	0.000	**0.003**	0.167	**0.000**
Egr4	1.0	1.7	0.9	6.5	0.006	**0.013**	0.503	**0.004**
Gadd45b	1.0	1.7	1.2	6.2	0.007	**0.003**	0.299	**0.027**
Tiparp	1.0	1.5	1.2	4.2	0.029	**0.013**	0.211	0.138
RGD15646	1.0	1.4	0.9	6.6	0.006	**0.025**	0.275	**0.003**

Fig. 5 Analysis of ERK/MAPK regulation of conditioning induced LA gene expression. (a) Schematic of the behavioral protocol. Rats were habituated to handling for 4 days. On the day of training one group of rats received an intraperitoneal (i.p.) injection of SL327. Another group of rats received an i.p. injection of the vehicle. One hour after the injections, the rats received F.C. and were killed 30 min later. A third 'naïve' group of rats received i.p. injections of the vehicle and were killed 1.5 h after the injections. (b) Gene expression analysis via qRT-PCR for 13 plasticity-associated genes were performed on LA tissue comparing the F.C.-SL327 group, F.C.-vehicle group, and the naïve-vehicle group. Graph of qRT-PCR average Ct differences among F.C.-SL327 group, F.C.-vehicle group, and naïve-vehicle group (naïve-vehicle group normalized to zero), Error bars represent SEM. (c) Associated data table for experiment depicted in (b) with average fold changes of the F.C.-SL327 and F.C.-vehicle groups compared to the naïve-vehicle group. The ANOVA revealed a significant effect for group ($n = 8$, $p < 0.05$) for all 13 plasticity associated genes tested, with the fear conditioned-SL327 group being significantly different from the naïve-vehicle group ($p < 0.05$; Duncan's test). 10 plasticity-associated genes tested exhibited significant differences between the fear conditioned-SL327 group and the fear conditioned-vehicle group ($p < 0.05$; Duncan's test). Notably Gadd45g exhibited no reduction in gene expression with SL327 administration. Atf3 and Trib1 exhibited a non-significant trend toward reduction of gene expression with SL327 administration.

exhibited significant differences between the fear conditioned-SL327 group and the fear conditioned-vehicle group ($p < 0.05$; Duncan's test). Notably, Gadd45g exhibited no reduction in gene expression with SL327 administration. Atf3 and Trib1 exhibited a non-significant trend toward reduction of gene expression with SL327 administration. These findings are consistent with the hypothesis that Pavlovian fear conditioning regulates many of these genes in an ERK/MAPK-dependent fashion.

Discussion

Most recent studies aimed at defining the cellular and molecular mechanisms of Pavlovian fear conditioning have focused on protein kinase signaling pathways (Schafe and LeDoux 2000; Schafe *et al.* 2001) and the transcription factor CREB (Josselyn *et al.* 2001; Malkani *et al.* 2004; Han *et al.* 2007, 2009) that promote fear memory consolidation in the LA. Despite this progress, relatively little remains known

about the downstream genes required for long-term fear memory formation. The experiments in the present study were designed to identify a set of genes that are induced during the first wave of transcription following Pavlovian fear conditioning within the LA.

In the present study, our goal was to identify a list of genes whose expression could be reliably reproduced within brain circuits relevant to learning and memory. In our experiments, we chose to first use microarray techniques to identify genes regulated by LTP in the dentate gyrus, an experimental model of synaptic plasticity that is highly robust and reproducible, that exhibits a high signal-to-noise ratio, and that shares many biochemical features in common with fear learning in the LA. While a number of other studies have examined plasticity-induced gene expression within the hippocampus (Nedivi *et al.* 1993; Hevroni *et al.* 1998; Matsuo *et al.* 2000; Park *et al.* 2006; Havik *et al.* 2007; Coba *et al.* 2008), there have been no published reports of a genome wide microarray analysis of dentate gyrus following *in vivo* LTP induction in the anesthetized rat. By utilizing hippocampal LTP induction as a tool, we were able to identify 34 plasticity-associated genes that were robustly expressed following HFS of the perforant path. Notably, 24 of these plasticity-associated genes were also found to be induced within the LA following Pavlovian fear conditioning. These data are encouraging since we not only identified a number of genes previously implicated in learning and memory, including Fos (Yasoshima *et al.* 2006), Egr1 (Jones *et al.* 2001; Malkani *et al.* 2004), Per1 (Sakai *et al.* 2004), Arc/Arg3.1 (Plath *et al.* 2006; Ploski *et al.* 2008), Nr4a2 (Colon-Cesario *et al.* 2006), and Ptgs2 (Cowley *et al.* 2008), but we also identified many others that have not been previously shown to be regulated by learning and memory. Further, only 10 of the 34 plasticity-associated genes that we identified have been previously been found to be up-regulated within the amygdala following fear conditioning (Ressler *et al.* 2002; Keeley *et al.* 2006) (see Fig. S6).

Many of the genes that we examined in the LA appeared to be regulated in a training-specific manner, exhibiting enhanced expression in paired rats relative to those exposed to either shock alone, tone alone, or the training context alone. Nonetheless, while all of the genes we examined were found to be increased in paired animals relative to those receiving tone alone, not all were found to be increased relative to immediate shock controls. This pattern of findings may indicate that there are two populations of genes in our sample – those that are associatively regulated and therefore correlated with fear learning, and those that are not correlated with learning *per se*. In practice, however, this interpretation is not as straightforward as it would appear. One important caveat of the use of an immediate shock control, for example, is that it assumes (possibly incorrectly) that the expression of a given gene does not reach a maximum induction or 'ceiling effect'. For example, if differences are not detected between the immediate shock group and the fear conditioned groups, it may be because the gene is maximally transcriptionally activated and further 'learning' induced gene expression is not possible. If this is the case, then little can be interpreted by the lack of difference between these two groups. Further, it is likely the case that the 'ceiling' for a particular gene is sensitive to factors such as the strength of the training protocol, suggesting that the use of a uniform training protocol may not be sufficient to tease apart differences in learning versus shock-induced gene expression for a large sample of genes. In our own qRT-PCR experiments, for example, we found no detectable difference in the expression of Egr1 between immediate shock controls and animals that had received fear conditioning. This negative finding stands in contrast to previous reports that have shown that Egr1 expression in the LA is necessary for fear learning and/or memory consolidation (Malkani and Rosen 2000; S. A. Maddox, M. S. Monsey, J. E. Ploski and G. E. Schafe, unpublished observations). Collectively, this pattern of findings suggests that while the approach of attempting to correlate changes in gene expression with behavioral outcomes is important, it can never be sufficient. Ultimately, specific gene knockdown or manipulation of each of these genes in the LA will be required to unambiguously determine their individual roles in fear learning and memory.

Previous studies have shown that fear conditioning in the LA requires calcium entry via NMDAR and L-type voltage-gated calcium channels (Miserendino *et al.* 1990; Rodrigues *et al.* 2001; Bauer *et al.* 2002), ERK activation (Schafe *et al.* 2000), and the transcription factor CREB (Josselyn *et al.* 2001; Han *et al.* 2007, 2009). In the present study, we also showed that a large subset of the plasticity-associated genes regulated by fear conditioning in the LA are ERK-dependent. Given this pattern of findings, it is reasonable to speculate that the genes found to be regulated by fear learning in the LA in the present study are likely be downstream of CREB activation and regulated by neuronal activity dependent increases in intracellular calcium. To examine this hypothesis, we analyzed two publically available gene expression datasets (GSE62540 and GSE11256) designed to investigate neural activity and calcium mediated increases in gene expression in rat or mouse cortical neurons grown in culture (Xiang *et al.* 2007; Lin *et al.* 2008). Notably, 25 of the 34 plasticity-associated genes we identified in this study were found to be neuronal activity-dependent, where, in most cases, gene expression was dependent on NMDAR and L-type voltage-gated calcium channels (Xiang *et al.* 2007). Further, 22 of the 34 plasticity-associated genes we identified have been previously shown to contain a full cAMP response element (CRE) within their promoters, and/or have been shown to bind CREB directly (Impey *et al.* 2004; Zhang *et al.* 2005). In addition to these 22 genes, 10 more genes contain an un-conserved half-site CRE within their promoter genes (Zhang *et al.* 2005). Further research is required to determine if these are CREB-regulated genes (see Fig. S6).

Collectively these data support the hypothesis that the majority of the plasticity-associated genes we identified are calcium, ERK/MAPK and CREB-dependent.

Interestingly, previous studies have suggested that some of these genes may have paradoxical effects on learning when they are deleted. For example, mice engineered to have a conditional forebrain specific Egr2 deletion do not show learning deficits and in some cases exhibit memory enhancements (Poirier *et al.* 2007). Importantly, caution should be used when assigning a particular role to a gene, such as enhancing or perturbing memory, since biological adaptations can occur to compensate for the deletion of a particular gene. In addition, some of these genes likely modulate homeostatic plasticity mechanisms that contribute to the proper functioning of the neuron by maintaining precise control of cellular excitability (Turrigiano 2008). For example, the protein Arc/Arg3.1 functions to endocytose α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors, which at first glance would appear to be a counterintuitive mechanism for memory formation (Chowdhury *et al.* 2006; Shepherd *et al.* 2006). However, when these findings are considered in light of with Arc's well established role in memory consolidation (Guzowski *et al.* 2000; Plath *et al.* 2006; Ploski *et al.* 2008), they underscore the importance of regulating synaptic strength and cellular excitability of neurons for proper memory formation.

Exactly how these individual genes function to contribute to neuronal plasticity and memory formation is largely unknown. Notably, approximately one-third of the plasticity-associated genes that we have identified are transcription factors, which supports the hypothesis that there is at least one more wave of transcription during the consolidation phase of memory. These transcription factors are, at least in part, likely to transcribe necessary synaptic proteins that are needed to stabilize potentiated synapses and support the morphological changes during learning and memory that occur at dendritic spines. In addition, some of the plasticity associated genes are likely to contribute to mechanisms of homeostatic plasticity and other yet discovered mechanisms. Future studies will be designed to answer how each of these genes contributes to neural plasticity and fear memory consolidation.

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Supporting information

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

Figure S1. Data table similar to Table 1, with additional information.

Figure S2. Custom designed qRT-PCR oligonucleotide primers listed. Headings are self-explanatory.

Figure S3. Qiagen qRT-PCR oligonucleotide primers.

Figure S4. Oligonucleotide primers used to PCR amplify DNA templates for *in situ* hybridization riboprobes.

Figure S5. Analysis of gene expression induced within the LA following training chamber exposure and Pavlovian fear conditioning with and without prior chamber habituation.

Figure S6. A comparison of genes identified in this study with genes identified in other relevant studies.

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References

Atkins C. M., Selcher J. C., Petraitis J. J., Trzaskos J. M. and Sweatt J. D. (1998) The MAPK cascade is required for mammalian associative learning. *Nat. Neurosci.* **1**, 602–609.

Bailey D. J., Kim J. J., Sun W., Thompson R. F. and Helmstetter F. J. (1999) Acquisition of fear conditioning in rats requires the synthesis of mRNA in the amygdala. *Behav. Neurosci.* **113**, 276–282.

Bauer E. P., Schafe G. E. and LeDoux J. E. (2002) NMDA receptors and L-type voltage-gated calcium channels contribute to long-term potentiation and different components of fear memory formation in the lateral amygdala. *J. Neurosci.* **22**, 5239–5249.

Blair H. T., Schafe G. E., Bauer E. P., Rodrigues S. M. and LeDoux J. E. (2001a) Synaptic plasticity in the lateral amygdala: a cellular hypothesis of fear conditioning. *Learn Mem* **8**, 229–242.

Blair H. T., Schafe G. E., Bauer E. P., Rodrigues S. M. and LeDoux J. E. (2001b) Synaptic plasticity in the lateral amygdala: A cellular hypothesis of fear conditioning. *Learning & Memory* **8**(5), 229–242.

Bliss T. V. and Lomo T. (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J. Physiol.* **232**, 331–356.

Chowdhury S., Shepherd J. D., Okuno H., Lyford G., Petralia R. S., Plath N., Kuhl D., Huganir R. L. and Worley P. F. (2006) Arc/Arg3.1 interacts with the endocytic machinery to regulate AMPA receptor trafficking. *Neuron* **52**, 445–459.

Cirelli C., Gutierrez C. M. and Tononi G. (2004) Extensive and divergent effects of sleep and wakefulness on brain gene expression. *Neuron* **41**, 35–43.

Coba M. P., Valor L. M., Kopanitsa M. V., Afinowi N. O. and Grant S. G. (2008) Kinase networks integrate profiles of N-methyl-D-aspartate receptor-mediated gene expression in hippocampus. *J. Biol. Chem.* **283**, 34101–34107.

Colon-Cesario W. I., Martinez-Montemayor M. M., Morales S. *et al.* (2006) Knockdown of Nurr1 in the rat hippocampus: implications to spatial discrimination learning and memory. *Learn. Mem.* **13**, 734–744.

Cowley T. R., Fahey B. and O'Mara S. M. (2008) COX-2, but not COX-1, activity is necessary for the induction of perforant path long-term potentiation and spatial learning in vivo. *Eur. J. Neurosci.* **27**, 2999–3008.

Davis M. (1992) The role of the amygdala in fear-potentiated startle: implications for animal models of anxiety. *Trends Pharmacol. Sci.* **13**, 35–41.

Davis S., Vanhoutte P., Pages C., Caboche J. and Laroche S. (2000) The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo. *J. Neurosci.* **20**, 4563–4572.

Dolmetsch R. E., Pajvani U., Fife K., Spotts J. M. and Greenberg M. E. (2001) Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. *Science* **294**, 333–339.

Duvarci S., Nader K. and LeDoux J. E. (2008) De novo mRNA synthesis is required for both consolidation and reconsolidation of fear memories in the amygdala. *Learn Mem* **15**, 747–755.

Fanselow M. S. (1980) Conditioned and unconditional components of post-shock freezing. *Pavlov J. Biol. Sci.* **15**, 177–182.

Fanselow M. S. and LeDoux J. E. (1999) Why we think plasticity underlying Pavlovian fear conditioning occurs in the basolateral amygdala. *Neuron* **23**, 229–232.

Frantz S. (2005) An array of problems. *Nat. Rev. Drug Discov.* **4**, 362–363.

Guzowski J. F., Lyford G. L., Stevenson G. D., Houston F. P., McGaugh J. L., Worley P. F. and Barnes C. A. (2000) Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. *J. Neurosci.* **20**, 3993–4001.

Han J. H., Kushner S. A., Yiu A. P. *et al.* (2007) Neuronal competition and selection during memory formation. *Science* **316**, 457–460.

Han J. H., Kushner S. A., Yiu A. P. *et al.* (2009) Selective erasure of a fear memory. *Science* **323**, 1492–1496.

Havik B., Rokke H., Dagyte G., Stavrum A. K., Bramham C. R. and Steen V. M. (2007) Synaptic activity-induced global gene expression patterns in the dentate gyrus of adult behaving rats: induction of immunity-linked genes. *Neuroscience* **148**, 925–936.

Hevroni D., Rattner A., Bundman M. *et al.* (1998) Hippocampal plasticity involves extensive gene induction and multiple cellular mechanisms. *J. Mol. Neurosci.* **10**, 75–98.

Impey S., Obrietan K., Wong S. T., Poser S., Yano S., Wayman G., Deloulme J. C., Chan G. and Storm D. R. (1998) Cross talk between ERK and PKA is required for Ca2+ stimulation of CREB-dependent transcription and ERK nuclear translocation. *Neuron* **21**, 869–883.

Impey S., McCorkle S. R., Cha-Molstad H. *et al.* (2004) Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. *Cell* **119**, 1041–1054.

Jones M. W., Errington M. L., French P. J. *et al.* (2001) A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat. Neurosci.* **4**, 289–296.

Josselyn S. A., Shi C., Carlezon Jr. W. A., Neve R. L., Nestler E. J. and Davis M. (2001) Long-term memory is facilitated by cAMP response element-binding protein overexpression in the amygdala. *J. Neurosci.* **21**, 2404–2412.

Keeley M. B., Wood M. A., Isiegas C., Stein J., Hellman K., Hannenhalli S. and Abel T. (2006) Differential transcriptional response to nonassociative and associative components of classical fear conditioning in the amygdala and hippocampus. *Learn Mem* **13**, 135–142.

LeDoux J. E. (2000) Emotion circuits in the brain. *Annu. Rev. Neurosci.* **23**, 155–184.

Lin Y., Bloodgood B. L., Hauser J. L., Lapan A. D., Koon A. C., Kim T. K., Hu L. S., Malik A. N. and Greenberg M. E. (2008) Activity-dependent regulation of inhibitory synapse development by Npas4. *Nature* **455**, 1198–1204.

Malenka R. C. and Nicoll R. A. (1999) Long-term potentiation – a decade of progress? *Science* **285**, 1870–1874.

Malkani S. and Rosen J. B. (2000) Specific induction of early growth response gene 1 in the lateral nucleus of the amygdala following contextual fear conditioning in rats. *Neuroscience* **97**, 693–702.

Malkani S., Wallace K. J., Donley M. P. and Rosen J. B. (2004) An egr-1 (zif268) antisense oligodeoxynucleotide infused into the amygdala disrupts fear conditioning. *Learn Mem* **11**, 617–624.

Maren S. and Quirk G. J. (2004) Neuronal signalling of fear memory. *Nat. Rev. Neurosci.* **5**, 844–852.

Maren S., Ferrario C. R., Corcoran K. A., Desmond T. J. and Frey K. A. (2003) Protein synthesis in the amygdala, but not the auditory thalamus, is required for consolidation of Pavlovian fear conditioning in rats. *Eur. J. Neurosci.* **18**, 3080–3088.

Matsuo R., Murayama A., Saitoh Y., Sakaki Y. and Inokuchi K. (2000) Identification and cataloging of genes induced by long-lasting long-term potentiation in awake rats. *J. Neurochem.* **74**, 2239–2249.

McHugh T. J., Jones M. W., Quinn J. J. *et al.* (2007) Dentate gyrus NMDA receptors mediate rapid pattern separation in the hippocampal network. *Science* **317**, 94–99.

Miklos G. L. and Maleszka R. (2004) Microarray reality checks in the context of a complex disease. *Nat Biotechnol* **22**, 615–621.

Miserendino M. J., Sananes C. B., Melia K. R. and Davis M. (1990) Blocking of acquisition but not expression of conditioned fear-potentiated startle by NMDA antagonists in the amygdala. *Nature* **345**, 716–718.

Nedivi E., Hevroni D., Naot D., Israeli D. and Citri Y. (1993) Numerous candidate plasticity-related genes revealed by differential cDNA cloning. *Nature* **363**, 718–722.

Newton S. S., Collier E. F., Hunsberger J., Adams D., Terwilliger R., Selvanayagam E. and Duman R. S. (2003) Gene profile of electroconvulsive seizures: induction of neurotrophic and angiogenic factors. *J. Neurosci.* **23**, 10841–10851.

Park C. S., Gong R., Stuart J. and Tang S. J. (2006) Molecular network and chromosomal clustering of genes involved in synaptic plasticity in the hippocampus. *J. Biol. Chem.* **281**, 30195–30211.

Plath N., Ohana O., Dammermann B. *et al.* (2006) Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. *Neuron* **52**, 437–444.

Ploski J. E., Newton S. S. and Duman R. S. (2006) Electroconvulsive seizure-induced gene expression profile of the hippocampus dentate gyrus granule cell layer. *J. Neurochem.* **99**, 1122–1132.

Ploski J. E., Pierre V. J., Smucny J., Park K., Monsey M. S., Overeem K. A. and Schafe G. E. (2008) The activity-regulated cytoskeletal-associated protein (Arc/Arg3.1) is required for memory consolidation of pavlovian fear conditioning in the lateral amygdala. *J. Neurosci.* **28**, 12383–12395.

Poirier R., Cheval H., Mailhes C., Charnay P., Davis S. and Laroche S. (2007) Paradoxical role of an egr transcription factor family member, egr2/krox20, in learning and memory. *Front. Behav. Neurosci.* **1**, 6.

Ressler K. J., Paschall G., Zhou X. L. and Davis M. (2002) Regulation of synaptic plasticity genes during consolidation of fear conditioning. *J. Neurosci.* **22**, 7892–7902.

Rodrigues S. M., Schafe G. E. and LeDoux J. E. (2001) Intra-amygdala blockade of the NR2B subunit of the NMDA receptor disrupts the acquisition but not the expression of fear conditioning. *J. Neurosci.* **21**, 6889–6896.

Rodrigues S. M., Farb C. R., Bauer E. P., LeDoux J. E. and Schafe G. E. (2004) Pavlovian fear conditioning regulates Thr286 autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II at lateral amygdala synapses. *J. Neurosci.* **24**, 3281–3288.

Sakai T., Tamura T., Kitamoto T. and Kidokoro Y. (2004) A clock gene, period, plays a key role in long-term memory formation in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **101**, 16058–16063.

Schafe G. E. and LeDoux J. E. (2000) Memory consolidation of auditory pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala. *J. Neurosci.* **20**, RC96.

Schafe G. E., Atkins C. M., Swank M. W., Bauer E. P., Sweatt J. D. and LeDoux J. E. (2000) Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of pavlovian fear conditioning. *J. Neurosci.* **20**, 8177–8187.

Schafe G. E., Nader K., Blair H. T. and LeDoux J. E. (2001) Memory consolidation of Pavlovian fear conditioning: a cellular and molecular perspective. *Trends Neurosci.* **24**, 540–546.

Schafe G. E., Doyere V. and LeDoux J. E. (2005) Tracking the fear engram: the lateral amygdala is an essential locus of fear memory storage. *J. Neurosci.* **25**, 10010–10014.

Shepherd J. D., Rumbaugh G., Wu J., Chowdhury S., Plath N., Kuhl D., Huganir R. L. and Worley P. F. (2006) Arc/Arg3.1 mediates homeostatic synaptic scaling of AMPA receptors. *Neuron* **52**, 475–484.

Shi L., Reid L. H., Jones W. D. *et al.* (2006) The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat. Biotechnol.* **24**, 1151–1161.

Stork O., Stork S., Pape H. C. and Obata K. (2001) Identification of genes expressed in the amygdala during the formation of fear memory. *Learn. Mem.* **8**, 209–219.

Tan P. K., Downey T. J., Spitznagel Jr E. L., Xu P., Fu D., Dimitrov D. S., Lempicki R. A., Raaka B. M. and Cam M. C. (2003) Evaluation of gene expression measurements from commercial microarray platforms. *Nucleic Acids Res.* **31**, 5676–5684.

Taubenfeld S. M., Stevens K. A., Pollonini G., Ruggiero J. and Alberini C. M. (2002) Profound molecular changes following hippocampal slice preparation: loss of AMPA receptor subunits and uncoupled mRNA/protein expression. *J. Neurochem.* **81**, 1348–1360.

Turrigiano G. G. (2008) The self-tuning neuron: synaptic scaling of excitatory synapses. *Cell* **135**, 422–435.

Xiang G., Pan L., Xing W. *et al.* (2007) Identification of activity-dependent gene expression profiles reveals specific subsets of genes induced by different routes of Ca(2 +) entry in cultured rat cortical neurons. *J. Cell. Physiol.* **212**, 126–136.

Yasoshima Y., Sako N., Senba E. and Yamamoto T. (2006) Acute suppression, but not chronic genetic deficiency, of c-fos gene expression impairs long-term memory in aversive taste learning. *Proc. Natl. Acad. Sci. USA* **103**, 7106–7111.

Zhang X., Odom D. T., Koo S. H. *et al.* (2005) Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. *Proc. Natl Acad. Sci. USA* **102**, 4459–4464.

