

Temporal change in gene expression in the rat dentate gyrus following passive avoidance learning

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Abstract

A learning event initiates a cascade of altered gene expression leading to synaptic remodelling within the hippocampal dentate gyrus, a structure vital to memory formation. To illuminate this transcriptional program of synaptic plasticity we used microarrays to quantify mRNA from the rat dentate gyrus at increasing times following passive avoidance learning. Approximately, 500 known genes were transcriptionally regulated across the 24 h post-training period. The 0–2 h period saw up-regulation of genes involved in transcription while genes with a role in synaptic/cytoskeletal structure increased 0–6 h, consistent with structural rearrangements known to occur at these times. The most striking feature was the profound down-regulation, across all functional groups, 12 h post-training. Bioinformatics analysis

identified the likely transcription factors controlling gene expression in each post-training period. The role of NFκB, implicated in the early post-training period was subsequently confirmed with activation and nuclear translocation seen in dentate granule neurons following training. mRNA changes for four genes, LRP3 (0 h), alpha actin (3 h), SNAP25 and NSF (6–12 h), were validated at message and/or protein level and shown to be learning specific. Thus, the memory-associated transcriptional cascade supports the cardinal periods of synaptic loosening, reorganisation and selection thought to underpin the process of long-term memory consolidation in the hippocampus.

Keywords: dentate gyrus, memory consolidation, microarray, transcription factor.

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When acquired initially, newly learned information is held in a fragile state becoming robust over time (McGaugh 2000). This consolidation process is driven by the hippocampus and requires *de novo* RNA translation and protein synthesis (Stork and Welzl 1999; Igaz *et al.* 2002), processes that provide the materials to allow the necessary structural changes in synaptic connections between neurons (Lamprucht and LeDoux 2004). In the hippocampus, such synaptic plasticity involves three key phases: synaptic loosening, synaptic reorganisation and synaptic selection. Synaptic loosening occurs when adhesive elements, such as neural cell adhesion molecule (NCAM), are selectively internalised and degraded (Foley *et al.* 2000). Synaptic reorganisation is evidenced by changes in synaptic number, synaptic curvature, size of synaptic elements and synaptic perforations (Marrone and Petit 2002). For example, quantitative electron

microscopic analysis of the rat hippocampal dentate gyrus has revealed an increase in dendritic spine and synaptic density, 6–9 h following either avoidance conditioning or spatial learning (O'Malley *et al.* 1998, 2000; Eyre *et al.*

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Abbreviations used: EST, expressed sequence tag; IEG, immediate early gene; Lrp, low density lipoprotein receptor related protein; NFκB, nuclear factor kappa B; NSF, *N*-ethylmaleimide sensitive factor; PAGE, polyacrylamide gel electrophoresis; SNAP25, synaptosomal-associated protein 25 kDa; SNARE, soluble NSF-attachment protein receptor; TF, transcription factor; TFBS, transcription factor binding site.

2003). This increase in synaptic number is transient, however, returning to basal levels by 24–48 h post-training. These latter observations are indicative of a period of synaptic selection and elimination thought to be essential for the retention of only relevant connections in the memory circuit (Regan 2004).

Several groups have used the large-scale screening of mRNA by microarrays to study the consolidation process in different learning paradigms at a transcriptional level (Cavallaro *et al.* 2002; D'Agata and Cavallaro 2003; Ressler *et al.* 2002; Leil *et al.* 2003; Igaz *et al.* 2004). These changes detected by microarray are usually considered indicative of transcriptional regulation but it must be noted that they could be because of altered RNA processing and post-transcriptional control (Mata *et al.* 2005). These studies have identified lists of genes that could be grouped into functional families including; transcription/translation, signal cascade enzymes, structural proteins and neural transmission. Although complex, this data begins to offer a picture of the transcriptional events that occur during memory consolidation, however, several issues remain to be addressed. Firstly, to fully understand the cascade of processes occurring during memory consolidation a more complete temporal picture of gene expression is required. In particular, the periods of synaptic loosening and reorganisation, 2–6 h post-training, and the period of synaptic selection and elimination, 10–24 h post-training, remain poorly understood. Secondly, because of the heterogeneity of plasticity mechanisms in different hippocampal sub-fields, focussing on the gateway structure, the dentate gyrus, alone may be more informative than analysis of the whole hippocampus. Finally, instead of simply reporting the 'most changed genes', which fails to utilise the true power of microarrays, the underlying mechanism of transcriptional control can be elucidated by studying the overall pattern of gene expression (Schulze and Downward 2001).

To address these issues, we studied the gene expression profile following passive avoidance training at six carefully chosen time points, up to and including 24 h post-training. This transcriptional profile was carried out in the hippocampal dentate gyrus, a structure in which physical changes are known to occur during memory consolidation (O'Malley *et al.* 1998, 2000; Eyre *et al.* 2003). Finally, the overall pattern of gene expression and the upstream elements involved in their control were studied, generating a comprehensive map of the transcriptional events underlying memory consolidation.

Materials and methods

Animal maintenance and training

Post-natal day 80 male Wistar rats (330–350 g) were individually housed on a 12 : 12 light/dark cycle, with *ad libitum* access to food and water. Animals were assessed for 3 days prior to passive avoidance training by observing their open field behaviour. The one-

trial, step-through, light-dark version of the passive avoidance paradigm was employed as we have described previously (Fox *et al.* 1995). The smaller illuminated compartment was separated from the larger dark compartment by a shutter that contained a small entrance. The floor of the training apparatus consisted of a grid of stainless steel bars that would deliver a scrambled shock (0.75 mA every 0.5 ms) for 5 s when the animal entered the dark chamber. A parallel group of animals were tested for recall of the task to demonstrate robust learning. In order to determine the sequential expression of mRNA in the rat dentate gyrus during memory consolidation, animals were trained in the passive avoidance paradigm and then sacrificed at discrete time points 0, 2, 3, 6, 12 and 24 h following training. Naïve littermates were treated precisely as their trained animals except they were not exposed to the passive avoidance box prior to sacrifice. For the real-time PCR and western immunoblot confirmation studies a second series of animals were trained and sacrificed at these same post-training times along with passive and yoked control animals at 3 and 12 h. Passive controls were exposed to the avoidance apparatus for a time matched to trained counterparts but never received a foot-shock. Yoked controls received an unavoidable foot shock of 5 s duration, without chamber partition. All animals were killed by cervical dislocation, the dentate gyrus rapidly dissected. The dentate gyrus was readily separated from the CA regions of the hippocampus with the use of a forceps inserted into the natural fissure between the suprapyramidal surface of the dentate and the overlying CA1 structure. Our own histological analysis of this dissected tissue has shown it to contain only the intact dentate gyrus cut at the ends of the dentate granular cell blades. All experimental procedures were approved by the Animal Research Ethics Committee of the Biomedical Facility at University College, Dublin, and were carried out by individuals who held the appropriate licence issued by the Minister for Health and Children.

Microarray and real-time sample preparation

Total RNA was extracted from each dentate gyrus by homogenisation in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and following the TRIzol protocol. The resulting RNA samples were purified using an RNeasy mini kit (Qiagen, UK). RNA concentration was determined spectrophotometrically, and RNA integrity was confirmed by agarose gel electrophoresis.

Double-stranded DNA was synthesised from total RNA (15 µg) using the Superscript Choice System (Invitrogen) and a HPLC-purified T7-d(T)₂₄ primer (Sigma Genosys; 5' –GGCCAGT-GAATTGTAATACGACTCAC TATAGGGAGGCGG-(dT)₂₄ – 3'). *In vitro* transcription using double-stranded cDNA (1 µg) as a template in the presence of biotinylated UTP and CTP was carried out using BioArray™ high yield RNA transcript labelling kit (Enzo Diagnostics, Farmingdale, NY, USA). The biotin-labelled cRNA was then purified, quantified by absorbance at 260 nm and fragmented by incubation at 94°C for 35 min in 40 mmol/L Tris–acetate, pH 8.1, 100 mmol/L potassium acetate, and 80 mmol/L magnesium acetate.

Microarray analysis

After hybridisation to Gene Chip test 3 arrays, to test quality of the targets, each sample ($2 \leq n \leq 3$ per post-training time point) was hybridised to an Affymetrix rat genome U34A array using the

protocol outlined in the GeneChip Expression Analysis Technical Manual (Affymetrix Inc., Santa Clara, CA, USA). The arrays were washed and stained using a fluidics system with streptavidin–phycoerythrin (Molecular Probes, Invitrogen), amplified with biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, CA, USA), and then scanned with a Gene Array Scanner (Affymetrix Inc.). The image data were analysed using Affymetrix microarray suite version 4.0 (MAS4; Affymetrix Inc.). The data was then normalised, filtered and analysed using GeneSpring™ version 4.2 software (Silicon Genetics, Redwood City, CA, USA).

All the raw data underwent two rounds of normalisation. For the per chip normalisation, each measurement for each gene was divided by the 50th percentile of all measurements. For the per gene normalisation, each sample was normalised to an average of the naïve samples ($n = 3$). This allowed the analysis of the temporal expression regulation relative to the naïve value. An average fold change of greater than 1.9 was used as the cut off for significant differences in gene expression. Genes that were switched ‘ON’ or ‘OFF’ following training were also included in this study. Genes that switched OFF following training were genes that were deemed present in all the naïve samples but were deemed absent in at least one of the post-training time points. Similarly, genes that switched ON following training were genes that were deemed absent in the naïve samples but were present in at least one of the post-training times.

Quantitative real-time PCR

Real-time PCR was carried out using TaqMan technology on an ABI Prism 7700HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). cDNAs, from 1 µg of DNase treated RNA from each animal ($3 \leq n \leq 8$ per group) were produced using SuperScript II RNase H Reverse Transcriptase Kit (Invitrogen) and 50–250 ng random primers (Invitrogen). cDNA (2 µL) from each animal was amplified using specific primers (Table 1). Relative quantitation was determined by constructing a standard curve for each primer and probe set, using pooled DNA from all the samples. A ribosomal RNA control primer and probe set (Applied Biosystems) was used for normalisation purposes.

Protein sample preparation

Each dentate gyrus ($n = 3$) from naïve and trained animals at 0, 2, 4, 6, 12 and 24 h post-training, was homogenised in ice-cold 0.32 mol/L sucrose. Protein concentrations were determined according to the method of Bradford (1976). Samples, of equal protein concentrations, were prepared in reducing sample buffer [3X Blue loading

buffer with 10% (v/v) dithiothreitol (DTT) (New England Biolabs)] and boiled at 100°C.

SDS-PAGE and immunoblotting

Normalised proteins samples were separated on polyacrylamide minigels and electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Equal protein loading was confirmed by ponceau S staining of the membrane (not shown). The nitrocellulose was blocked in 5% non-fat milk in 10 mmol/L Tris–HCl, 150 mmol/L NaCl, and 0.05% (vol/vol) Tween-20 (TBS-T) for 1 h at 20°C. Monoclonal antibodies to Lrp (American Diagnostica, Stamford, CT, USA), SNAP25 (Sigma, St Louis, MO, USA), NSF (Calbiochem, Temecula, CA, USA) and smooth muscle alpha-actin (Sigma) were diluted in 5% milk and incubated overnight at 4°C. The membranes were then incubated with appropriate secondary horseradish peroxidase-linked antibodies and visualised by Super-Signal Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and exposed to X-ray film (Kodak, New Haven, CT, USA) for appropriate times. Films were digitised and quantitative densitometry was performed using NIH Image software (Version 1.61) to determine amount of target protein (SNAP25, NSF or smooth muscle alpha-actin) electrophoresed per sample. Target protein expression changes, across all post-training time points, were calculated as a percentage of naïve.

Statistical analysis

Statistical significance of post-training mRNA and protein expression modulations were determined using the Student’s two-tailed unpaired *t*-test. A *p*-value less than 0.05 was considered to be significant.

Bioinformatic transcription factor identification

Significantly regulated genes were grouped according to time-point. Since transcription factors can operate as both activators and repressors, genes that were up-regulated, down-regulated and switched either on or off were all grouped together for the purpose of the analysis. The human, mouse and rat sequences were extracted from Ensembl database (Hubbard *et al.* 2005). The 5 prime UTR plus the 5 prime flanking region of 5000 bp was extracted for all annotated genes in each of the genomes: Human (Assembly NCBI34), Mouse (NCBIM33), Rat (RGSC3.1). The homologue database (Wheeler *et al.* 2006) was used to cross-reference the rat genes to the equivalent human and mouse genes. Repeats were then masked from the sequence using the tool Repeatmasker (Smit *et al.*

Table 1 Summary of primer and probe information used in real-time PC validation of selected gene changes

Gene	Forward primer	Probe	Reverse primer
Lrp3 (AB009463)	GTGACCACGTCT GGATCTTCTTC	CGCCTCCAGTTCGGCCAGO	TCGGATATAGGAGAGGCGAAAA
SNAP25 (BC087699)	GTGGATGAACGGGAGCAGAT	CATCAGTGOTGGCTTCATCCGC	CCGGGCATCGTTTGTTACC
NSF (AF091834)	GAAGGGCCGACTGCAGATC	CATCCACACAGCAAGGATGCGAGG	CCACATCCGCAGACAGTAGCT
Alpha-actin (M22757)	GGAAGGTAGATAGAGAAGCCAGGAT	AGCCGCCGATCCAGACAGAATATTTGC	GATCAAGATTATTGCTCCTCCAGAA

1996–2004). The sequences were aligned using the AVID software (Bray *et al.* 2003). The alignments were then parsed to extract the conserved regions between the species for transcription factor binding site (TFBS) analysis. The Transfac public databases v6.0 (Wingender *et al.* 1996) was used as the source of the transcription factor binding site motifs. The position weight matrices used in Transfac were converted to log-odds matrices for use with the MAST application (Bailey and Gribskov 1998). In total 232 matrices corresponding to 148 different mammalian transcription factors were analysed. The application MAST was used to scan the conserved upstream regions of each gene in the rat genome for TFBSs. The output of MAST was parsed to calculate the number of genes containing binding sites for each transcription factor. The binomial test for over-representation was constructed as follows:

x_i is the number of genes with a conserved TFBS for transcription factor i ; N the total number of genes in genome; p_i the probability of finding a binding site for transcription factor i in 5 kbp upstream region of a gene $p_i = \frac{x_i}{N}$; k_{ij} represents the number of co-regulated genes with a conserved TFBS for transcription factor i at timepoint j ; and K_j is the total number of co-regulated genes at time point j .

Binomial test calculate probability of getting k_{ij} out of K_j genes with TFBS for transcription factor i at time j given probability of success p_i .

The value of the binomial distribution function was calculated using function *pbinom* in software R (Ihaka and Gentleman 1996). The p -value is then calculated as follows:

$$p \text{ val} = 1 - p \text{ binom}(k_{ij}, K_j, p_i)$$

Cryosectioning and immunofluorescence

Approximately, 12 μ m ventral hippocampal sections were taken at -7.1 with respect to bregma from naïve, 3 h trained and passive control brains that had been immediately fresh frozen in OCT following decapitation ($n = 4$). Sections were adhered to glass slides coated with poly-L-lysine. Sections were made permeable using a solution of 0.1% Triton X in phosphate-buffered saline (PBS) for 30 min, fixed in 70% ethanol for 30 min and incubated overnight (18 h) with the primary antibody MAB3026 (Chemicon), which binds to an epitope overlapping the nuclear location signal on the p65 subunit of the NF κ B heterodimer thus selectively binding to the activated form of NF κ B. Following two 10 min washes in PBS, sections were incubated for 3 h with an anti-mouse IgG secondary antibody labelled with the fluorescent marker FITC. The secondary antibody was then washed off and sections were dipped in propidium iodide (PI) for 5 s in order to stain the nuclei red. Each section was mounted in Citifluor® (Agar, UK), a fluorescence-enhancing medium, and the slides were stored in darkness at 4°C. Confocal images were taken using the 40 \times /0.8 W water-dipping lens (Zeiss Achroplan, Hertfordshire, UK). Images were captured from two distinct regions of the hippocampus, the dentate granule neurons and hilar polymorphic cells.

Image analysis

Approximately, 50 nuclei per image were randomly selected for analysis. Using the confocal software package LSM Pascal, an 18 μ m line was drawn across each nucleus and the fluorescence intensities for NF κ B and PI staining obtained at each point along the length of the line. In this manner, nuclear NF κ B fluorescence intensities were measured as well as the intensity of staining at

increasing distances from the nucleus. The nuclear boundaries were defined as the point at which the PI fluorescence intensity value dropped to 20% of the maximum for each individual cell analysed. The cytoplasmic regions were defined as the area lying at least 2 μ m either side of the nuclear boundary. NF κ B activity was measured as the ratio of nuclear to cytoplasmic NF κ B-associated fluorescence. This was calculated by taking the average nuclear and cytoplasmic fluorescence intensities for each cell and the ratio calculated according to the equation:

$$(F_n - F_c)/F_c$$

where F_n is the average nuclear fluorescence intensity and F_c the average cytoplasmic fluorescence intensity. The mean value for each animal was then normalised to the mean value for the naïve animal group.

Results

Passive avoidance training

Parallel to those employed in the microarray study, an additional animal group was trained in passive avoidance and subsequently tested for task recall 24 h post-learning. On recall, most trained animals remained in the light box for the criterion latency time of 600 s (592.2 ± 7.8 s, mean \pm SEM time to enter dark chamber, $n = 5$) while passive control animals had a latency time of 10.2 ± 1.6 s (mean \pm SEM; $p = 0.0079$ vs. trained animals, Mann-Whitney U -test). The robust recall of these trained animals supports solid learning in the groups analysed by microarray and real time PCR. These latter animals were not tested for their latency to enter the dark chamber prior to sacrifice in order to avoid contamination of consolidation transcriptional events with those that would undoubtedly occur during a 600 s recall session.

Microarray analysis

To generate an initial picture of hippocampal dentate gene changes in the first 24 h period following avoidance learning, gene expression was measured using oligonucleotide-based DNA microarrays (U34A chip, Affymetrix) that express 8740 probe sets corresponding to approximately 7000 annotated rat genes and approximately 1700 expressed sequence tags (ESTs). Using a 1.9-fold change, genes turned ON or genes turned OFF criteria, the mRNA levels of 597 genes/ESTs (6.8%) were found to have increased or decreased at some point during the 24-h period following training with respect to expression levels in the naïve controls. Of these, approximately 80 were ESTs with no known function.

A master list of the ~ 500 known genes was further analysed based on gene function using literature searches on the web available database Entrez Gene (<http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene>). The genes were categorised based on their reported or suggested functions and placed into one of ten broad functional groups: regulation of

Table 2 Representative gene regulations in the dentate gyrus following passive avoidance learning

Time post-training (h)						Gene name	Accession number
0	2	3	6	12	24		
<i>Transcription/translation</i>							
↑	–	–	–	–	–	Translation initiation factor (EiEbl)	NM_172029
↑	–	–	–	–	–	Heterogeneous nuclear ribonucleoprotein (hnRNP)	XR_008097
–	–	–	↑	–	–	RNA binding motif protein 9 (Rtm9)	XM_343281
–	–	–	↑	–	–	Ribosomal protein L5 (Rpl5)	NM_031099
–	–	–	–	↓	–	Translation elongation factor (Eef2)	AA892801
<i>Cellular structure</i>							
↑	–	–	–	–	–	Synuclein, alpha (Snca)	S73007
–	↑	–	–	–	–	Tenascin R	Z18630
↓	–	↑	–	↓	–	<i>Smooth muscle alpha-actin (Acta)</i>	M22757
–	–	–	–	–	↓	Myelin-associated oligodendrocytic basic protein (Mobp)	D28110
–	–	–	–	–	↓	Myelin-associated oligodendrocytic basic protein (Mobp)	D28111
<i>Cellular trafficking</i>							
↑	–	–	–	–	–	<i>Low density lipoprotein receptor-related protein 3 (Lrp3)</i>	AB009463
–	↓	–	↓	↓	–	Dynamin I-like protein (Dlpl)	AF020211
–	–	–	↓	↓	–	ADP-ribosylation factor 3 (ArG)	L12382
–	–	–	↓	↓	–	Kinesin family member 3C(Kif3c)	AF083330
–	–	–	–	↓	–	Kinesin family member 3C(Kif3c)	NM_053486
<i>Neurotransmission/ion conductance</i>							
–	–	–	↓	↓	–	<i>Synaptosomal-associated protein, 25 kDa (SNAP25)</i>	BC087699
–	–	–	↓	↓	–	<i>N-ethylmaleimide-sensitive factor (NSF)</i>	AF091834
–	–	–	–	↓	–	Synaptotagmin IV (Syt4)	U14398
–	–	–	–	↓	–	ATPase, NA+K+ transporting, alpha 1 (Atplal)	M74494
–	–	–	–	↓	–	ATPase, NA+K+ transport ing, beta2 (AtplbZ)	J03754
<i>Second messenger signals</i>							
–	↑	–	–	–	–	Calcium/calmodulin-dependent protein kinase II beta	M16112
–	–	–	–	↓	–	Mitogen activated protein kinase 1 (MAPK1)	M64300
–	–	–	–	↓	–	Mftogen activated protein kinase kinase 1 (MAPKK1)	D14591
–	–	–	–	↓	–	Protein kinase, cAMP-dependent, catalytic, beta	XM_215070
–	–	–	–	↓	–	Protein kinase, cAMP-dependent regulatory, beta	M12492
<i>Protein processing</i>							
–	–	–	–	↓	–	Ubiquitin-conjugating enzyme E21 (Ube2i)	U54632
–	–	–	–	↓	–	Calnexin (Canx)	L18889
–	–	–	–	↓	–	CathepsinD (CtsD)	BC062032
–	–	–	–	↓	–	Vasolin-containing protein (Vcp)	BC060518
–	–	–	–	↓	–	DnaJ homolog, subfamily b, member 9 (Dnajb9)	A1011998
<i>Metabolism</i>							
–	↓	–	–	–	–	ATP citrate lyase (Acly)	J05210
–	↓	–	–	–	–	Brain acyl-CoA hydrolase (Bach)	Y09332
–	–	–	–	↓	–	Sterol-Coenzyme A desaturase 2 (Scd2)	U67995
–	–	↓	↓	↓	–	Sterol-Coenzyme A desaturase 2 (Scd2)	AF036761
–	–	–	–	↓	–	Lipoprotein lipase (Lpl)	BC081836
<i>Cell growth factors/immune signalling</i>							
↑	–	–	–	–	–	Brain derived neurotrophic factor (BDNF)	D10938
↓	–	–	–	↓	–	Insulin-like growth factor 2 (Igf2)	X17012
↓	–	–	↓	↓	–	Insulin-Ike growth factor binding protein (Igfb2)	J04486
–	–	–	–	↓	–	Fibroblast growth factor receptor 1 (Fgfrl)	D12498
–	–	–	–	↓	–	Fibroblast growth factor receptor 1 (Fgfrl)	S54008
<i>Cell surface receptors</i>							
–	↓	–	–	–	–	CD9 cell surface glycoprotein (Cd9)	X76489
–	↓	–	–	–	–	Thy cell surface glycoprotein (Cd7)	X02002
–	–	–	–	–	↓	G-protein coup led receptor (Lrg4)	AF061443

Table 2 Continued

Time post-training (h)						Gene name	Accession number
0	2	3	6	12	24		
↑	–	–	–	↑	–	Muscarinic receptor m2 (Chrm2)	AB017655
–	–	–	–	–	↑	Chemokine (C-C) receptor 5 (Cmrkb5)	Y12009

↑, Up-regulated; –, no change; ↓, down-regulated.

Genes printed in bold italics were verified by real-time PCR.

transcription/translation, cellular structure, cell adhesion, intercellular trafficking, neurotransmission and ion conductance, second messenger signals, protein processing, energy metabolism, cell growth factors/immune signals and cell surface receptors. Examples of each of these classifications can be seen in Table 2. It should be noted that a small proportion of genes could of course fit into more than one category.

Temporal map of gene changes associated with passive avoidance learning: Implications for memory-associated synaptic plasticity

Functional analysis identified the numbers of genes up- and down-regulated and facilitated identification of the overall molecular events at each post-training time point (Fig. 1a). Regulators of transcription were found to be increased at very early times post-training with ~22% of genes up-regulated 0–2 h having a role in transcription/translation (Fig. 1b). Further, there is evidence of a second, later wave of transcription/translation at 6 h post-training. Large numbers of signalling molecules also modulate 0–2 h post-training with the end of this period marked, in particular, by down-regulation of genes involved in metabolism, of which ~60% function explicitly in fatty acid metabolism (Fig. 1b). During the subsequent period, 2–6 h post-training, ~22% of genes up-regulated were found to have a role in cellular structuring or adhesion (Fig. 1b). Later, at 12 h post-training, there was profound down-regulation across all functional groups. Of particular note, almost all (~87%) the regulated genes in the protein-processing group had decreased expression at this specific time (Fig. 1b). Finally, across gene regulations at 24 h post-training, the best-represented functional group is that of cellular restructuring, particularly those regulating myelination.

Validation of microarray data

As well as the usual potential difficulties that accompany microarray studies where mRNA change, protein level change and functional relevance remain to be validated for any given mRNA change seen on the array, in our study the broad temporal approach necessitated relatively low numbers per time point and presents the possibility that gene changes are not specific to the learning event. For

example, gene expression changes seen during the 24 h period may simply reflect circadian rhythms. Thus, for any gene change in our study it is at least necessary to validate the mRNA change and show learning specificity.

The expression of four genes were chosen to be validated; synaptosomal-associated protein 25 (SNAP25), *N*-ethylmaleimide sensitive factor (NSF), smooth-muscle alpha-actin and low density lipoprotein receptor-related protein 3 (Lrp3). These genes were selected as they modulated at different times and represented different functional groups defined in the microarray study, namely: neurotransmission, cellular structuring and cellular trafficking. The changes in mRNA across the time points were studied using quantitative real-time PCR. This independent analysis, carried out on a new sample set distinct from that used for the microarray study, confirmed the transcriptional changes observed following passive avoidance training. The microarray analysis deemed the Lrp3 mRNA absent in the naïve group and all post-training times except immediately following training when mRNA expression increased to levels that allowed a present call (Fig. 2). Real-time PCR analysis verified this expression profile. Specifically, Lrp3 mRNA levels significantly increased 0–2 h after training, returning to basal levels by 3 h. Previously published results from our group demonstrate this mRNA expression increase translating into protein level alterations 2–4 h following avoidance learning, is specific to learning as it is not observed in passive controls and, moreover, that LRP function is required at this precise time window for memory consolidation (Fig. 2; Conboy *et al.* 2005).

The structural protein alpha-actin microarray result showed relatively small down regulations at 0 and 12 h but a substantial increase in mRNA levels at 3 h post-training (Fig. 2). While the decreases failed to replicate with real time PCR, the 3 h increase was clearly validated and, moreover, proved to be learning specific as time-matched passive and yoked control animals exhibited alpha-actin mRNA levels indistinguishable from those of the naïve group. In contrast, while alpha-actin protein levels did increase 2–24 h post-training, due to high variability, none of these increases proved significant.

The SNARE complex proteins, SNAP25 and NSF, were both predicted by the microarray to decrease markedly at message level 6–12 h following passive avoidance training

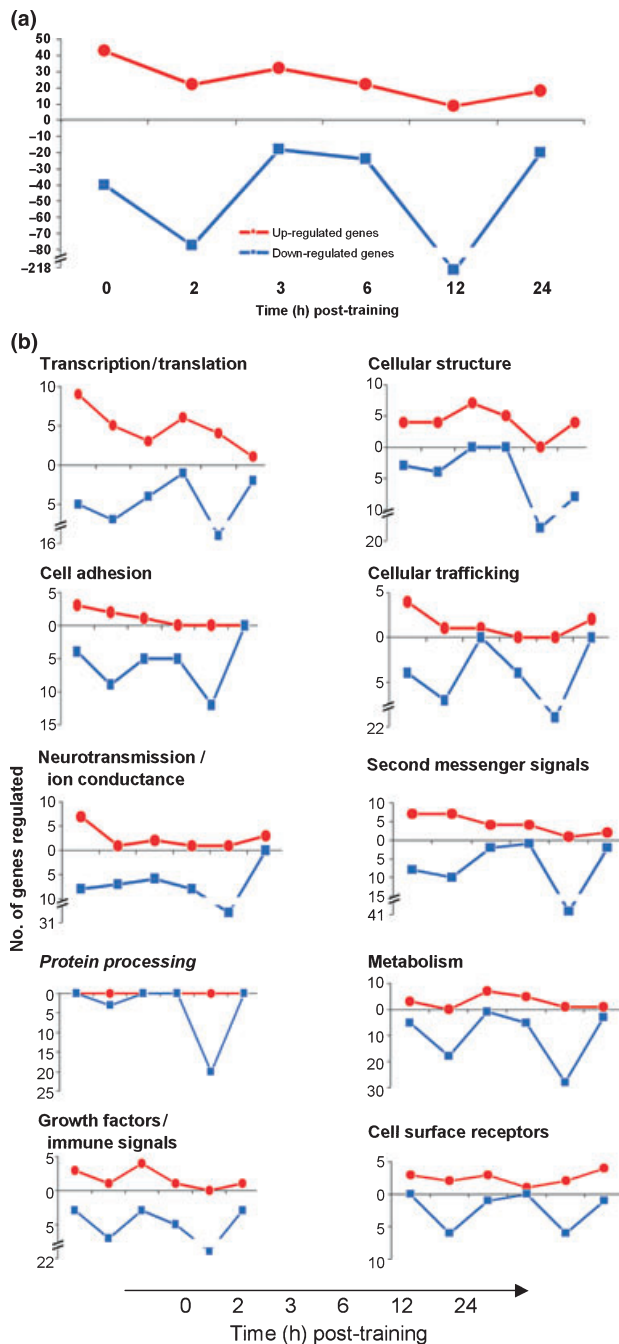


Fig. 1 Temporal profile of gene changes across the first 24 h following passive avoidance training. Panel a graphs the total number of genes regulating at each post-training time. Panel b breaks this down into gene changes for each functional grouping.

(Fig. 3). As with both *Lrp3* and α actin, real-time PCR confirmed this temporal pattern of mRNA expression for SNAP25 and NSF. Moreover, western immunoblot analysis demonstrated corresponding protein level decreases for these SNARE components. Crucially, both mRNA and protein

expression changes were shown to be learning specific as neither was observed in time-matched passive and yoked control animals at the 12 h time point.

Temporal mapping of transcriptional control

We were interested to investigate the nature of transcriptional control at different post-training time-points. Identifying common transcriptional elements controlling a group of co-regulating genes is made difficult by the simple fact that transcription factor binding motifs are small and degenerate and so any given stretch of DNA is likely to have putative binding sites for many transcription factors. Here, we took transcription factor binding site (TFBS) motifs from the Transfac public databases v6.0 (Wingender *et al.* 1996) and scanned across the 5 kb upstream regions for all genes in the genome for these TFBSs. We reduced the noise in our motif analysis by using orthologous DNA sequences from 3 species, rat, human and mouse to determine conserved motifs, as such evolutionarily conserved motifs are likely to be most functionally relevant (Fig. 4). Differentially expressed genes from the microarray experiment were then analysed and Table 3 details the statistically over-represented motifs present on the gene groups regulating at each post-training time. Over half (35/67) of these motifs bind transcription factors already known to be involved in brain development and synaptic plasticity. The remaining TFBSs have not previously been implicated in synaptic plasticity or memory formation. Several of this latter group, however, do exhibit highly restricted tissue expression patterns in the mouse brain supportive of a role in hippocampal function and synaptic plasticity. By way of example, *FoxJ2* expression has recently been shown to be highly restricted to the adult mouse hippocampus (Wijchers *et al.* 2005).

Validation of TFBS analysis

To validate a role for transcription factors implicated by the above bioinformatics analysis in memory consolidation, we used confocal imaging and a customised image analysis approach, to quantify NF κ B activity in different cellular compartments within the dentate gyrus, namely the dentate granule neurons and hilar polymorphic cells, following avoidance learning. Nuclear NF κ B activity increased in the dentate granule neurons 3 h post-training, the time at which the bioinformatics analysis predicted its involvement in transcriptional regulation (Fig. 5 and Table 3). This rise in nuclear NF κ B activity was reflected by a similar increase in the perinuclear region, suggesting a true activation/nuclear re-localisation process (data not shown). Importantly, the increased NF κ B activity was not observed in passive control animals. In addition, increased NF κ B activity following learning was specific to the granule neurons as it was not observed in the polymorphic cell population of the hilus (Fig. 5).

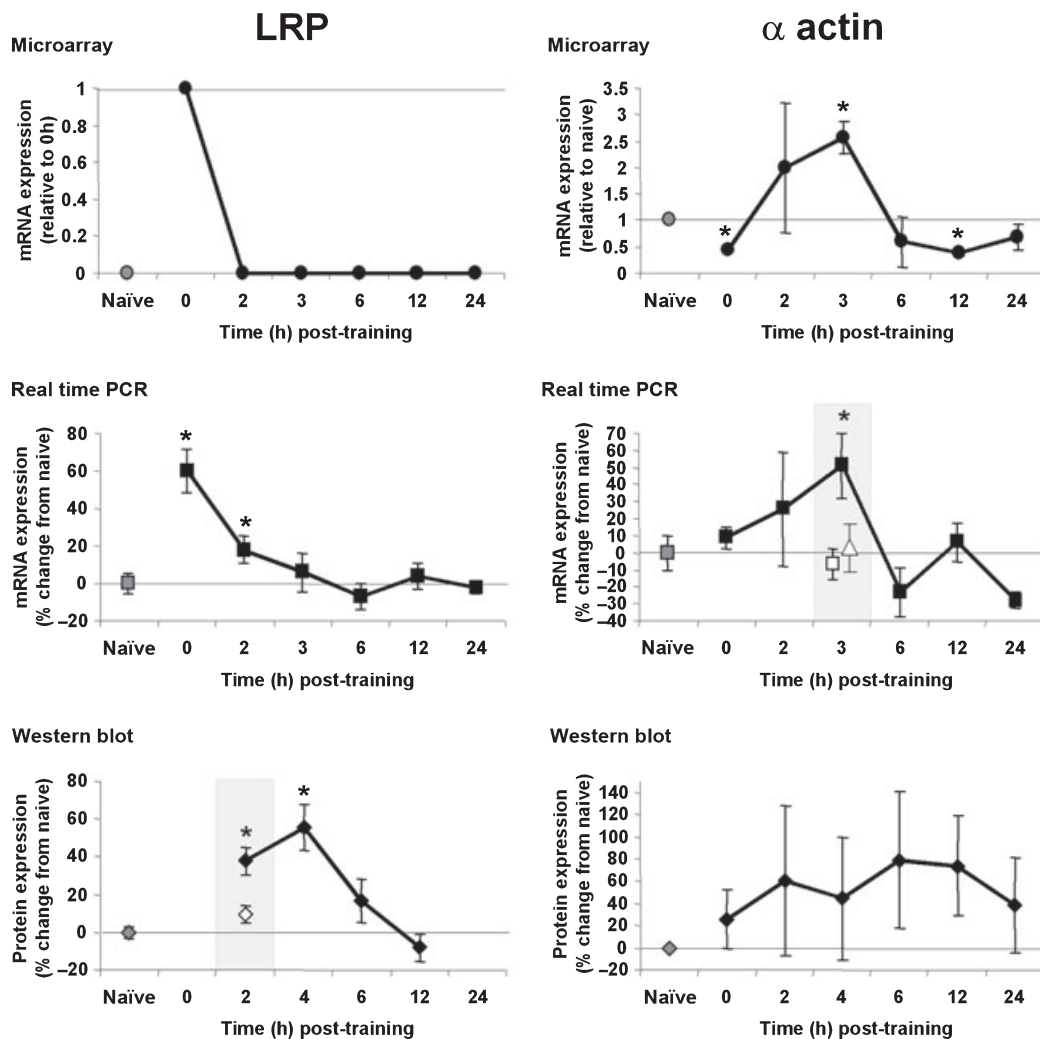


Fig. 2 Learning specific regulation of *Lrp3* and α actin. For each gene, the graphs report the microarray and real-time PCR mRNA expression and analysis of protein expression by densitometric western-immunoblotting. Microarray results are expressed as the mean \pm SEM ($2 \leq n \leq 3$) value normalised to the mean expression of that gene in the naïves ($n = 3$). An asterisk indicates the times where gene changes were deemed significant on the 1.9-fold change, genes turned ON or genes turned OFF criteria. Real-time and western-blot

results are expressed as the mean \pm SEM percent change from naïve ($3 \leq n \leq 8$) and values significantly different from naïve and corresponding passive and yoked controls where present ($p < 0.05$, Student's two tailed *t*-test) are indicated by an asterisk. For real-time results, passive controls are represented by open squares, yoked controls by open triangles. LRP protein expression data are adapted and extended from Conboy *et al.* 2005.

Discussion

Sensitivity to transcriptional and translational inhibitors clearly shows memory consolidation to require alterations at the level of gene and protein expression (Davis and Squire 1984; Freeman *et al.* 1995). These, in turn, likely regulate synaptic remodelling in the hippocampus and the reorganisation and selection of neuronal connections (O'Malley *et al.* 1998, 2000; Eyre *et al.* 2003). While many proteins involved have been identified, many more remain to be elucidated. This study, along with others in the literature, move us towards a more comprehensive molecular picture of

memory consolidation by demonstrating that hippocampal-based memory paradigms, such as passive avoidance, have a significant and varied effect on the expression of a large number of genes in the dentate gyrus over the initial 24 h period following a learning event. Moreover, the overall pattern of gene expression corresponds very closely with discrete functional periods known to be important in memory consolidation.

Earlier studies that employed transcription inhibitors to assess the importance of *de novo* gene transcription during memory formation revealed two critical time windows for up-regulated gene expression, the first immediately following

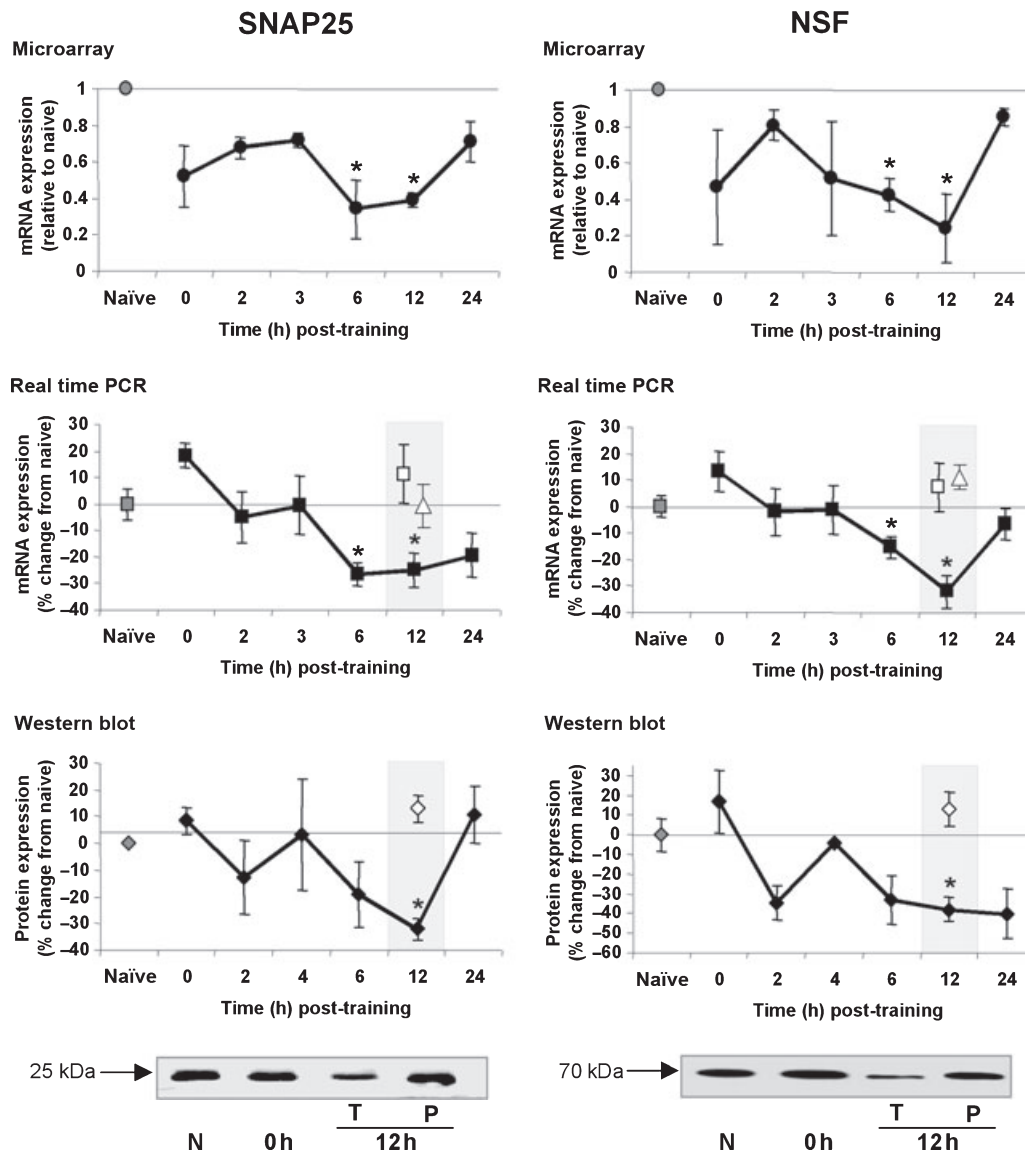


Fig. 3 Learning specific regulation of SNAP25 and NSF. For each gene, the graphs report the microarray and real-time PCR mRNA expression and analysis of protein expression by densitometric western-immunoblotting. Microarray results are expressed as the mean \pm SEM ($2 \leq n \leq 3$) value normalised to the mean expression of that gene in the naïves ($n = 3$). An asterisk indicates the times where gene changes were deemed significant on the 1.9-fold change, genes turned ON or genes turned OFF criteria. Real-time and western-blot results are expressed as the mean \pm SEM percent change from naïve

($3 \leq n \leq 8$) and values significantly different from naïve and corresponding passive and yoked controls where present ($p < 0.05$, Student's two tailed t -test) are indicated by an asterisk. For real-time results, passive controls are represented by open squares, yoked controls by open triangles. Finally, representative immunoblots illustrate training specific down-regulations in both SNAP25 and NSF in trained animals (T) at 12 h following learning compared to time-matched passive controls (P). N: naïve.

the learning event (0 h post-training), the other during the early stage of memory consolidation, 2–6 h post-training (Quevedo *et al.* 1999; Igaz *et al.* 2002). It has been proposed that the first transcriptional wave immediately following training pertains to increased expression of transcription factors (TFs) and immediate early genes (IEGs) (Igaz *et al.* 2002). In our study, several of the early up-regulated genes in the transcription/translation group, which comprise almost a

quarter of all increased genes at these times (0–2 h), code for TFs and IEGs already known to be functionally important in the brain. Furthermore, among the transcription factors implicated by analysis of the promoter structures of genes regulated at 0 h post-training we find cAMP-responsive element binding protein (CRE-BP1) and serum response factor (SRF) to be significantly over-represented. These TFs are known to have a role in the initiation of memory

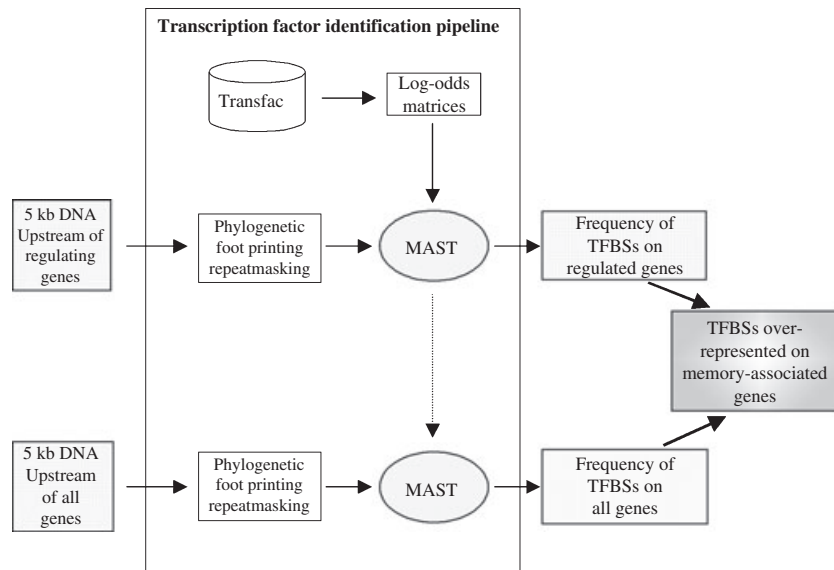


Fig. 4 Summary of the method used to identify over-represented transcription factor binding sites (TFBSs). The NCBI locuslink/Entrez gene identifier for the rat gene was used to identify the homologs in mouse and human via the Homologene database. The 5 kb upstream sequence (from the transcription start site) for the orthologous human, mouse and rat genes were then extracted and their sequences aligned (ENSEMBL; AVID). Regions of DNA conserved between the species were filtered for repeat DNA (REPEATMASKER) giving considerable reduction in potential transcription binding sites. Position weight

matrices (PWMs) for consensus binding motifs of known transcription factors (Transfac) were converted into log odds matrices and used with the MAST application to identify DNA motifs for transcription factor binding. The list of transcription factors for a set of genes was then compared to the occurrence seen in the entire genome (subject to the same conservation analysis as above). The binomial test of proportions was used to determine statistical significance. TFBSs having a $p > 0.95$ were determined to be highly likely to be involved in transcriptional regulation of the set of genes.

0 h	2 h	3 h	6 h	12 h	24 h
Cre-BP1	NF-Y	USF	Myogenin	Cre-BP1	Hox-1.3
MZF1	Arnt	NFkappaB	E4BP4	c-Jun	Lyf1
FoxD3	Gfi-1	TCF11	STAT3	Myogenin	MyoD
MIF	E4BP4	HEN1	FoxJ2	S8	c-Myb
SRF	HSF2	Max	HSF1	GATA2	HEN1
S8	Myogenin	RORalpha1	n-Myc	STAT3	c-Rel
GATA2		Arnt	Freac-7	Sp1	HNF-4alpha1
Pax5		Myogenin	Arnt	Egr4	Pou2F1
Pou2F1		E47	E47	GATA3	Myogenin
TCF-1		SREBP-1		FoxD3	NRSF
ER		NF-Y		Pax5	p300
Cart-1				NF-AT	
FoxJ2				FoxJ2	
NF-Y				Egr2	
c-Myb				Egr1	

The TFBS software pipeline identified between 6–15 TFBSs to be over-represented in the promoter regions of genes regulating at specific post-training times. TFBSs printed in bold italics have been shown to function in learning and memory or in neuronal development. TFBSs appear in order of descending p -value.

consolidation and their dysregulation has been implicated in Alzheimer's disease where we see marked memory deficits even before serious degeneration occurs (Pearson *et al.* 2005; Ramanan *et al.* 2005).

Among the genes regulated immediately following learning, mRNA levels for several cellular trafficking proteins were increased. These included Lrp3 from the low-density lipoprotein receptor-related protein (Lrp) family that function

Table 3 The mammalian transcription factor binding sites over-represented in the promoter regions of genes regulating at increasing times following passive avoidance training

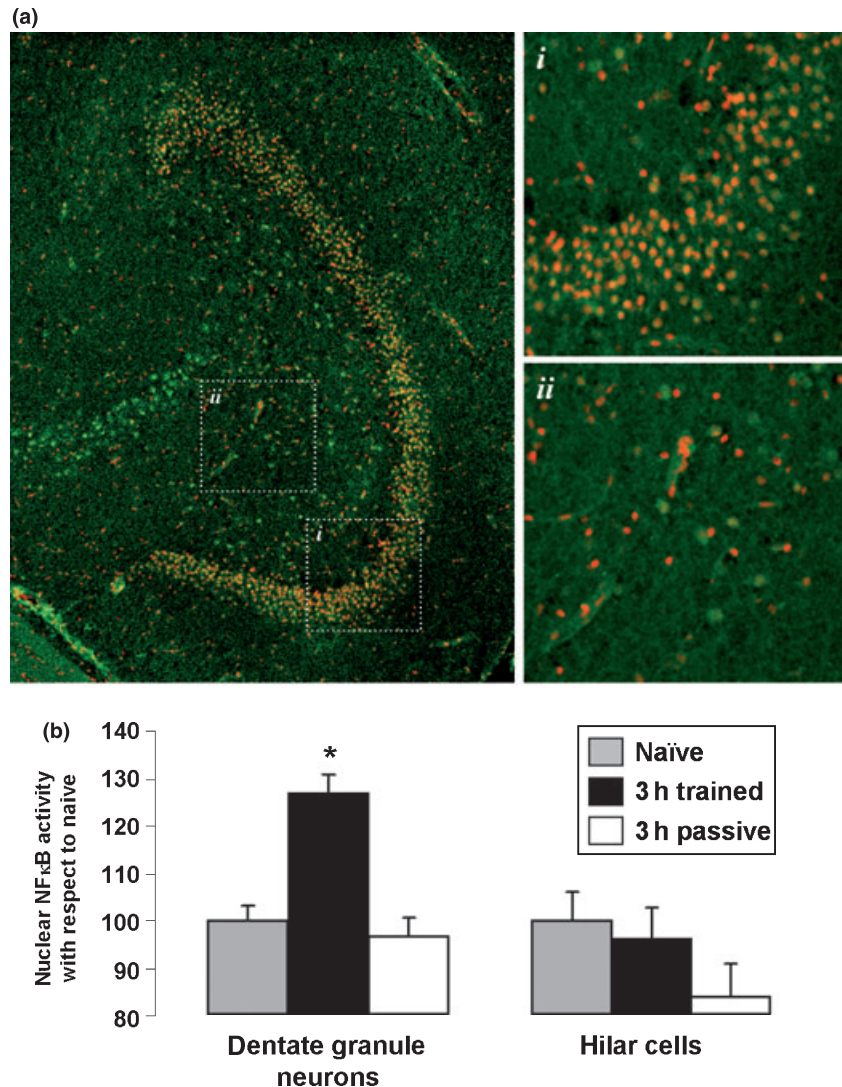


Fig. 5 Activation of NFκB in dentate granule neurons but not hilar cells 3 h post-training. Panel a: representative confocal images of active NFκB in the hippocampus identified by immunofluorescent labelling procedures. Nuclei are stained red with propidium iodide (PI) and active NFκB labelled green using FITC-conjugated secondary antibodies. The two high power images, *i* and *ii*, show representative dentate granule and hilar cells, respectively. Panel b: the graphs represent the relative levels of active nuclear NFκB in both dentate cell populations studied 3 h following passive avoidance training. Data are mean \pm SEM percent change from naïve ($n = 4$) and values significantly different from naïve and corresponding passive control ($p < 0.05$, Student's one-tailed *t*-test) are indicated by an asterisk.

in the internalisation of bulky trans-membrane proteins such as amyloid precursor protein (APP) (Kounnas *et al.* 1995; Cam *et al.* 2005). The increase in Lrp3 mRNA, validated by real-time PCR, agrees well with the up-regulation of Lrp protein expression seen previously, 2–4 h post-avoidance training (Conboy *et al.* 2005). The latter increase in protein expression was accompanied by internalisation and degradation of APP, likely promoting synaptic loosening, and, moreover, Lrp function was shown to be essential during the 2–4 h post-training time for memory consolidation to occur. Most importantly, this indicates that gene regulations identified through our microarray approach have the clear potential to flag key molecular players required for long-term memory formation.

The second wave of *de novo* gene expression previously observed, 2–6 h post-training, putatively relates to the regulation of structural genes likely underpinning the long-lasting synaptic remodelling required for long-term memory

formation (Bailey *et al.* 1996; Kandel 2001; Igaz *et al.* 2002). Again, our findings are in strong agreement as almost a quarter of genes up-regulated between 2–6 h have a role in cellular restructuring, with many functioning specifically in actin-cytoskeleton dynamics, a process fundamental to physical reorganisation of synaptic connections. Moreover, actin expression is enriched in dendritic spines, precisely the structures observed to remodel during memory consolidation (O'Malley *et al.* 1998, 2000). Here, while the transient up-regulation of alpha-actin mRNA at 3 h following training was validated by real-time PCR and was training specific, no alteration at protein level could be observed. Lack of change at the protein level may not be surprising, however, if the increased mRNA levels indeed facilitate actin remodelling as in this situation there would be parallel depolymerisation and degradation of existing actin structures. In addition, spines contain two distinct pools of actin filaments, one stable the other unstable (Smart and Halpain 2000). The stable pool

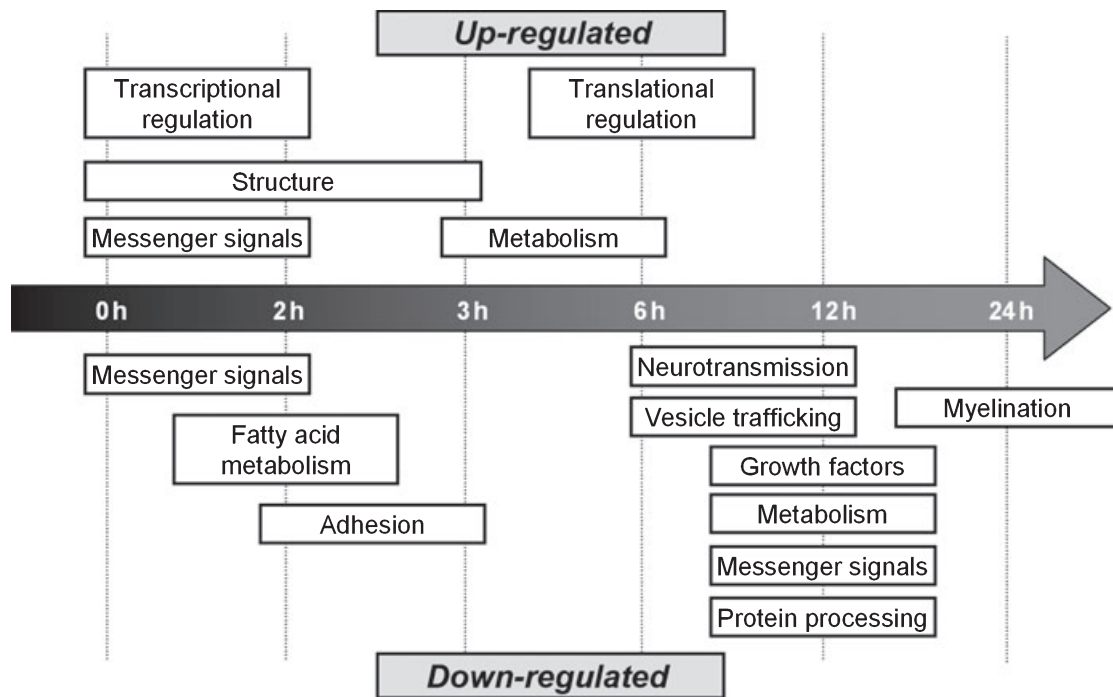


Fig. 6 Temporal profile of transcriptional events in the dentate gyrus over 24 h post-training. This panel highlights the key molecular events occurring during memory consolidation: The initial up-regulation of TFs and signalling molecules; early regulations in restructuring and adhesive genes; late global down-regulation and synaptic 'silencing'.

represents the supporting cytoskeletal structure while the unstable pool provides the potential for morphological plasticity under circumstances where adaptive changes in synaptic connections become appropriate (Matus 2000). Thus, during this critical period of memory consolidation, increased actin mRNA may facilitate generation of new actin protein to feed the unstable pool and to replace and remodel the degraded pre-existing spine cytoskeleton. In this pertinent scenario, no change in total alpha-actin protein would be observed. Moreover, the high variability seen in the alpha-actin protein analysis may, at least in part, reflect the actin dynamics proposed.

Another component gene group regulating early in memory consolidation are cell adhesion molecules. In contrast to the structural genes primarily involved in actin polymerisation and restructuring, the cell adhesion genes, such as CD9 and Thy1, are primarily down-regulated 2–6 h post-training (~20% of all genes down-regulated at these times). Even the few cell adhesion related genes which are up-regulated at these early times may contribute to synaptic de-adhesiveness – for example, G-protein 12 (Gna12) which has been shown to decrease E-cadherin-mediated cell-cell adhesion (Meigs *et al.* 2002). Moreover, previous observations have shown two adhesion molecules critical to synaptic integrity, APP and neural cell adhesion molecule (NCAM), to down regulate in the early time period following passive avoidance training (Foley *et al.* 2000; Conboy *et al.* 2005).

Along with these previous reports, we propose that the transcriptional down-regulation of neuronal adhesion molecules observed here, 2–6 h post-training, identify a cardinal molecular process of synaptic loosening facilitating subsequent synaptic modulation (Bailey *et al.* 1992; O'Malley *et al.* 1998; Foley *et al.* 2000).

Among the transcription factors implicated by the bio-informatics analysis of genes regulated 3 h post-training we find NFκB, a transcription factor previously suggested to play a role in learning and memory (Romano *et al.* 2006). As our analysis was based on the dentate gyrus, the predominate transcriptional signal likely comes from one or both of the major cell populations of this structure namely the dentate granule neurons or the mixed neural cell population of the hilus. Here, we report increased activation and nuclear re-localisation of NFκB 3 h following learning to be particular to the granule neurons of the dentate. These findings agree with recent work where selective blockade of NFκB has been shown to inhibit long-term memory of passive avoidance in mice (Freudenthal *et al.* 2005). In particular, activation of NFκB in the granule neurons supports a role for this cardinal transcription factor specifically in gene regulations required for the remodelling of perforant path-granule cell synaptic connections that accompanies memory consolidation (O'Malley *et al.* 1998, 2000).

The most striking finding of this microarray experiment is the profoundly decreased mRNA levels at 12 h post-training,

with over 40% of all modulating genes decreased at this time. This finding introduces a new aspect of transcriptional regulation during memory consolidation – a stage of late quiescence. This down-regulation was seen in genes involved in all of the functional groups studied, most notably: neurotransmission, cellular structure, second messenger signals, growth factors, protein processing and metabolism. This would indicate that 12 h post-training is a period of extensive inactivity within the cell and particularly at the synapse. It is important to note again that we cannot discount the possibility that many of these gene changes are not learning related but instead reflect circadian phenomena that would be most apparent at this time point. While 12 h post-training was not addressed in their study, however, Cavallaro and colleagues noted a similar dominance of gene down-regulation at 24 h following rabbit eye blink conditioning (Cavallaro *et al.* 2001). A significant component of the 12 h down-regulation is the MAPK pathway, long established to play a key role in plastic events needed for the establishment of long-term memory through the activation of transcription factor targets (Impey *et al.* 1999). Results from this microarray study indicate that several components of the MAPK pathway, including MAPK1 and MAPKK1, have decreased expression 12 h post-training. Coincident with this MAPK modulation, the TFBS study identified three members of the early growth response (Egr) transcription factor family: Egr1, Egr2 and Egr4, as over-represented motifs. These TFs, which are regulated by the MAPK pathway, also have clear roles in mediating gene expression required for learning and memory processes (Jones *et al.* 2001; Li *et al.* 2005). Decreased expression of these IEGs, perhaps brought about by reduced MAPK activity, implicates MAPK signalling as a key player in this proposed later stage of synaptic quiescence.

This ‘quietening’ of the synapse is perhaps best supported by the decreased neurotransmission potential likely brought about by reduction in the relative abundance of proteins involved in mediating synaptic vesicle exocytosis. The SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptors) complex proteins, including SNAP25 and NSF, have a role in regulating synaptic plasticity via control of synaptic transmission. Verderio *et al.* (2004) have recently used knockdown and knock-in methods to demonstrate that SNAP25 negatively regulates neuronal calcium responsiveness to stimuli. Thus, coordinated decreases in expression of both SNAP25 and NSF likely function to modulate neuronal excitability during the 12 h post-training period. Overall, given its timing, late in memory consolidation and coincidence with the period of synaptic elimination (O’Malley *et al.* 1998, 2000), this ‘quietening’ of transcription may well be a prerequisite to allow synaptic selection to proceed.

In conclusion, this study provides evidence that memory consolidation involves coordinated waves of transcriptional

control: initial transcription/translation, followed by increased restructuring and decreased adhesion, and finally, profoundly decreased activity 12 h post-training (Fig. 6). The timing and identities of genes showing regulation support the cardinal periods of synaptic loosening, reorganisation and selection thought to underpin the process of long-term memory consolidation driven by the hippocampus.

Acknowledgement

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