The Pharmacogenomics Journal (2005) 5, 278–291

www.nature.com/tpj

ORIGINAL ARTICLE

Gene expression profiling in the hippocampus of learned helpless and nonhelpless rats

R Kohen[1,2]
S Kirov[3]
GP Navaja[1,2]
H Kevin Happe[4]
MW Hamblin[1,2]
JR Snoddy[3]
JF Neumaier[1,5]
F Petty[4]

[1]*Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, WA, USA;* [2]*Veterans Affairs Puget Sound Health Care System, University of Washington, Seattle, WA, USA;* [3]*Genome Science and Technology, Oak Ridge National Laboratory-University of Tennessee, Oak Ridge, TN, USA;* [4]*Department of Psychiatry and Omaha VAMC, Creighton University, Omaha, NE, USA;* [5]*Harborview Medical Center, The University of Washington, Seattle, WA, USA*

Correspondence:
Dr R Kohen, Department of Psychiatry and Behavioral Sciences, University of Washington, GRECC (182B), VAPSHCS, 1660 South Columbian Way, Seattle, WA 98108, USA.
Tel: +1 206 764 2308 ext. 6-3073
Fax: +1 206 764 2569
E-mail: ruko@u.washington.edu

Received: 29 September 2004
Revised: 27 April 2005
Accepted: 31 May 2005
Publlished online 12 July 2005

ABSTRACT

In the learned helplessness (LH) animal model of depression, failure to attempt escape from avoidable environmental stress, LH, indicates behavioral despair, whereas nonhelpless (NH) behavior reflects behavioral resilience to the effects of environmental stress. Comparing hippocampal gene expression with large-scale oligonucleotide microarrays, we found that stress-resilient (NH) rats, although behaviorally indistinguishable from controls, showed a distinct gene expression profile compared to LH, sham stressed, and naïve control animals. Genes that were confirmed as differentially expressed in the NH group by quantitative PCR strongly correlated in their levels of expression across all four animal groups. Differential expression could not be confirmed at the protein level. We identified several shared degenerate sequence motifs in the 3′ untranslated region (3′UTR) of differentially expressed genes that could be a factor in this tight correlation of expression levels among differentially expressed genes.
The Pharmacogenomics Journal (2005) **5,** 278–291. doi:10.1038/sj.tpj.6500322; published online 12 July 2005

Keywords: gene expression profiling; animal model; learned helplessness; stress; depression; regulation of gene expression

INTRODUCTION

Depression is one of the most prevalent and disabling of all medical disorders.[1] Environmental stressors play a strong role in the etiology of the disease, yet not all humans become depressed after exposure to similar environmental stressors. This indicates that vulnerability to the depression-inducing effects of environmental stress may be at least in part genetically determined.[2,3] In this study we explored possible genetic factors mediating stress resilience in the learned helplessness (LH) animal model of depression.[4] In this model, animals are exposed to inescapable stress on day 1 and then given an escapable stress test, shuttle box testing, 24 h later on day 2. Following exposure to inescapable environmental stress some animals demonstrate escape deficits during shuttle box testing performed on the following day, that is, they no longer attempt to avoid escapable stressors (LH animals). In contrast, other animals exposed to the same inescapable stressor on day 1 do not develop LH (NH animals) and instead show behavioral responses in the escapable stress test similar to nonstressed or sham stressed controls. Thus, the LH model can provide for study of differences in individual susceptibility to development of behavioral depression after inescapable stress, and to resilience to the adverse effects of inescapable stress.

Our study is the first to compare large-scale gene expression patterns between rats showing either one or the other of the two different behavioral outcomes of

LH training (LH and NH), along with sham stressed and naïve control animals. A number of studies have previously demonstrated that LH is a syndrome of changes in brain activity involving multiple neurotransmitter systems and brain regions. Among the brain regions implicated are the dorsal raphe nucleus,[5,6] the nucleus accumbens,[7] prefrontal cortex, hippocampus and striatum.[8] We chose to investigate the hippocampus because it is one of the brain areas most heavily implicated in mediating the effects of environmental stress on affective state. Chronic depression and post-traumatic stress disorder have been associated with hippocampal volume loss in human subjects[9–12] and a number of animal studies have described changes in the expression of different classes of genes in the hippocampus following stress exposure, among them immediate early genes,[13] glucocorticoid and adrenocorticoid receptors and different classes of neurotransmitter receptors.[14–18] Likewise, hippocampal neurogenesis has been shown to be required for the behavioral effect of antidepressant drugs.[19]

Our investigation expands on a number of previous studies that have compared neurotransmitter receptor binding and gene expression in the hippocampus of learned helpless and nonhelpless (NH) animals. Among the findings previously described are an upregulation of β-adrenergic and 5-HT_{1B} serotonin receptors in the hippocampus of LH compared to NH and control animals[20,21] and a decrease in 5-HT_{2A} receptor density in the dorsal hippocampus of NH rats.[22] Rats selectively bred for high or low vulnerability to LH[23] show differences in mRNA expression of hippocampal neuropeptide Y, CREB, the α-catalytic subunit of protein kinase A (PKA-Cα) and glycogen-synthase kinase 3, subtype β (GSK-3β).[24,25]

RESULTS

Intensity and Variability of Microarray Gene Expression Data (Figure 1)

Using Resolver, we calculated the confidence level (*P*-value) for each gene indicating whether the corresponding mRNA was expressed above background level. If $P \leqslant 0.05$, the corresponding mRNA was considered to be expressed above background, a criterion met by around 5300 (62%) of the approximately 8600 transcripts represented on the rat genome U34A array. For each transcript, the degree of interindividual variability is reflected by its coefficient of variation, the standard deviation of expression levels divided by the average level of gene expression, among naïve animals. We found the coefficient of variation to be correlated with the intensity of gene expression, as highly expressed genes showed a statistically significant trend towards less variability in expression levels (Figure 1).

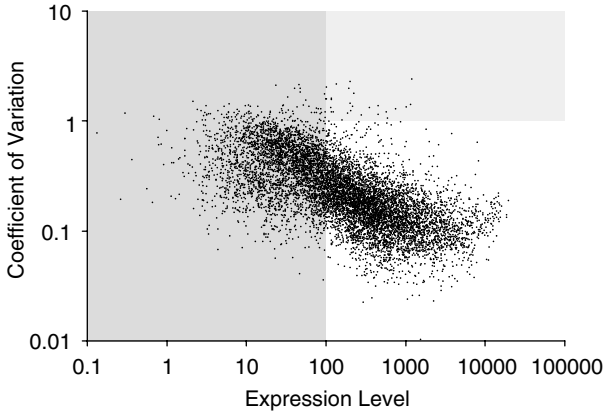


Figure 1 Gene expression in naïve animals. Coefficient of variation (STD/mean) over the mean level of gene expression in naïve control animals ($n = 6$) for ~8000 genes is shown. The axes are scaled logarithmically. Expression levels of below 100 were near background (dark gray overlay). Coefficients of variation of 1 or higher (STD ⩾ mean) in transcripts expressing above background were in each case due to a single outlier, likely reflecting a technical fault of the array hybridization (light gray overlay). The mean coefficient of variation in transcripts entering further analysis (white, unshaded area of the graph, ~5500 transcripts) was 0.18. For these transcripts the correlation coefficient between $\log_{10}$ (mean level of gene expression) and $\log_{10}$(coefficient of variation) was −0.50 at a significance level of $P \leqslant 0.01$.

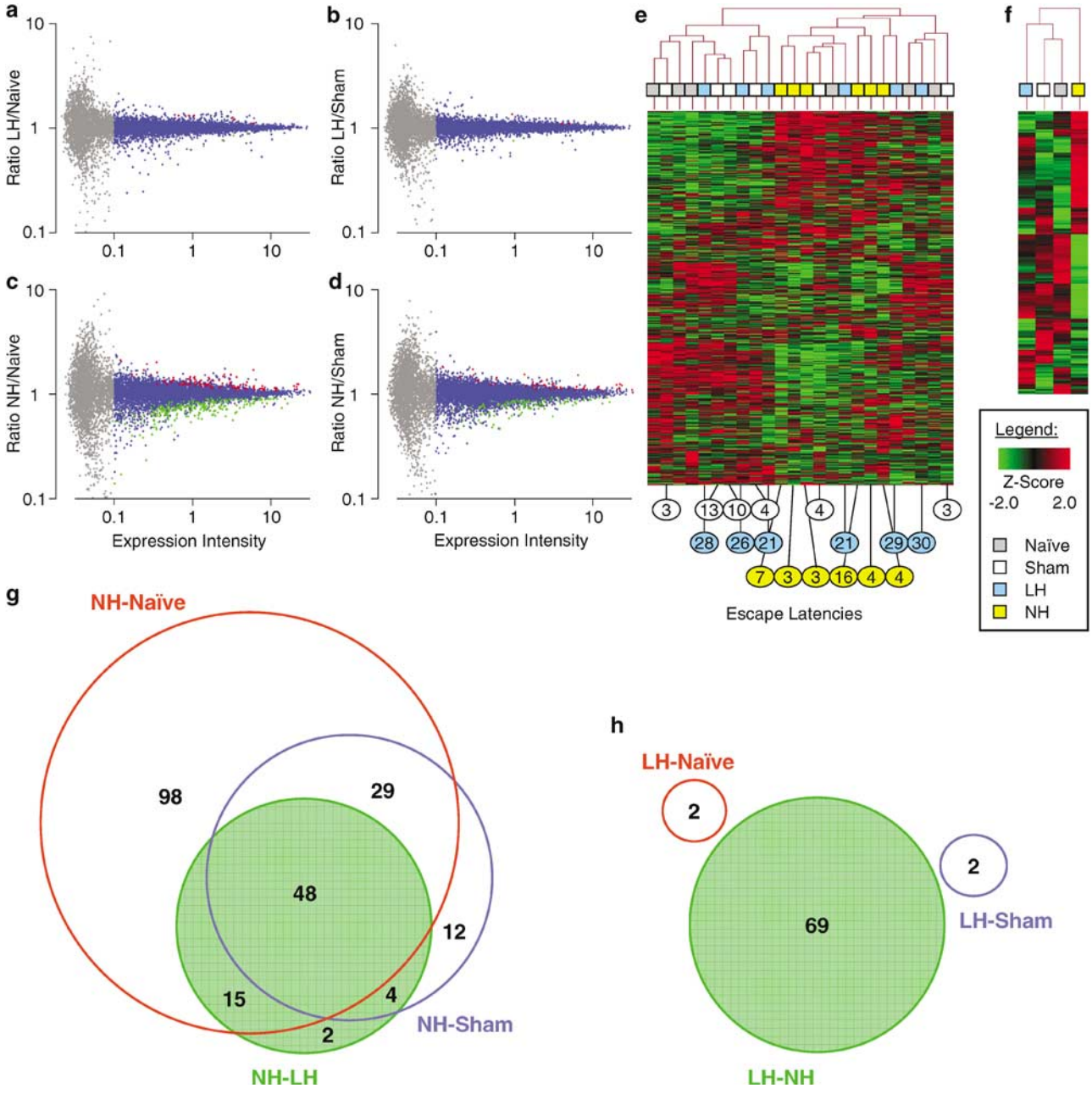
Comparison of Gene Expression Patterns between Animal Groups (Figure 2, Tables 1–3)

Comparing patterns of gene expression on microarray among the four animal groups (naïve, sham stressed, LH and NH), we selected a cutoff of at least 25% (1.25-fold) change in gene expression and a statistical significance level of $P \leqslant 0.01$ on ANOVA. In total, 208 genes differed significantly in their expression levels between NH animals and one or more of the other three animal groups (Figure 2). In total, 48 of these genes were differentially expressed in NH animals compared to all other three groups (Table 1). In contrast, while LH animals differed from NH animals in the expression of 69 genes, they showed no consistent pattern of gene expression changes compared to the two control groups, naïve and sham stressed animals. Thus, while the LH animals were, by definition, behaviorally distinct from the other three animal groups, this was not reflected by their pattern of hippocampal gene expression. In contrast, the stress-resilient NH animals, while behaviorally indistinguishable from sham stressed controls, were characterized by a distinct pattern of gene expression changes in the hippocampus. Hierarchical clustering did not identify NH animals as a group distinct from the other three, unless expression data from the six animals per group were averaged into one 'average group animal'. The 'average' naïve and sham stressed animals resembled each other most closely, followed by the 'average LH animal', with the 'average NH animal' being the most dissimilar. Hence, gene expression profiling in the hippocampus did not reveal any changes in expression level that could explain the altered behavior of the LH animals. Rather, the comparison of expression patterns among animal groups unexpectedly defined NH animals as the outlier.

All but two of these 48 genes differentially expressed on microarray in the NH group were downregulated in the stress-resilient animals. Effect sizes in this core group of 48

genes were small, as no changes larger than 2.5-fold could be observed. We chose 16 of these 48 genes for confirmation of expression changes by quantitative PCR. Criteria for the selection of individual genes were the direction of fold change, effect sizes, and existing knowledge about the gene in question. The only two genes that appeared upregulated in NH animals based on the microarray data, System N1 Na+ and H+-coupled glutamine transporter (Hnrpu), and Serine protease inhibitor, Kazal type 1II (Spink1) could not be confirmed as differentially expressed by quantitative PCR. We deliberately chose genes with large fold changes as well as some with small differences in gene expression to show that quantitative PCR confirmation worked as well for small fold changes as for large ones. 12 of the 16 genes, chosen for quantitative RT–PCR analysis were confirmed as differentially expressed by showing at least one statistically significant expression difference between animal groups. Effect sizes as observed on microarray and by RT-PCR were generally in good agreement. Due to the small effect sizes, however, differences in gene expression changes observed with quantitative RT–PCR were not always statistically significant ($P \leqslant 0.05$) across all three comparisons (Table 2).

The surprising finding that all genes differentially expressed in the NH group were downregulated in these animals prompted us to investigate whether the level of



expression between these genes might be correlated. We therefore compared the levels of expression among genes investigated by quantitative RT–PCR across all animals and within each animal group. Investigating bivariate correlations across all 24 animals regardless of group membership, we found that the expression levels of each of the 12 confirmed differentially expressed genes correlated with expression levels of each of the 11 other ones at a significance level of $P \leqslant 0.001$, with Pearson's correlation coefficients of 0.75 and over. Moreover, we observed no correlation between the level of expression among our 12 confirmed genes and a randomly chosen gene, cytochrome *b* (Cyt*b*), investigated in the same manner (not shown). Since the group of genes identified as differentially expressed in the NH animals cannot explain the behavioral phenotype, which is identical to that of the sham stressed animals, we did not find a relationship between the levels of expression in these genes and escape latencies (not shown).

The high correlation between the expression levels in our differentially expressed genes may reflect the fact that these genes were chosen by virtue of being downregulated in NH animals, and this communality could inflate the correlations observed among gene expression levels within the entire group of animals. This fallacy can be avoided by comparing correlation coefficients between our differentially expressed genes within each animal group, as shown in Table 3. The 264 correlations (4×66) shown in Table 3 are subject to the statistical fallacy of multiple comparisons, however, as the statistically significant correlation between Cyt*b* and gamma-aminobutyric acid A receptor, gamma 2 subunit (Gabrg2) expression in sham stressed animals indicates. Nonetheless, the overall pattern indicates a high number of significant correlations within each animal group that is dependent on the application of and response to environmental stress. In total, 18% of the correlations between our 12 differentially expressed genes were significant in naïve animals, 52% in sham stressed animals, 73% in LH animals and 85% in the NH group. Moreover, there was a trend for the tightness of the correlations, as indicated by correlation coefficients close to 1.0, to increase as the number of statistically significant correlations increased from group to group. These data suggest the presence of a regulatory factor that is invoked upon exposure to stress, and which brings into synch the expression levels of genes that are differentially expressed in NH animals.

Western Blot Analysis of Protein Expression (Figure 3)

Stress activated protein kinase alpha II (Mapk9), *N*-ethylmaleimide sensitive factor (Nsf), Neurotensin receptor 3 (Nt3, also known as sortilin) and Synaptotagmin 1 (Syt1) were investigated by Western blot analysis of hippocampus protein homogenate. In contrast to gene expression, which was downregulated in NH animals for all confirmed differentially expressed genes, protein expression of Mapk9 and Nt3 was significantly upregulated in NH animals compared to two or three of the other animal groups. Signal intensities on Western blot were higher in NH animals compared to LH by 17% for Mapk9 and 16% for Nt3. No statistically significantly changes across animal groups could be observed for protein levels of Nsf and Syt1.

Investigation of Promotor and UTR Regions in Differentially Expressed Genes (Figure 4)

As the high degree of correlation between levels of expression among differentially expressed genes suggested the presence of a common regulatory factor, we investigated

Figure 2 Comparison of gene expression between animal groups. (a–d) Resolver analysis of gene expression transformed raw expression levels into expression intensities, with an expression intensity of 1 roughly corresponding to a raw expression level of 1000. The ratio of gene expression intensities between two groups of animals is shown over the average intensity of gene expression in both groups together (naïve = naïve control animals, sham = sham stressed control animals, LH = learned helpless animals, NH = nonhelpless animals, $n = 6$ per group). The axes are scaled logarithmically. Genes expressing near background (expression intensity below 0.1) are shown in gray, not differentially expressed genes above background in blue, upregulated genes ($P \leqslant 0.01$) are shown red, downregulated genes ($P \leqslant 0.01$) green. Nonhelpless animals differed more strongly from controls (naïve and sham stressed) than helpless animals, as evidenced by the greater number of differentially expressed genes (red and green) and the wider spread of the blue cones indicating a greater difference in gene expression below statistically significant levels. (e–f) Cluster analysis of gene expression data, using agglomerative hierarchical clustering for genes expressing above background (~5500 transcripts). Expression intensities were transformed into *z*-scores, with upregulated genes shown red and downregulated genes green. (e) Each lane corresponds to a single animal, its group membership indicated by the color of the tile shown above the lane (gray = naïve control, white = sham stressed control, blue = learned helpless, yellow = nonhelpless). For shuttle box tested animals (sham stressed, learned helpless and nonhelpless animals) mean escape latencies are indicated in circles below the lanes. Single animals did not cluster by group membership or by escape latency. (f) Expression intensities were averaged in each animal group before clustering analysis was performed on the averaged expression intensities. The branching pattern of the dendogram indicates that naïve and sham stressed controls were most similar to each other, followed by learned helpless animals, with nonhelpless animals being the most dissimilar from animals in the other three groups. (g, h) Venn diagrams comparing the number of genes differentially expressed with at least 1.25-fold change in expression level between at least two of the animal groups ($P \leqslant 0.01$). (g) In total, 208 genes were differentially expressed in nonhelpless animals. Of these, 190 were differentially expressed between NH and naïve animals, 93 between NH and sham stressed animals, and 69 between NH and LH animals. The colored circles indicate the degree of overlap between these groups, leading to the identification of a core group of 48 genes that were differentially expressed in NH animals compared to each of the other three animals groups. (h) In total, 73 genes were differentially expressed in learned helpless (LH) animals, but only four of these genes were differentially expressed in comparison to either one of the two control groups. There was no overlap between the three NH differential expression data sets, indicating the absence of a consistent pattern of gene expression changes in the hippocampus of LH animals.

Table 1 Gene expression changes by microarray

Accession number	*Gene symbol*	*Gene name*	*Fold change in NH group compared to other groups*			*PCR*
			Naïve	*Sham*	*LH*	
AI231354	Mapk9	Stress-activated protein kinase alpha II	−2.49	−2.17	−1.99	+
AF089839	Nsf	*N*-ethylmaleimide sensitive factor	−2.36	−1.98	−1.95	+
AF023621	Nt3	Neurotensin receptor 3	−1.98	−1.84	−1.87	+
AA800908	Kctd12	Potassium channel tetramerisation domain containing 12	−1.89	−1.90	−1.75	
AI043631	Oazi	Ornithine decarboxylase antizyme inhibitor	−1.87	−1.80	−1.53	
D21869	Pfkm	Phosphofructokinase, muscle	−1.87	−1.64	−1.64	+
D12524	Kit	C-kit receptor tyrosine kinase	−1.85	−1.54	−1.64	
X52772	Syt1	Synaptotagmin 1	−1.82	−1.64	−1.62	+
AA108277	Hsp105	Heat shock protein 105 (mouse)	−1.78	−1.54	−1.57	
AF020212	Dnm1l	Dynamin 1-like	−1.74	−1.69	−1.56	
M74494	Atp1a1	ATPase, Na+K+ transporting, alpha 1 polypeptide	−1.66	−1.39	−1.38	+
AA893328	Canx	Calnexin	−1.66	−1.51	−1.59	
L08497	Gabrg2	Gamma-aminobutyric acid A receptor, gamma 2	−1.65	−1.72	−1.60	+
AF048828	Vdac1	Voltage-dependent anion channel 1	−1.65	−1.39	−1.44	+
AA685903	Tra1	Tumor rejection antigen gp96 (mouse)	−1.60	−1.32	−1.35	
U75920	Mapre1	Microtubule-associated protein, RP/EB family, member 1	−1.58	−1.50	−1.36	
D14419	Ppp2r2a	Protein phosphatase 2, regulatory subunit B, alpha isoform	−1.56	−1.38	−1.37	+
X63375	Atp1b1	ATPase Na+/K+ transporting beta 1 polypeptide	−1.55	−1.43	−1.41	+
H31734	Doc2b	Double C2, beta (mouse)	−1.53	−1.68	−1.57	
M25638	Nfl	Neurofilament, light polypeptide	−1.53	−1.39	−1.49	−
AI044508	Bsmrb	Brain-specific mRNA b	−1.46	−1.39	−1.29	
AI177503	H3f3b	H3 histone, family 3B	−1.46	−1.36	−1.24	
U56862	Znf146	Zinc-finger protein 260	−1.44	−1.38	−1.39	
X13905	Rab1b	Rab1b, member RAS oncogene family (human)	−1.44	−1.37	−1.34	
L27075	Acly	ATP citrate lyase	−1.42	−1.31	−1.34	
AI073056	Klc1	Kinesin light chain 1	−1.40	−1.23	−1.27	
Z12158	Pdha1	Pyruvate dehydrogenase E1 alpha 1	−1.40	−1.30	−1.29	
AA924326	Aldoa	Aldolase A	−1.38	−1.25	−1.20	
AA926149	Cat	Catalase	−1.37	−1.37	−1.33	
M26686	Pcmt1	Protein-L-isoaspartate *O*-methyltransferase 1	−1.37	−1.27	−1.23	
AI008074	Hspcb	Heat shock protein 1, beta (mouse)	−1.36	−1.28	−1.27	−
AI237731	Lpl	Lipoprotein lipase	−1.36	−1.22	−1.25	
AI170776	Grb2	Growth factor receptor bound protein 2 (mouse)	−1.35	−1.21	−1.19	
E03859	Rab11a	RAB11a, member RAS oncogene family	−1.35	−1.21	−1.29	
AF071225	Ppib	Cyclophilin B	−1.33	−1.20	−1.16	
AA899935	Pafah1b2	Platelet-activating factor acetylhydrolase alpha 2 subunit	−1.33	−1.26	−1.22	
U95727	Dnaja2	DnaJ (Hsp40) homolog, subfamily A, member 2	−1.32	−1.23	−1.26	
AF001953	Gnb5	Guanine nucleotide binding protein beta 5	−1.32	−1.26	−1.27	
U75932	Prkar1a	Protein kinase, cAMP-dependent regulatory, type I, alpha	−1.32	−1.25	−1.20	
AI228738	Fkbp1a	FK506-binding protein 1a	−1.31	−1.30	−1.18	
X55812	Cnr1	Cannabinoid receptor 1	−1.31	−1.26	−1.27	+
U75917	Ap2s1	Clathrin-associated protein 17	−1.29	−1.21	−1.20	
X74402	Gdi1	Guanosine diphosphate dissociation inhibitor 1	−1.29	−1.20	−1.21	
M76426	Dpp6	Dipeptidylpeptidase 6	−1.28	−1.26	−1.25	
AI070848	Actb	Actin, beta	−1.25	−1.22	−1.20	
D14418	Ppp2r1a	Protein phosphatase 2, regulatory subunit A, alpha isoform	−1.25	−1.24	−1.22	+
D14048	Hnrpu	System N1 Na+ and H+-coupled glutamine transporter	1.27	1.22	1.19	−
AA858673	Spink1	Serine protease inhibitor, Kazal type 1II	2.09	1.77	1.67	−

List of all 48 genes differentially expressed in nonhelpless animals (NH). Fold changes in NH animals compared to naïve controls (Naïve), sham stressed controls (Sham) and learned helpless animals (LH) are shown, all $P \leqslant 0.01$, Resolver analysis without post-test. All but two genes (Hnrpu and Spink1, neither one of them confirmed by quantitative PCR) were downregulated in NH animals. Confirmation of gene expression changes by quantitative RT-PCR is indicated as positive (+), negative (−) or not done (blank).

Table 2 Confirmation of microarray gene expression changes by quantitative RT-PCR

Accession number	*Gene symbol*	*Fold change in NH group compared to other groups*						*Confirmed*
		Microarray			*RT-PCR*			
		Naïve	*Sham*	*LH*	*Naive*	*Sham*	*LH*	
AI231354	Mapk9	**−2.49**	**−2.17**	**−1.99**	**−1.85**	**−1.73**	−1.51	Yes
AF089839	Nsf	**−2.36**	**−1.98**	**−1.95**	**−2.40**	−1.95*	−1.92*	Yes
AF023621	Nt3	**−1.98**	**−1.84**	**−1.87**	**−2.15**	−1.83*	−1.56	Yes
L08497	Gabrg2	**−1.65**	**−1.72**	**−1.6**	−1.88*	−1.82*	−1.61	Yes
X55812	Cnr1	**−1.31**	**−1.26**	**−1.27**	**−2.50**	−2.00*	−1.82	Yes
D21869	Pfkm	**−1.87**	**−1.64**	**−1.64**	**−1.44**	**−1.38**	−1.30*	Yes
X52772	Syt1	**−1.82**	**−1.64**	**−1.62**	**−2.08**	**−1.86**	**−1.77**	Yes
AF048828	Vdac1	**−1.65**	**−1.39**	**−1.44**	**−1.42**	−1.35*	−1.35*	Yes
M74494	Atp1a1	**−1.66**	**−1.39**	**−1.38**	**−1.81**	−1.61*	−1.48	Yes
X63375	Atp1b1	**−1.55**	**−1.43**	**−1.41**	**−1.47**	−1.41*	−1.39*	Yes
D14419	Ppp2r2a	**−1.56**	**−1.38**	**−1.37**	**−1.73**	**−1.52**	−1.47*	Yes
D14418	Ppp2r1a	**−1.25**	**−1.24**	**−1.22**	**−1.24**	−1.18	−1.20*	Yes
M25638	Nfl	**−1.53**	**−1.39**	**−1.49**	−1.13	−1.20	−1.21	No
AI008074	Hspcb	**−1.36**	**−1.28**	**−1.27**	−1.05	1.09	−1.12	No
D14048	Hnrpu	**1.27**	**1.22**	**1.19**	**−1.32**	−1.20	−1.17	No
AA858673	Spink1	**2.09**	**1.77**	**1.67**	—	—	—	No

From among 48 genes differentially expressed in nonhelpless animals (NH) compared to naïve controls (Naïve), sham stressed controls (Sham), and learned helpless animals (LH), 16 genes were chosen for confirmation analysis by RT-PCR. For gene names see Table 1. Fold changes in NH animals compared to the other animal groups ($n = 6$ per group) are shown, as indicated above the columns. All gene expression changes identified by microarray analysis were statistically significant ($P \leqslant 0.01$, Resolver analysis without post-test, bold numbers). Fold changes seen with RT-PCR (ANOVA with Bonferroni post-test) were either significant at $P \leqslant 0.01$ (bold numbers), at $P \leqslant 0.05$ (indicated by *) or not significant. Differential gene expression was confirmed in 12 of 16 investigated genes with at least one RT-PCR comparison between NH and other animal groups showing a significant fold change. In the remaining four genes, confirmation failed either because changes observed with RT-PCR were not significant (Nfl and Hspcb), were statistically significant in another direction (down-, instead of upregulated in NH animals, Hnrpu), or a PCR product could not be amplified with multiple different primer pairs, indicating a microarray artifact in the absence of gene product (Spink1).

the promotor and untranslated regions of our group of coregulated genes for common regulatory elements. The input data set for this analysis consisted of rat, mouse and human orthologs of our 12 genes confirmed by quantitative PCR (Table 2), plus the three among our set of differentially expressed genes (Table 1) that showed the highest correlation of expression levels in microarray analysis (not shown), Dnaja2, Oazi, and Pcmt1 (see Table 1 for full gene names). We could exclude the possibility that their synchronized expression pattern was guided by a locus control mechanism, because the genes we identified as differentially expressed are scattered throughout the genome.

Batch sequence analysis of the upstream regions of the differentially expressed genes did not reveal any common sequence motifs that could possibly bring in sync the transcription rate of this group, however, and neither did analysis of the 5′UTR regions (data not shown).

Batch sequence analysis of the 3′UTRs was able to predict 11 degenerate sequence motifs, which could be putative targets for regulatory proteins or micro-RNAs (Figure 4). The predicted motifs were able to recognize all of the genes included in the initial data set *vs* the entire 3′UTR database, yet Mapk9 was only predicted at a low *e*-value (1.8, motif 11) and not by all motifs in combination. The motifs were unable to predict any of the other genes among the group of 48 found by comparison of microarray data, with the exception of lipoprotein lipase (Lpl), which was predicted by motif 5 with an *e*-value of 8.7.

To control for the possibility that the identification of common motifs might be an artifact generated by overall high sequence similarity, possibly due to evolutionary conservation, we compared the 3′UTRs of the different genes in this data set using the BLAST sequence alignment tool. The degree of sequence similarity detected in the input data set after 'all against all' pairwise alignment gave no evidence for high overall sequence similarity, however, thus rejecting the possibility the motifs found could be a result of common evolutionary origin (data not shown). Next, we compared our results of the 3′UTR analysis to a set of simulations, where the initial data set was a randomly generated gene list, subject to the same analysis. While some of the random simulations resulted in a motif(s) prediction, these motifs were not able to discriminate between the initial data set and the entire 3′UTR database. Out of 20 repeats just one simulation returned more than 25% of the initial genes and none returned more than 50%, our threshold for successful analysis (not shown).

DISCUSSION

In this study we used large-scale gene expression analysis to investigate groups of rats that responded to LH training with different behavioral outcomes (LH *vs* NH) and two control

Table 3 Correlation of quantitative RT-PCR expression levels among differentially expressed genes

Naïve animals

Nsf	0.53											
Nt3	0.61	0.14										
Gabrg2	0.64	0.88*	0.14									
Cnr1	0.35	**0.95**	0.14	0.77								
Pfkm	0.64	0.66	0.78	0.54	0.71							
Syt1	0.50	0.77	0.19	0.55	0.82*	0.70						
Vdac1	0.59	0.76	0.63	0.56	0.70	0.82*	0.52					
Atp1a1	0.63	0.44	0.46	0.75	0.40	0.58	0.31	0.29				
Atp1b1	0.44	0.88*	0.17	0.75	**0.95**	0.74	0.92*	0.56	0.53			
Ppp2r1a	0.71	0.67	0.71	0.58	0.71	**0.97**	0.80	0.72	0.64	0.81		
Ppp2r2a	0.72	**0.95**	0.21	**0.96**	0.85*	0.66	0.75	0.67	0.64	0.85*	0.72	
Cyt*b*	0.26	−0.34	0.35	−0.07	−0.27	0.18	0.06	−0.37	0.54	0.03	0.30	−0.09
	Mapk9	Nsf	Nt3	Gabrg2	Cnr1	Pfkm	Syt1	Vdac1	Atp1a1	Atp1b1	Ppp2r1a	Ppp2r2a

Sham stressed animals

Nsf	**1.0**											
Nt3	0.62	0.66										
Gabrg2	0.87*	0.88*	0.66									
Cnr1	0.81*	0.85*	0.91*	0.82*								
Pfkm	0.91*	0.89*	0.44	0.78	0.58							
Syt1	0.82*	0.85*	0.87*	0.69	**0.94**	0.64						
Vdac1	0.91*	0.90*	0.38	0.68	0.62	**0.92**	0.73					
Atp1a1	0.85*	0.87*	0.63	0.74	0.83*	0.58	0.75	0.63				
Atp1b1	0.85*	0.88*	0.84*	0.79	**0.93**	0.74	**0.97**	0.79	0.68			
Ppp2r1a	**0.96**	**0.94**	0.46	0.85*	0.65	**0.94**	0.64	0.88*	0.78	0.70		
Ppp2r2a	0.81*	0.86*	0.77	0.71	**0.94**	0.59	**0.96**	0.74	0.81	**0.93**	0.63	
Cyt*b*	−0.68	−0.68	−0.60	−0.88*	−0.64	−0.58	−0.44	−0.36	−0.67	−0.51	−0.72	−0.41
	Mapk9	Nsf	Nt3	Gabrg2	Cnr1	Pfkm	Syt1	Vdac1	Atp1a1	Atp1b1	Ppp2r1a	Ppp2r2a

Learned helpless animals

Nsf	**0.98**											
Nt3	**0.96**	0.91*										
Gabrg2	**0.97**	**0.99**	0.91*									
Cnr1	**0.96**	**0.97**	**0.93**	**0.99**								
Pfkm	**0.97**	**0.99**	0.87*	**0.99**	**0.97**							
Syt1	0.89*	**0.92**	0.81	**0.94**	**0.96**	**0.95**						
Vdac1	0.88*	0.90*	0.77	0.92*	**0.94**	**0.94**	**0.99**					
Atp1a1	**0.97**	**0.95**	**0.94**	**0.92**	0.90*	0.90*	0.77	0.76				
Atp1b1	0.88*	0.91*	0.89*	**0.93**	0.91*	0.89*	0.82*	0.76	0.83*			
Ppp2r1a	0.73	0.78	0.54	0.76	0.67	0.83*	0.69	0.71	0.65	0.69		
Ppp2r2a	0.82*	0.89*	0.76	**0.92**	**0.94**	0.88*	**0.95**	**0.92**	0.76	0.81	0.58	
Cyt*b*	−0.29	−0.44	−0.13	−0.46	−0.35	−0.48	−0.41	−0.38	−0.23	−0.53	−0.75	−0.43
	Mapk9	Nsf	Nt3	Gabrg2	Cnr1	Pfkm	Syt1	Vdac1	Atp1a1	Atp1b1	Ppp2r1a	Ppp2r2a

Nonhelpless animals

Nsf	**0.99**											
Nt3	**0.97**	**0.98**										
Gabrg2	**0.97**	**0.98**	**1.0**									
Cnr1	**0.97**	**1.0**	**0.97**	**0.97**								
Pfkm	**1.0**	**0.99**	**0.98**	**0.98**	**0.97**							
Syt1	**0.98**	**0.96**	**0.99**	**0.99**	**0.94**	**0.98**						
Vdac1	**0.93**	0.92*	**0.97**	**0.96**	0.89*	**0.95**	**0.98**					
Atp1a1	**0.95**	**0.93**	**0.96**	**0.95**	0.92*	**0.93**	**0.96**	0.90*				
Atp1b1	**0.96**	**0.98**	**0.99**	**0.98**	**0.98**	**0.97**	**0.96**	**0.94**	**0.94**			
Ppp2r1a	0.75	0.80	0.81	0.84*	0.83*	0.76	0.75	0.74	0.69	0.81		
Ppp2r2a	**0.93**	0.89*	0.87*	0.89*	0.86*	**0.94**	0.90*	0.88*	0.80	0.83*	0.74	
Cyt*b*	0.44	0.36	0.33	0.34	0.30	0.48	0.43	0.48	0.24	0.26	0.10	0.68
	Mapk9	Nsf	Nt3	Gabrg2	Cnr1	Pfkm	Syt1	Vdac1	Atp1a1	Atp1b1	Ppp2r1a	Ppp2r2a

Expression levels determined by quantitative RT-PCR were normalized to a panel of housekeeping genes and compared between animals within each group ($n = 6$ per group). For full gene names, see Table 2. Pearson's correlation coefficients are shown. Correlations highlighted by * are significant at $P \leqslant 0.05$. Bold numbers indicate correlations significant at $P \leqslant 0.01$. Correlations between differentially expressed genes and cytochrome *b* (Cyt*b*), a nondifferentially expressed gene, are shown as control.

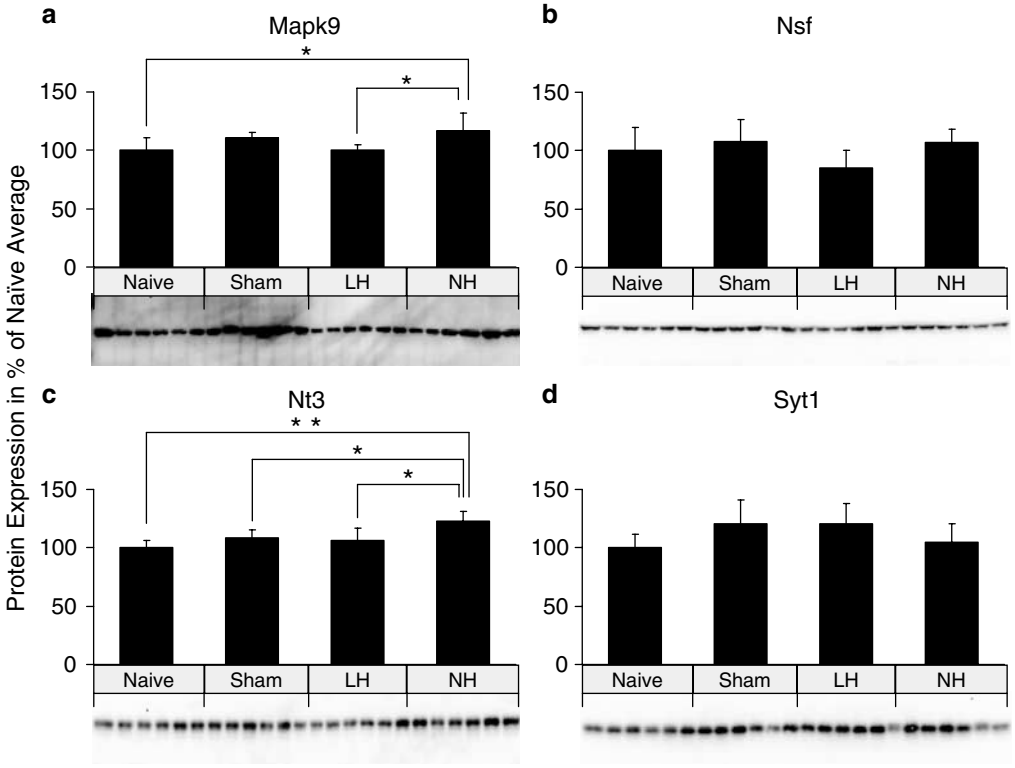


Figure 3 Western blot analysis of protein expression. One representative of three immunoblots is shown for each protein investigated. (a) Mapk9, probed with anti-SAPKα-antibody (1 : 250); (b) Nsf, probed with anti-Nsf antibody (1 : 5000); (c) Nt3, probed with anti-Nt3 antibody (1 : 250); (d) Syt1 probed with anti-Syt1 antibody (1 : 250); see Table 1 for full gene names. In total, 30 μg of total protein extract from hippocampus homogenate of individual animals were loaded in each lane. Animal groups are indicated below the lanes (naïve control animals, naive; sham stressed controls, sham; learned helpless animals, LH; nonhelpless animals, NH; $n = 6$ per group). Bar graphs show the average level of protein expression in percent of average protein expression in naïve animals, as determined by densitrometry of three separate Western blots. Statistically significant differences in protein expression are highlighted (*$P < 0.05$; **$P < 0.001$). Due to the variability in signal intensity between replicate Western blots, the examples of individual blots shown below the bar graphs do, in some instances, not appear to correlate well with the averaged data shown above (eg Mapk9—sham stressed animals). For the same reason, the error bars appear small in relation to the variations in signal intensity seen on one blot alone.

groups, sham stressed and naïve animals. LH and NH animals can be seen as models for vulnerability or resilience to behavioral depression following exposure to inescapable environmental stress. LH animals are phenotypically characterized by an increased latency (escape failure) in their attempts to escape an avoidable environmental stressor that is presented on the day following inescapable stress exposure. Investigating gene expression patterns in the hippocampus of these animals we expected to find changes in gene expression that could explain the behavioral phenotype. Instead, the reverse was found: NH, that is, stress-resilient animals, showed differential expression of 208 transcripts in the hippocampus while LH animals were indistinguishable from controls in terms of their hippocampal pattern of gene expression. Hence, the changes in gene expression we found cannot explain the behavioral phenotype. Rather, they suggest that successful coping with environmental stress is associated with gene expression changes in the hippocampus.

In choosing a cutoff level of 1.25-fold for differential expression, we were guided by studies showing that behavioral stimulation is likely to induce changes in gene expression that lie well below the traditionally chosen cutoff of two-fold changes in gene expression.[26] Of the 208 transcripts thus identified, 48 were differentially expressed in NH animals compared to each of the other three animal groups. For 12 of 16 of these genes significant differential expression was confirmed by quantitative real-time PCR, in keeping with our previous observations that real-time PCR is a powerful method for detecting even small fold changes in gene expression.[25] Expression of all differentially expressed genes was downregulated in NH compared to the other animal groups, with the exception of two genes which could not be confirmed by quantitative RT–PCR. The expression levels of genes assessed by quantitative PCR showed a high number of significant correlations that were highest within the NH group, thus indicating the possible presence of a regulatory factor mediating stress resilience, which brought

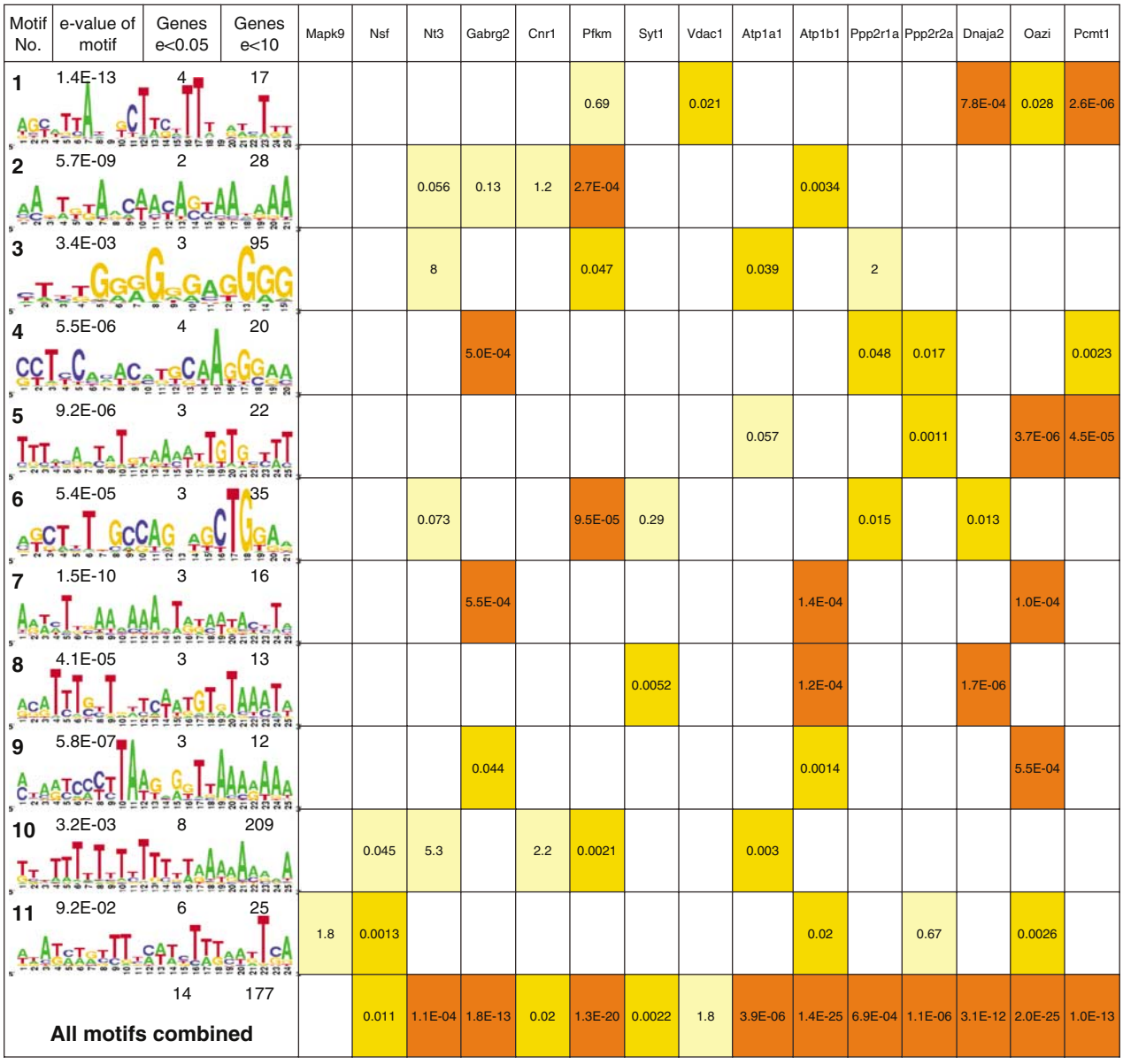


Figure 4 Motif predictions for 3′UTRs. Motifs predicted by batch sequence analysis as regions of possible regulatory significance in the 3′UTR of coregulated genes and their predictions for members of the input data set are shown. The leftmost column shows the motif consensus sequence in weblogo format[60] (http://weblogo.berkeley.edu/), where each logo consists of stacks of symbols, one stack for each position in the sequence. The height of symbols within the stack indicates the relative frequency of each nucleic acid at that position. Shown above the weblogo figure of each motif are its *e*-value and the number of genes in the 31K 3′UTR database predicted by this motif at an *e*-value of $e<0.05$ and $e<10$, respectively. Almost all genes predicted by the motifs either in isolation or combined (lowermost row) at an *e*-value of 0.05 or less are members of the input data set. For each gene, only the data for one of the three homologs (human, rat, and mouse) are shown and *e*-values for motif predictions in individual genes (columns to the right of the weblogo figures) are for the homologs predicted at the lowest *e*-value. The color overlay indicates the level of significance ($e<0.001$ brown, $e<0.05$ orange, $e<10$ yellow). The quality of prediction depends in part on the number of homologs available in the database for any given gene, ranging from 1 to 3. For example, for Mapk9, only one homolog was available for analysis.

into synch the expression levels of these genes. For example, a recent study has shown that micro-RNA's can down-regulate large number of target mRNAs by pairing to their 3′UTR via conserved consensus sequences.[27] Searching for possible shared regulatory elements within a subgroup of our gene set that might account for the synchronicity of their expression levels, we were able to detect a group of 11 specifically enriched 3′UTR sequence motifs. Most of the motifs we found are TA-rich and thus resemble binding sites for the Hu group of proteins that regulate mRNA turnover

and that have been implicated in the control of neuronal development and plasticity.[28] Our inability to find a single DNA motif shared by all coregulated genes could be accounted for by the low-signal-to-noise ratios making computational analysis of degenerate motifs difficult, especially in higher organisms.[29] Problems created by the signal-to-noise ratio in current computational approaches also prevented us from exploring the distant possibility that a signal exists further than 5 Kbp upstream, or that the signal is scattered in different noncoding sequences in different genes.

Changes in gene expression could not be confirmed at the protein level, which calls into question the functional significance of the observed downregulation at the mRNA level. On the contrary, among the four proteins we investigated, Mapk9 and Nt3 were slightly upregulated in stress-resilient animals. Rats were killed 24 h after shuttle box testing to allow comparison of our results with those of previous studies reporting changes in receptor binding and neurotransmitter release in the brain of rats killed at that time point,[20,22,30,31] yet it is possible that a different time point might have been more suitable to capture changes in gene expression.

An argument can be made that the behavioral response of the NH group is due to a priori genetic differences in these animals, which could also explain the differences from all other groups. In this context, the 24 h time point may represent steady-state levels of expression rather than expression responses to the challenges of the LH paradigm. However, the expression profile of the NH group differed more from that of the naïve and sham stressed animals than from that of the LH group. If the differences in gene expression were the results of pre-existing genetic differences between animal groups, rather than induced by exposure to LH training, one would expect our naïve and sham stressed groups of animals—which were randomly drawn from the same pool as the rats exposed to inescapable shock and shuttle box testing—to exhibit gene expression profiles that were intermediate between those shown by the two animal groups with distinct behavioral outcomes.

A number of previous animal and human studies have implicated some of the genes we identified as differentially expressed in stress-resilient animals as potential participants in the regulation of affect. Among those are aldolase A, and the Na+K+ transporting ATPase alpha 1.[32,33] Nsf[34] and synaptotagmin 1[35,36] have both been implicated in the regulation of neurotransmitter release. Crispino *et al*[37] described a modulation of Syt1 mRNA levels in area CA3 of the hippocampus during the estrous cycle with highest levels occurring during diestrus, while Jenkins *et al*[38] showed that rats in diestrus were more vulnerable to develop LH, thus supporting our observations that lowered levels of Syt1 are accompanied by increased stress resilience.

GABA receptors have previously been shown to be downregulated in the prefrontal cortex or hippocampus following electroconvulsive therapy and antidepressant drug treatment[39,40] and have been implicated in the development of LH.[31,41] In this context, our observation of cannabinoid receptor 1 downregulation in stress-resilient animals is particularly intriguing, as in the hippocampus Cnr1 is expressed exclusively by a subpopulation of inhibitory GABA-ergic interneurons where the receptor is thought to regulate GABA release from presynaptic boutons (reviewed in Wilson and Nicoll[42]).

The neurotensin-3 receptor not only binds neurotensin but also Lpl with high affinity,[43] which is intriguing in view of our observation that Lpl was among the genes showing downregulated expression in NH animals. Moreover, Nt3, in acting as a neurotrophin receptor, can mediate proapoptotic signaling.[44] Among the genes we found to be differentially expressed in the hippocampus of stress-resilient animals, several others have also been implicated in proapoptotic signaling, among them Vdac1,[45] Mapk9,[46] and protein phosphatase 2A.[47] Taken together, these findings indicate that regulation of GABA-ergic neurotransmission, synaptic vesicle exocytosis and apoptosis in the hippocampus may jointly contribute to the development of resilience or vulnerability to environmental stress in an animal.

MATERIALS AND METHODS

Animals

Adult, male Sprague–Dawley rats weighing 300–350 g at the start of the behavioral procedure were used in all experiments. Rats were housed in a temperature- (22°C) and humidity-controlled environment with free access to food and water and were maintained on a 12 h light/dark cycle, with experimentation occurring during the light part of the cycle. Animals were acclimated to these housing conditions for at least 2 weeks before any experimental manipulation. Care was taken to minimize animal discomfort during all procedures. All procedures were in accordance with protocols approved by the Dallas and Omaha Veterans Affairs Animal Care and Use Committees and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

LH Training and Behavioral Testing

In the LH model as currently used by us and other investigators,[48] rats are exposed to an inescapable stressor (tail shock while restrained) or sham stress (restraint only) on day 1. The escapable stressor, administered in a shuttle box on day 2, requires a rat to cross from one compartment of the cage to another to terminate electrical foot shock, which ends automatically after 30 s if the rat fails to perform the task. In about 50% of the animals stress exposure on day 1 induces LH, that is, escape failure from subsequent stressors on day 2. NH animals, on the other hand, behave similarly to control animals in the shuttle box escape task, indicating their resilience to the inescapable environmental stressor offered on the previous day.

Procedures for stress exposure and shuttle-escape testing were as previously described.[22] Rats were randomly assigned either to be exposed to uncontrollable tail shock, sham stress, or to remain in their home cages (naïve control). The inescapable shock session consisted of 100, 5 s, 1.0 mA tail shocks on a 1 min variable-interval schedule (range 25–90 s)

with the rats confined in Plexiglass tubes. Sham stressed rats were restrained for an equivalent period of time as rats exposed to uncontrollable stress, but received no tail shocks.

Rats were tested for escape performance in a shuttle box with an electrified floor grid 24 h after stress or sham stress treatment. Naïve controls were not tested. The test began with five trials during which one shuttle crossing terminated shock (FR-1 trials), followed by 25 trials during which the rat had to cross the shuttle box twice to terminate shock (FR-2 trials). Shock intensity was 0.8 mA with an average intertribal interval of 60 s. Shocks terminated automatically 30 s after onset if escape had not occurred. Escape latencies were recorded automatically by a computer. Rats that had previously received uncontrollable stress were classified as learned helpless (LH) if the mean escape latency for the 25 FR-2 trials was $\geqslant 20$ s or as NH if the latency was <20 s. The FR-1 trials serve to insure that the tested animal has normal motor function in response to foot shock. FR-1 escape latency is unaffected by prior stress exposure. Thus, most animals have an escape latency of <10 s on the FR-1 trials, whether or not they test as learned helpless or NH on the subsequent FR-2 trials. The FR-1 trials can therefore be used to identify animals with overall slow motor response, which could otherwise mimic the escape deficit of LH.[48]

Microarray Analysis

Six animals in each group (naïve controls, sham stressed controls, LH and NH) were used for gene expression analysis. At 24 h after the test session animals were killed by decapitation, their brains removed and the left and right hippocampus dissected free of the brain. Hippocampi were stored in RNAlater (Ambion) to prevent degradation of RNA. RNA was extracted from hippocampal tissue using RNeasy kits (Qiagen) and following the manufacturers' instructions. The quality of total RNA was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies) in order to determine the ratios of 28S–18S ribosomal RNA band intensities, which were 1.9 on average, without evidence for degradation in any of the samples. In total, 15 μg total RNA was then used to generate biotinylated probes for hybridization to Affymetrix U34A rat genome arrays, following the manufacturer's protocol. Quality of the probes was confirmed by Agilent 2100 Bioanalyzer analysis before hybridization to individual Affymetrix U34A microarrays.

Gene expression profiles of individual animals were normalized to all probe sets on the array and then compared using the Rosetta Resolver analysis package (Rosetta Biosoftware) and Microsoft Excel. Variability in gene expression on microarray that occurred independently of the experimental paradigm was analyzed using Microsoft Excel, comparing average level and standard deviation of gene expression among naïve animals only. For comparison between animal groups, we used Rosetta Resolver as well as Microsoft Excel. Using Resolver, we first identified transcripts that were expressed significantly above background level ($P\leqslant 0.05$) in all 24 array hybridizations. These transcripts were then compared for statistical differences in expression intensities between animal groups, using one-way ANOVA. The *P*-value for significant difference in gene expression between animal groups was set to $P\leqslant 0.01$ to minimize the number of false positive results. Due to the high number of comparisons made (>5000) using a post-test to control for statistical errors introduced by multiple comparisons would have been overly restrictive.[49] Raw lists of differentially expressed transcripts produced by Resolver were further analyzed using Microsoft Excel. Duplicate reports of genes represented by multiple probe sets on the U34A array were consolidated into one listing. Genes that were reported as differentially expressed due to a clear technical fault of the array hybridization were manually excluded from further analysis, as were genes reported as differentially expressed in different directions as reported by multiple probe sets (total number of thus excluded genes = 7). Finally, genes that were differentially expressed with an effect size of less than 1.25-fold in all comparisons were excluded from the final report.

Quantitative PCR

Confirmation of microarray results for 16 selected genes was done as previously described.[25] Briefly, 2 μg of total RNA per animal were treated with DNase I, using DNA-free kits (Ambion), then reverse transcribed into first strand cDNA in the presence of 500μM dNTPs (Amersham), 500 ng oligo(dT)$_{12–18}$ primer (Gibco) and 20 U SUPERaseIn (Ambion), using 200 U of SuperScript II (Gibco) and following the manufacturer's instructions. Quantitative PCR was carried out using gene-specific primers that were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi),[50] with the following input parameters: 22-mer primers with T_m between 57 and 63°C optimum T_m of 60°C with a maximum T_m difference between the two members of a primer pair of 2°C, optimum GC percentage of the primers 50%, optimum product size 200 bp, all other inputs per program default. Quantitative PCR was carried out on a Roche LightCycler (10 min incubation step at 95°C followed by 35 cycles of 95°C for 15 s, 60°C for 10 s, and 72°C for 10 s) in the presence of 0.5 μM primers and 3 mM $MgCl_2$ and using LightCycler FastStart DNA Master SYBR Green I kits (Roche).

All assays were carried out in duplicate and results averaged across both assays. Relative concentrations of amplified PCR product were determined with the help of mock standards prepared by serial dilutions (1 : 1, 1 : 2, 1 : 4, 1 : 10 and 1 : 20) of a cDNA mix composed of aliquots from all samples. Relative expression levels for each gene of interest were normalized by dividing them by the average expression levels across a panel of four housekeeping genes. Housekeeping genes were glyceraldehyde-3-phosphate dehydrogenase (Gapdh), acidic ribosomal protein P0 (Arbp), ribosomal protein S2 (Rps2) and ribosomal protein S4, X-linked (Rps4×). The ribosomal proteins were chosen as members of the housekeeping gene panel on the basis of their high and stable levels of expression that were independent of rat group membership (naïve control, sham stressed, LH or NH) in the microarray analysis (coefficients of variation <0.07). Data were evaluated statistically with

SPSS Base11.0, and using one-way ANOVA followed by Bonferroni *post hoc* analysis.

Western Blot Analysis

A different group of 24 animals than those used for the gene expression studies, with six animals in each group (naïve controls, sham stressed controls, LH and NH) were used for protein expression analysis. Using an Omni TH115 homogenizer (Omni International), frozen hippocampus samples were homogenized in three volumes of ice-cold modified RIPA buffer (1 × phosphate-buffered saline, pH 7.6, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) containing the following protease inhibitors (all from Sigma): 1 mM Pefablock, 2 μg/ml leupeptin, 3 μg/ml aprotenin, 1 μg/ml pepstatin, 40 μg/ml soybean trypsin inhibitor and 100 μg/ml benzamidine. After incubation on ice for 30 min, homogenates were centrifuged twice at 10 000 *g* to produce a clear lysate. Protein concentrations were determined using a BCA protein assay kit (Pierce). Protein samples (30 μg) were mixed with an equal volume of 2 × sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 0.1% bromphenol blue, and 5% β-mercaptoethanol) and denatured by boiling for 10 min. Samples were fractionated on 10% Tris-HCl gels and blotted onto PVDF membranes, using the Criterion precast gel system (BioRad) and following the manufacturer's instructions.

Membranes were blocked for 1 h at room temperature with 5% dried milk (Carnation) in 1 × Tris-buffered saline. Blots were then probed for 1 h with primary antibody dilutions optimized so that the densitometric values of immunoreactive bands fell within the linear range of scanned images. Following three washes in wash buffer (1 × Tris-buffered saline/0.1% Tween-20), blots were incubated for 30 min with appropriate HRP-conjugated secondary antibodies. Antibody combinations used were as follows: antistress activated protein kinase alpha II (Mapk9; Chemicon) 1 : 250 and anti-rabbit (Sigma) 1 : 5000; anti-*N*-ethylmaleimide sensitive factor (Nsf; BD Biosciences) 1 : 5000 and anti-mouse (Sigma) 1 : 5000; anti-neurotensin receptor 3 (Nt3; BD Biosciences) 1 : 250 and anti-mouse 1 : 5000; anti-synoptotagmin 1 (Syt1; BD Biosciences) 1 : 250 and anti-mouse 1 : 5000. Blots were again washed four times in wash buffer and then exposed to chemiluminescence detection solution (ECL plus Western Blotting Detection System, Amersham). Blots were scanned on a Kodak DS Image Station 400 CF and analyzed by densitometry, using the Kodak 1D program. All Western blots were carried out in triplicate. For each blot, protein expression was normalized to percent of average protein expression from naïve animals, to allow for comparison and statistical analysis of Western blots with different overall signal intensities. Data were evaluated statistically with SPSS Base11.0 as described above.

Analysis of Common Elements among Coexpressed Genes

We looked for possible control elements within the UTR and the upstream regions of differentially expressed genes through a high-throughput sequence analysis pipeline, using phylogenetic footprinting.[51] This pipeline uses sequence databases for human, mouse and rat, assembled from ensembl database release 20 (human genome build 34, mouse genome build 32 and rat genome build 3)[52] (http://www.ensembl.org/). We retrieved sequence data for differentially expressed genes as well as for a control set of 100 randomly chosen genes, using the ensembl application programming interface. For each gene, rat, mouse and human orthologs, if known, were used to enhance the specificity of the analysis by allowing us to identify regions that are conserved across species, as nonconserved regions are unlikely to serve an important regulatory function.

For analysis of the promoter regions, a sequence database with includes upstream regions and containing more than 39K genes from different species was used. Since cis-regulatory elements tend to occur more often within 5 kb of the transcription start site, which corresponds well to the average size of the upstream regions with high similarity between human and mouse, we restricted our analysis of the promoter regions to this area.[53]

For the analysis of untranslated mRNA regions (3′UTR and 5′UTR), we used a database featuring approximately 32K genes from different species. This sequence database was assembled based on the genome annotation, available from ensembl. Where alternative transcripts are described we used the most upstream ending transcript for analyzing 5′UTRs, and the most downstream one for 3′UTRs.

Sequence repeats were masked first with sequential RepeatMasker (http://www.repeatmasker.org/) and Tandem Repeats Finder analysis,[54] (http://c3.biomath.mssm.edu/trf.html/). Next we identified conserved regions by additive pairwise alignment of rat, mouse and human sequences, using the BLAST sequence alignment tool, bl2seq, with the following parameters: gap penalty 1, gap extension penalty 1, penalty for mismatch 2, window word size 9.[55] Regions of high similarity were defined as follows: (a) single blast hit should have at least 22 identities (conserved nucleotides); (b) the sum of overall blast hits similarity should be more than 5% of the length of the analyzed sequence. If conserved regions could not be identified, the raw, non-masked sequence was used for further analysis.

The promotor regions of differentially expressed and control genes were searched for common transcription factor binding sites or novel, shared motifs, using Bioperl methods[56] (http://www.bioperl.org/) and utilizing the databases Transfac 6.0 professional release[57] (http://www.gene-regulation.com/) and Jaspar[58] (http://jaspar.cgb.ki.se/cgi-bin/jaspar_db.pl).

The upstream (5′UTR) and downstream regions (3′UTR) of differentially expressed genes were analyzed for shared motifs using the MAST and MEME analysis tools[59] (http://meme.sdsc.edu/meme/website/).

Motifs were predicted with MEME, using the following analysis parameters: $\text{maxw} = n/2 + 1$ and $\text{minsites} = 3 + \text{int}(n/7)$, where n is the number of the genes in the analysis, then filtered then on the basis of their *e*-value (<1) and complexity ($<80\%$ single base consensus). A motif's *e*-value indicates the probability of its chance occurrence among all genes in the database, whereas the *e*-value of

prediction indicates the level of significance at which the existence of the motif is predicted for a given gene. Predicted motifs were stored in a database for future reference and for evaluation of the MEME predictions. (GeneKeyDB, http://genereg.ornl.gov/gkdb). Evaluation of MEME-predicted motifs was carried out using MAST to search the 32 K UTR database of for occurrence of the predicted motifs in rat, mouse and human genes. Motifs were considered of possible regulatory significance if they were specifically enriched among differentially expressed genes.

ACKNOWLEDGEMENTS

We thank the staff of the University of Washington Center for Expression Arrays for their expert assistance with our microarray experiments and Gerald Kramer for technical assistance. We would also like to thank the VA Medial Research Service for supporting this study and Daniel R Wilson, MD, Professor and Chair of Psychiatry, Creighton University, for administrative support.

DUALITY OF INTEREST

None declared.

REFERENCES

1 Charney DS, Manji HK. Life stress, genes, and depression: multiple pathways lead to increased risk and new opportunities for intervention. *Sci STKE* 2004; **re5**: 2004.
2 Kendler KS, Karkowski LM, Prescott CA. Causal relationship between stressful life events and the onset of major depression. *Am J Psychiatry* 1999; **156**: 837–841.
3 Kendler KS, Thornton LM, Prescott CA. Gender differences in the rates of exposure to stressful life events and sensitivity to their depressogenic effects. *Am J Psychiatry* 2001; **158**: 587–593.
4 Seligman ME, Rosellini RA, Kozak MJ. Learned helplessness in the rat: time course, immunization, and reversibility. *J Comp Physiol Psychol* 1975; **88**: 542–547.
5 Grahn RE, Watkins LR, Maier SF. Impaired escape performance and enhanced conditioned fear in rats following exposure to an uncontrollable stressor are mediated by glutamate and nitric oxide in the dorsal raphe nucleus. *Behav Brain Res* 2000; **112**: 33–41.
6 Neumaier JF, Petty F, Kramer GL, Szot P, Hamblin MW. Learned helplessness increases 5-hydroxytryptamine1B receptor mRNA levels in the rat dorsal raphe nucleus. *Biol Psychiatry* 1997; **41**: 668–674.
7 Newton SS, Thome J, Wallace TL, Shirayama Y, Schlesinger L, Sakai N *et al.* Inhibition of cAMP response element-binding protein or dynorphin in the nucleus accumbens produces an antidepressant-like effect. *J Neurosci* 2002; **22**: 10883–10890.
8 Carlson JN, Fitzgerald LW, Keller Jr RW, Glick SD. Lateralized changes in prefrontal cortical dopamine activity induced by controllable and uncontrollable stress in the rat. *Brain Res* 1993; **630**: 178–187.
9 Frodl T, Meisenzahl EM, Zetzsche T, Born C, Groll C, Jager M *et al.* Hippocampal changes in patients with a first episode of major depression. *Am J Psychiatry* 2002; **159**: 1112–1118.
10 MacQueen GM, Campbell S, McEwen BS, Macdonald K, Amano S, Joffe RT *et al.* Course of illness, hippocampal function, and hippocampal volume in major depression. *Proc Natl Acad Sci USA* 2003; **100**: 1387–1392.
11 Vythilingam M, Heim C, Newport J, Miller AH, Anderson E, Bronen R *et al.* Childhood trauma associated with smaller hippocampal volume in women with major depression. *Am J Psychiatry* 2002; **159**: 2072–2080.
12 Villarreal G, Hamilton DA, Petropoulos H, Driscoll I, Rowland LM, Griego JA *et al.* Reduced hippocampal volume and total white matter volume in posttraumatic stress disorder. *Biol Psychiatry* 2002; **52**: 119–125.
13 Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ. Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience* 1995; **64**: 477–505.
14 Paskitti ME, McCreary BJ, Herman JP. Stress regulation of adrenocorticosteroid receptor gene transcription and mRNA expression in rat hippocampus: time-course analysis. *Brain Res (Mol Brain Res)* 2000; **80**: 142–152.
15 Montpied P, Weizman A, Weizman R, Kook KA, Morrow AL, Paul SM. Repeated swim-stress reduces GABAA receptor alpha subunit mRNAs in the mouse hippocampus. *Brain Res (Mol Brain Res)* 1993; **18**: 267–272.
16 Bartanusz V, Aubry JM, Pagliusi S, Jezova D, Baffi J, Kiss JZ. Stress-induced changes in messenger RNA levels of *N*-methyl-D-aspartate and AMPA receptor subunits in selected regions of the rat hippocampus and hypothalamus. *Neuroscience* 1995; **66**: 247–252.
17 Schwendt M, Jezova D. Gene expression of two glutamate receptor subunits in response to repeated stress exposure in rat hippocampus. *Cell Mol Neurobiol* 2000; **20**: 319–329.
18 Lopez JF, Chalmers DT, Little KY, Watson SJ. A.E. Bennett Research Award. Regulation of serotonin1A, glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: implications for the neurobiology of depression. *Biol Psychiatry* 1998; **43**: 547–573.
19 Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S *et al.* Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 2003; **301**: 805–809.
20 Martin JV, Edwards E, Johnson JO, Henn FA. Monoamine receptors in an animal model of affective disorder. *J Neurochem* 1990; **55**: 1142–1148.
21 Edwards E, Harkins K, Wright G, Henn FA. 5-HT1b receptors in an animal model of depression. *Neuropharmacology* 1991; **30**: 101–105.
22 Wu J, Kramer GL, Kram M, Steciuk M, Crawford IL, Petty F. Serotonin and learned helplessness: a regional study of 5-HT1A, 5-HT2A receptors and the serotonin transport site in rat brain. *J Psychiatric Res* 1999; **33**: 17–22.
23 Edwards E, King JA, Fray JC. Increased basal activity of the HPA axis and renin-angiotensin system in congenital learned helpless rats exposed to stress early in development. *Int J Dev Neurosci* 1999; **17**: 805–812.
24 Lachman HM, Papolos DF, Weiner ED, Ramazankhana R, Hartnick C, Edwards E *et al.* Hippocampal neuropeptide Y mRNA is reduced in a strain of learned helpless resistant rats. *Brain Res (Mol Brain Res)* 1992; **14**: 94–100.
25 Kohen R, Neumaier JF, Hamblin MW, Edwards E. Congenitally learned helpless rats show abnormalities in intracellular signaling. *Biol Psychiatry* 2003; **53**: 520–529.
26 Irwin LN. Gene expression in the hippocampus of behaviorally stimulated rats: analysis by DNA microarray. *Brain Res (Mol Brain Res)* 2001; **96**: 163–169.
27 Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J *et al.* Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 2005; **433**: 769–773.
28 Perrone-Bizzozero N, Bolognani F. Role of HuD and other RNA-binding proteins in neural development and plasticity. *J Neurosci Res* 2002; **68**: 121–126.
29 Benos PV, Lapedes AS, Stormo GD. Is there a code for protein-DNA recognition? Probab(ilistical)ly…. *Bioessays* 2002; **24**: 466–475.
30 Edwards E, Kornrich W, van Houtten P, Henn FA. *In vitro* neurotransmitter release in an animal model of depression. *Neurochem Int* 1992; **21**: 29–35.
31 Kram ML, Kramer GL, Steciuk M, Ronan PJ, Petty F. Effects of learned helplessness on brain GABA receptors. *Neurosci Res* 2000; **38**: 193–198.
32 Kinnunen AK, Koenig JI, Bilbe G. Repeated variable prenatal stress alters pre- and postsynaptic gene expression in the rat frontal pole. *J Neurochem* 2003; **86**: 736–748.
33 Looney SW, el Mallakh RS. Meta-analysis of erythrocyte Na, K-ATPase activity in bipolar illness. *Depression Anxiety* 1997; **5**: 53–65.
34 Zinsmaier KE, Bronk P. Molecular chaperones and the regulation of neurotransmitter exocytosis. *Biochem Pharmacol* 2001; **62**: 1–11.
35 Drigues N, Poltyrev T, Bejar C, Weinstock M, Youdim MB. cDNA gene expression profile of rat hippocampus after chronic treatment with antidepressant drugs. *J Neur Transm* 2003; **110**: 1413–1436.
36 Thome J, Pesold B, Baader M, Hu M, Gewirtz JC, Duman RS *et al.* Stress differentially regulates synaptophysin and synaptotagmin expression in hippocampus. *Biol Psychiatry* 2001; **50**: 809–812.
37 Crispino M, Stone DJ, Wei M, Anderson CP, Tocco G, Finch CE *et al.* Variations of synaptotagmin I, synaptotagmin IV, and synaptophysin mRNA levels in rat hippocampus during the estrous cycle. *Exp Neurol* 1999; **159**: 574–583.

38 Jenkins JA, Williams P, Kramer GL, Davis LL, Petty F. The influence of gender and the estrous cycle on learned helplessness in the rat. *Biol Psychol* 2001; **58**: 147–158.
39 Altar CA, Laeng P, Jurata LW, Brockman JA, Lemire A, Bullard J *et al.* Electroconvulsive seizures regulate gene expression of distinct neurotrophic signaling pathways. *J Neurosci* 2004; **24**: 2667–2677.
40 Suzdak PD, Gianutsos G. Parallel changes in the sensitivity of gamma-aminobutyric acid and noradrenergic receptors following chronic administration of antidepressant and GABAergic drugs. A possible role in affective disorders. *Neuropharmacology* 1985; **24**: 217–222.
41 Drugan RC, Morrow AL, Weizman R, Weizman A, Deutsch SI, Crawley JN *et al.* Stress-induced behavioral depression in the rat is associated with a decrease in GABA receptor-mediated chloride ion flux and brain benzodiazepine receptor occupancy. *Brain Res* 1989; **487**: 45–51.
42 Wilson RI, Nicoll RA. Endocannabinoid signaling in the brain. *Science* 2002; **296**: 678–682.
43 Nielsen MS, Jacobsen C, Olivecrona G, Gliemann J, Petersen CM. Sortilin/neurotensin receptor-3 binds and mediates degradation of lipoprotein lipase. *J Biol Chem* 1999; **274**: 8832–8836.
44 Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS *et al.* Sortilin is essential for proNGF-induced neuronal cell death. *Nature* 2004; **427**: 843–848.
45 Shimizu S, Matsuoka Y, Shinohara Y, Yoneda Y, Tsujimoto Y. Essential role of voltage-dependent anion channel in various forms of apoptosis in mammalian cells. *J Cell Biol* 2001; **152**: 237–250.
46 Hochedlinger K, Wagner EF, Sabapathy K. Differential effects of JNK1 and JNK2 on signal specific induction of apoptosis. *Oncogene* 2002; **21**: 2441–2445.
47 Ruvolo PP, Clark W, Mumby M, Gao F, May WS. A functional role for the B56 alpha-subunit of protein phosphatase 2A in ceramide-mediated regulation of Bcl2 phosphorylation status and function. *J Biol Chem* 2002; **277**: 22847–22852.
48 Steciuk M, Kram M, Kramer GL, Petty F. Decrease in stress-induced c-Fos-like immunoreactivity in the lateral septal nucleus of learned helpless rats. *Brain Res* 1999; **822**: 256–259.
49 Slonim DK. From patterns to pathways: gene expression data analysis comes of age. *Nat Genet* 2002; **32**(Suppl): 502–508.
50 Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000; **132**: 365–386.
51 Duret L, Bucher P. Searching for regulatory elements in human noncoding sequences. *Curr Opin Struct Biol* 1997; **7**: 399–406.
52 Hubbard T, Barker D, Birney E, Cameron G, Chen Y, Clark L *et al.* The Ensembl genome database project. *Nucleic Acids Res* 2002; **30**: 38–41.
53 Wray GA, Hahn MW, Abouheif E, Balhoff JP, Pizer M, Rockman MV *et al.* The evolution of transcriptional regulation in eukaryotes. *Mol Biol Evol* 2003; **20**: 1377–1419.
54 Benson G. Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Res* 1999; **27**: 573–580.
55 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990; **215**: 403–410.
56 Stajich JE, Block D, Boulez K, Brenner SE, Chervitz SA, Dagdigian C *et al.* The Bioperl toolkit: perl modules for the life sciences. *Genome Res* 2002; **12**: 1611–1618.
57 Matys V, Fricke E, Geffers R, Gossling E, Haubrock M, Hehl R *et al.* TRANSFAC: transcriptional regulation, from patterns to profiles. *Nucleic Acids Res* 2003; **31**: 374–378.
58 Sandelin A, Alkema W, Engstrom P, Wasserman WW, Lenhard B. JASPAR: an open-access database for eukaryotic transcription factor binding profiles. *Nucleic Acids Res* 2004; **32**(Database issue): D91–D94.
59 Bailey TL, Gribskov M. Methods and statistics for combining motif match scores. *J Comput Biol* 1998; **5**: 211–221.
60 Crooks GE, Hon G, Chandonia JM, Brenner SE. WebLogo: a sequence logo generator. *Genome Res* 2004; **14**: 1188–1190.