Gene expression profiles during long-term memory consolidation

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Abstract

Changes in gene expression have been postulated to occur during long-term memory (LTM). We used high-density cDNA microarrays to assess changes in gene expression 24 h after rabbit eye blink conditioning. Paired animals were presented with a 400 ms, 1000 Hz, 82 dB tone conditioned stimulus that coterminated with a 100 ms, 60 Hz, 2 mA electrical pulse unconditioned stimulus. Unpaired animals received the same conditioned and unconditioned stimuli but presented in an explicitly unpaired manner. Differences in expression levels between paired and unpaired animals in the hippocampus and cerebellar lobule HVI, two regions activated during eye blink conditioning, indicated the involvement of novel genes as well as the participation of previously implicated genes. Patterns of gene expression were validated by *in situ* hybridization. Surprisingly, the data suggest that an underlying mechanism of LTM involves widespread decreased, rather than increased, gene expression. These results demonstrate the feasibility and utility of a cDNA microarray system as a tool for dissecting the molecular mechanisms of associative memory.

Introduction

Identifying the mechanisms responsible for memory formation and consolidation has long been a goal of behavioural neuroscience. For more than a century, two forms of memory have been distinguished by their duration: short-term memory (STM), which is rapidly formed and can outlast training for minutes or hours; and long-term memory (LTM), which lasts from hours to days, weeks or even years (James, 1890). In both vertebrates and invertebrates, STM is based on transient modification of pre-existing molecules, capable of rapidly altering the efficiency of synaptic transmission. By contrast, LTM can be blocked by inhibitors of transcription or translation, indicating that it is dependent on *de novo* gene expression (Davis & Squire, 1984; Stork & Welzl, 1999). Proteins newly synthesized during memory consolidation may contribute to restructuring processes at the synapse and thereby alter the efficiency of synaptic transmission beyond the duration of STM. Revealing the dependence of LTM on protein synthesis, however, provides no information on the identity and specificity of the required proteins.

The quantity of a particular protein is often reflected by the abundance of its mRNA and a variety of methods have been used to describe a limited number of differentially expressed mRNAs during LTM. For example, by RNA fingerprinting, we have previously identified several genes, including the ryanodine receptor type-2 and glutamate dehydrogenase genes, which were induced during spatial learning in the rat hippocampus (Cavallaro *et al.*, 1997; Zhao *et al.*, 2000). Successive large-scale screening, however, is required to uncover the spectrum of genes involved in distinct temporal domains of memory storage.

To begin a comprehensive survey of the molecular mechanisms that underlie LTM, we have used the unprecedented experimental opportunities that the genome sequences and the development of cDNA array technology now provide to perform genome-wide expression analysis after classical conditioning of the rabbit's nictitating membrane response (NMR), a uniquely well-controlled associative learning paradigm. Classical conditioning of the rabbit NMR involves the presentation of an innocuous stimulus, such as a tone, followed by a noxious stimulus, such as an air puff to, or electrical stimulation around, the eye (Gormezano *et al.*, 1962). Extensive lesion and recording data have implicated the cortex of the cerebellum and in particular, lobule HVI, in classical conditioning of the rabbit NMR (Yeo *et al.*, 1985; Berthier & Moore, 1986; Schreurs *et al.*, 1991; Gruart & Yeo, 1995; Gould & Steinmetz, 1996; Schreurs *et al.*, 1997a; Schreurs *et al.*, 1998). Although the hippocampus might not be necessary for NMR conditioning, recording data do show consistent eye blink conditioning-specific hippocampal changes (Coulter *et al.*, 1989; Sanchez-Andres & Alkon, 1991). Imaging studies have implicated both structures in human eye blink conditioning (Molchan *et al.*, 1994; Logan & Grafton, 1995; Blaxton *et al.*, 1996; Schreurs *et al.*, 1997b).

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Materials and methods

Classical conditioning

Twenty-nine adult male albino rabbits (*Oryctolagus cuniculus*) each weighing 2.0–2.2 kg were used. Rabbits were housed individually, given access to food and water, and maintained on a 12 h light : 12 h dark cycle. Animals were allocated randomly to three groups and given paired training (paired, *n* = 12), explicitly unpaired stimuli (unpaired, *n* = 12) or restraint (sit, *n* = 5). Paired and unpaired rabbits received 1 day of preparation and three sessions of stimulus presentation. Sit rabbits received 1 day of preparation and three sessions of restraint. On adaptation day, the rabbits were prepared for periorbital electrical stimulation and nictitating membrane recording (right side) and acclimated to training chambers for the time of subsequent sessions (80 min). Paired subjects received 80 daily presentations of a 400 ms, 1000 Hz, 82 dB tone conditioned stimulus (CS) that coterminated with a 100 ms, 60 Hz, 2 mA electrical pulse unconditioned stimulus (US) delivered, on average, every 60 s. Unpaired subjects received the same 80 CSs and 80 USs but presented in an explicitly unpaired manner, on average, every 30 s. Stimulus delivery and data collection were via a Pentium PC/Labview computer-controlled system modelled after systems developed by Gormezano *et al.* (1962). A response was scored as a conditioned response if it exceeded 0.5 mm in amplitude between CS onset and US onset. All animal procedures followed NIH guidelines and were approved by the NINDS Animal Care and Use Committee.

Differential gene expression

Twenty-four hours after the final paired or explicitly unpaired session, rabbits (*n* = 7 per group) were killed with sodium pentobarbital (30 mg/kg) and the right cerebellar lobule HVI (side of training) and right hippocampus were dissected, frozen on dry ice and poly(A) + RNA isolated. The synthesis of cDNA probes, cross-species hybridization with the mouse GEM-1 cDNA microarray and signal analysis were conducted by Incyte Genomics (Palo Alto, CA, USA), as described at http://www.incyte.com. The basic alignment search tool (BLAST) was used to investigate the cross-species similarity of the clones contained in the Mouse GEM1™ IncyteGenomic array with the *Oryctolagus cuniculus* sequences available in the GenBank, EMBL, DDBJ, and PDB databases. Similarity searches were perfomed with the 8734 sequences of the mouse GEM1 clones using the MegaBLAST service at http://www.ncbi.nlm.nih.gov/BLAST. The percentage of identity of the homologous genes found was 88.98 ± 3.7 (mean $\pm$ SD). The complete BLAST search is available online at http://www.brni-jhu.org/sebi/microarray-data/Cross species similarity.htm as supplementary information. For cross-species hybridization, Incyte Genomics required three times the quantity of mRNA than that used for within-species hybridization. More than 7000 genes and expressed sequence tags (ESTs) showed measurable hybridization signals with both the paired and unpaired probes. To define the validity of a 'spot' on the array, a minimum signal-to-background ratio of 2.5 and a minimum area value of 40% were used. The balance coefficients for the microarray analysis of cerebellar lobule HVI and hippocampal mRNA were 1.07 and 1.0, respectively, indicating the absence of variation between Cy5 and Cy3 channel signals. Clones that showed differential hybridization of more than twofold were analysed by BLAST homology search against all available sequence databases.

In situ *hybridization*

Brains from paired, unpaired and sit animals (*n* = 2) were rapidly frozen on dry ice, and cryostat sectioned (12 μm; –20 °C). *In situ* hybridization as described previously (Zhao *et al.*, 2000) was performed for eight of those genes with differential expression >2 and probe average signal (the intensity reading read by the scanner) for the element >600. Specific mouse antisense riboprobes labelled with [α-^{35}S] were produced using T3 primer and *Eco*RI linearized I.M.A.G.E. Consortium (LLNL) cDNA clone (Lennon *et al.*, 1996). The following are the I.M.A.G.E. Consortium clone ID/GenBank accession numbers: 313322/W10072.1; 640200/AI604485.1; 419957/AI430365.1; 634849/AA163500.1; 336497/W18585.1; 480196/AA058055.1; 617038/AA163432.1; 483643/AI552486.1. No signal was detected in control sections hybridized with sense riboprobe or pretreated with RNase before hybridization with the antisense probe. Signals were obtained with film autoradiography. Evaluation of hybridization signals was carried out using a computer-assisted image analysis system and NIH Image 1.49 software (Wayne Rasband, NIH, Bethesda, MD).

Quantitative RT-PCR

The right lobule HVI from paired, unpaired and sit animals (*n* = 3) was rapidly frozen on dry ice. Total RNA samples were reverse transcribed with oligo(dT)12–18 and SuperScript II Rnase H-reverse transcriptase (Gibco BRL). Aliquots of cDNA (0.1 and 0.2 μg) and known amounts of external standard [purified polymerase chain reaction (PCR) product, 102–108 copies] were amplified in parallel reactions using primers (5′-ATCTGGCTGCGACATCTGT-3′ and 5′-GGAGCGCAGCTACTCGTATA-3′) based on the ESTs sequence (Acc. No. AA434883.1) and generated a 198-bp product. To control for RNA integrity and differences attributable to errors in experimental manipulation, mRNA levels of phosphoglycerate kinase 1 were measured in similar reactions using the primers: 5′-AGGTGCTCAACAACATGGAG-3′ and 5′-TACCAGAGGCCACAGTAGCT-3′. Each PCR reaction (final volume 20 μL) contained 0.5 μM of primers, 2.5 mM Mg^{2+} and 1 × DNA SYBR Green master mix (Roche Molecular Biochemicals, Mannheim, Germany). To prevent nonspecific amplification, 'Hot Start' was performed by preincubating the DNA SYBR Green master mix with TaqStart antibody solution (Clontech) for 5 min at RT. PCR amplifications were performed with a Light-Cycler (Roche Molecular Biochemicals, Mannheim, Germany) using four cycles: (i) denature cDNA (1 cycle, 95 °C for 1 min); (ii) amplification (40 cycles, 95 °C for 0 s, 57 °C for 5 s 72 °C for 10 s); (iii) melting curve analysis (1 cycle, 95 °C for 0 s, 67 °C for 10 s, 95 °C for 0 s); and (iv), cooling (1 cycle, 40 °C for 3 min). The temperature transition rate was 20 °C/s except for the third segment of the melting curve analysis where it was 0.2 °C/s. The fluorimeter gain value was 7. Real-time detection of fluorometric intensity of SYBR Green I, indicating amount of PCR product formed, was measured at the end of each elongation phase. Quantification was performed by comparing the fluorescence of the PCR products of unknown concentration with the fluorescence of the external standards. Fluorescence values measured in the log-linear phase of amplification were considered using the second derivative maximum method of the Light Cycler Data Analysis software (Roche Molecular Biochemicals, Mannheim, Germany). The specificity of the PCR products obtained was characterized by melting curve analysis followed by gel electrophoresis and DNA sequencing.

RNA and probe preparation, microarray analyses, *in situ* hybridization, autoradiographic evaluation and quantitative reverse

transcription (RT)-PCR were all performed blinded to the behavioural condition of the animals.

Results

To relate changes in gene expression to a learning task we used pairings of a tone and periorbital electrical stimulation in a standard delay conditioning procedure (Schreurs, 1993), training rabbits to asymptotic levels of conditioning over 3 consecutive days. Paired rabbits ($n = 12$) acquired conditioned responses to the tone and reached a mean terminal level of 94.7% conditioned responses, whereas the unpaired control rabbits ($n = 12$) responded to the tone at mean levels of < 1.3% across the 3 days of stimulus presentations and sit control rabbits ($n = 5$) had spontaneous blink rates of < 1.0% ($P < 0.001$) (Fig. 1A). Without further training or testing, rabbits show a level of 80% conditioned responses for as long as 1 month after the 3 days of the stimulus pairings used in the present experiments (Schreurs, 1993). Consequently, harvesting cerebellar and hippocampal tissue 24 h after 3 days of pairings ensured that rabbits were still at an asymptotic level of conditioning.

Messenger RNA levels from cerebellar lobule HVI and hippocampus of unpaired and paired rabbits ($n = 7$ per group) were simultaneously analysed with high-density cDNA microarrays containing more than 8700 cDNA mouse clones with a length of 500–5000 bp, and with averages in the 1 kb region. A complete list of the differentially expressed genes is available online at http://www.brni-jhu.org/sebi/microarray-data/microarray-data.htm as supplementary information. When gene expression patterns were compared using a statistically significant change of more than twofold, mRNA levels of 79 (0.9%) and 17 (0.2%) genes differed in lobule HVI and hippocampus, respectively (Fig. 1B and C). Approximately 50% (eight) of the genes differentially expressed in the hippocampus were also differentially expressed in the HVI lobule, suggesting common mechanisms of memory storage in the two areas.

A majority of genes that differed by a factor > 2 were downregulated, whereas only two genes that differed by a factor > 2 were upregulated in lobule HVI of paired animals (Fig. 1B). Because LTM can be blocked by transcription and protein synthesis inhibitors, most previous reports have focused on the identification of proteins whose expression is upregulated (Davis & Squire, 1984; Stork & Welzl, 1999). The preponderant reduction of gene expression during LTM therefore would not have been predicted and provides new and unexpected insights into the molecular mechanisms that underlie it.

Although our data represented the average gene expression from separate microarray analyses of cerebellar and hippocampal tissue obtained from a group of paired and a group of unpaired rabbits, there could be differences in gene expression between individual rabbit-derived tissues or between trained and sit control animals. To address these questions and confirm the microarray results, we selected eight (~10%) of the genes that were expressed differently at high levels and performed double blind *in situ* hybridization in cerebellum and forebrain tissue sections from individual paired, unpaired and sit rabbits (Table 1). In addition to corroborating the microarray data, the *in situ* hybridization analysis revealed distinct spatial distribution patterns of the genes. Figure 2 shows the regional mRNA expression of EST W18585.1, insulin-like growth factor-I (IGF-I) and Bach 2. All these mRNAs were expressed abundantly in the cerebellar cortex and were reduced in lobule HVI of paired rabbits. In addition to this area, downregulation of EST W18585.1 was also found in other cerebellar lobules. In paired animals, a marked downregulation of Bach 2 was also revealed in the dentate gyrus, CA1, and CA3 areas of
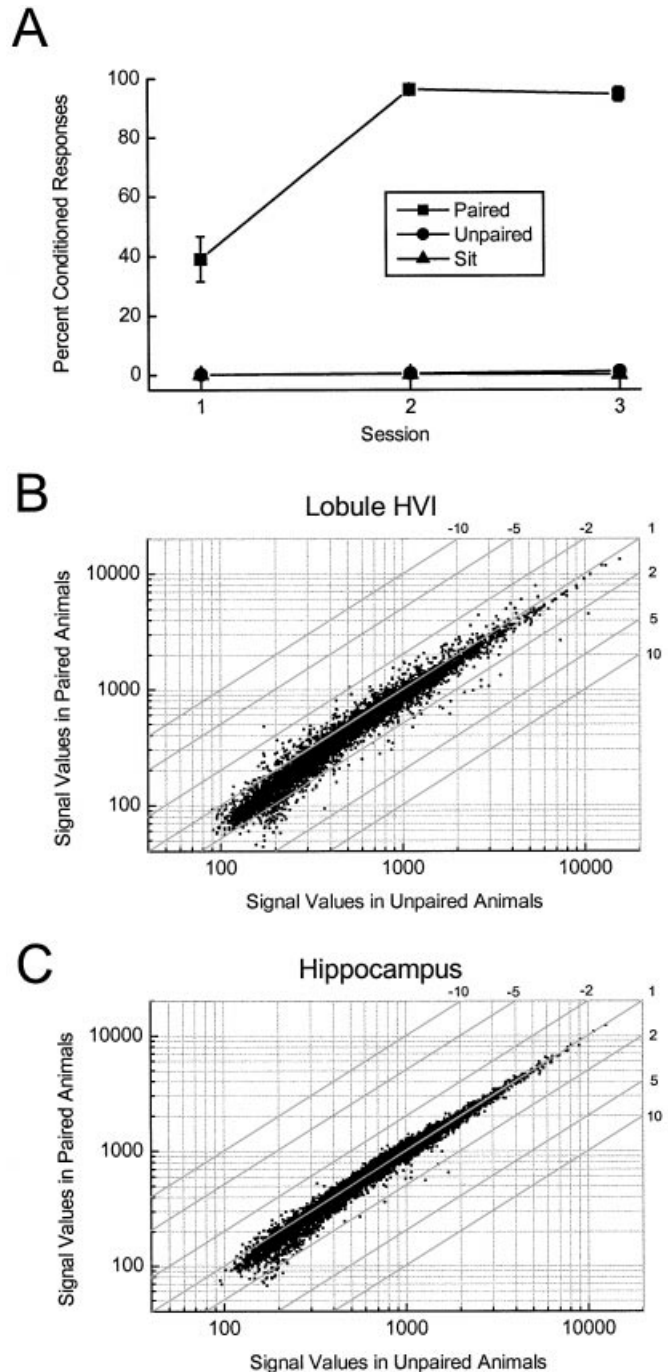

FIG. 1. Microarray analyses of eye blink conditioned rabbits reveal downregulation of gene expression following learning. (A) Mean percentage conditioned responses in paired, unpaired and sit control rabbits as a function of three training sessions. (B and C) Gene expression signal values for paired and unpaired animals in cerebellar lobule HVI (B) and hippocampus (C).

the hippocampus. Finally, to further validate the microarray results, a quantitative assessment of differential gene expression using quantitative RT-PCR was performed on one gene, 7SK. The mean number ± SEM of mRNA copies per ng of RNA was 16 094 ± 5450 in paired animals, 31 141 ± 6750 in unpaired animals, and 28 823 ± 5125 in sit animals (data points were

TABLE 1. Changes in gene expression in right cerebellar lobule HVI and hippocampus of unpaired vs. paired animals

Expression ratios >2 (unpaired vs. paired)		Gene name	Accession number	Confirmed by *in situ* hybridization
Hippocampus	HVI			
Signal transduction				
–	2.7	Insulin-like growth factor I	W10072.1	Yes
–	2.3	Growth differentiation factor 9	AA422377.1	–
–	2.3	Liprin-β 2	W12503.1	–
–	2.3	LAR receptor-linked tyrosine phosphatase	W16354.1	–
–	2.2	Phocein	W50771.1	–
–	2.2	Fibrinogen/angiopoietin-related protein	W13905.1	–
Protein modification				
–	3.2	ESTs, highly similar to CD156	AA470303.1	–
2.3	2.4	F-box protein FBX8	AA413956.1	–
–	2.3	ESTs, highly similar to hippostasin	W13212.1	–
DNA transcription regulation				
2.1	2.7	Bach protein 2	AI604485.1	Yes
–	2.6	ESTs, highly similar to WBSCR11	AA467436.1	–
–	2.5	Pirin	W08720.1	–
2.3	–	ESTs, Similar to C/EBP	AA473938.1	–
–	2.3	DRG11	AA387552.1	–
–	2.3	DCoH/PCD	W14332.1	–
–	2.1	7SK	AA058055.1	Yes*
–	2.1	Orphan receptor TR2	AA163245.1	–
Unknown				
2.6	2.8	ESTs, unknown	AA163432.1	No
–	2.6	ESTs, unknown	AI430365.1	Yes
2.3	2.5	ESTs, unknown	AI552486.1	Yes
–	2.2	ESTs, unknown	AA163500.1	Yes
–	2.2	ESTs, unknown	W18585.1	Yes

Significant changes (> twofold) are shown in the table. The inclusion of ESTs in front of a gene name indicates that the gene is similar but not identical in sequence to the named gene. Seven out of eight genes were confirmed by *in situ* hybridization (Yes). *Quantitative PCR was used to validate the microarray data for 7SK.

obtained from three individual animals, each run in duplicate; $P = 0.006$ paired vs. unpaired, $P = 0.007$ paired vs. sit, $P = 0.322$ unpaired vs. sit).

Discussion

A majority of differentially expressed genes identified in the present experiment have no currently recognized function and are not yet named. Indeed, 61 of the differentially expressed genes have no apparent homology to any gene the function of which is known. Complete nucleotide sequence determination, conceptual translation, expression monitoring, and biochemical analysis is currently underway and should provide a detailed functional understanding of these genes.

Seventeen genes have significant similarity to known genes and can be grouped into three different classes (Table 1): (i) signal transduction; (ii) protein modification; and (iii), DNA transcription regulation. It is important to note that some of these genes have been previously related to synaptic plasticity, memory or cognitive disorders.

The first group of genes encodes proteins involved in signal transduction and includes growth factors and proteins engaged in phosphorylation. One of the identified growth factors is IGF-I, a peptide with trophic and neuromodulatory actions. In the cerebellum, IGF-I is synthesized locally by Purkinje cells but also originates from climbing fibres, which are thought to convey information to the cerebellum about the reinforcing properties of the unconditioned stimulus. IGF-I modulates the size of dendritic spines on Purkinje cells (Nieto-Bona *et al.*, 1997), and inhibits the glutamate-induced release of γ-aminobutyric acid by Purkinje cells (Castro-Alamancos & Torres-Aleman, 1993). Interestingly, IGF-I levels have been correlated with cognitive test performance in ageing humans (Aleman *et al.*, 2000) and administration of IGF-I has been shown to

ameliorate age-related behavioural deficits in rats (Markowska *et al.*, 1998). Two differentially expressed growth factors the CNS functions of which are not known were growth differentiation factor-9 (Fitzpatrick *et al.*, 1998), a member of the transforming growth factor-β family, and a fibrinogen/angiopoietin-related protein (Kim *et al.*, 2000). In lobule HVI, we observed the combined down-regulation of a leucocyte common antigen-related (LAR) protein-tyrosine phosphatase and liprin-β2, a LAR-interacting protein-like gene. The LAR gene is a transmembrane protein tyrosine phosphatase (PTPase) with sequence similarity in the extracellular region to cell adhesion molecules such as the neural cell adhesion molecule NCAM (Zhang *et al.*, 1994). Liprins function to localize the LAR tyrosine PTPase at specific sites on the plasma membrane, possibly regulating their interaction with the extracellular environment and their association with substrates (Serra-Pages *et al.*, 1998). Although the extracellular ligands and physiological substrates of LAR-PTPase are not known, it could be part of specific signal transduction cascades that have effects on neuronal plasticity by functioning as signal transducers of cell contact phenomena. The final identified gene that could play a role in signal transduction is phocein, a protein that binds striatin, a Ca^{2+}/calmodulin-binding protein found mainly in dendritic spines where it is essential for the maintenance and growth of dendrites (Bartoli *et al.*, 1999).

The group of proteins involved in protein degradation includes a protein similar to CD156, a transmembrane glycoprotein with metalloprotease activity (Kataoka *et al.*, 1997), the F-box protein FBX8, a specificity-conferring component of the ubiquitin protein ligase SCFs complex that functions in phosphorylation-dependent ubiquitination of a wide array of regulatory molecules (Winston *et al.*, 1999) and hippostasin, a brain-related serine protease of unknown function. Although the substrates of these protein-degrading enzymes are unknown, their differential expression could play a critical role in synaptic plasticity and axonal remodeling.
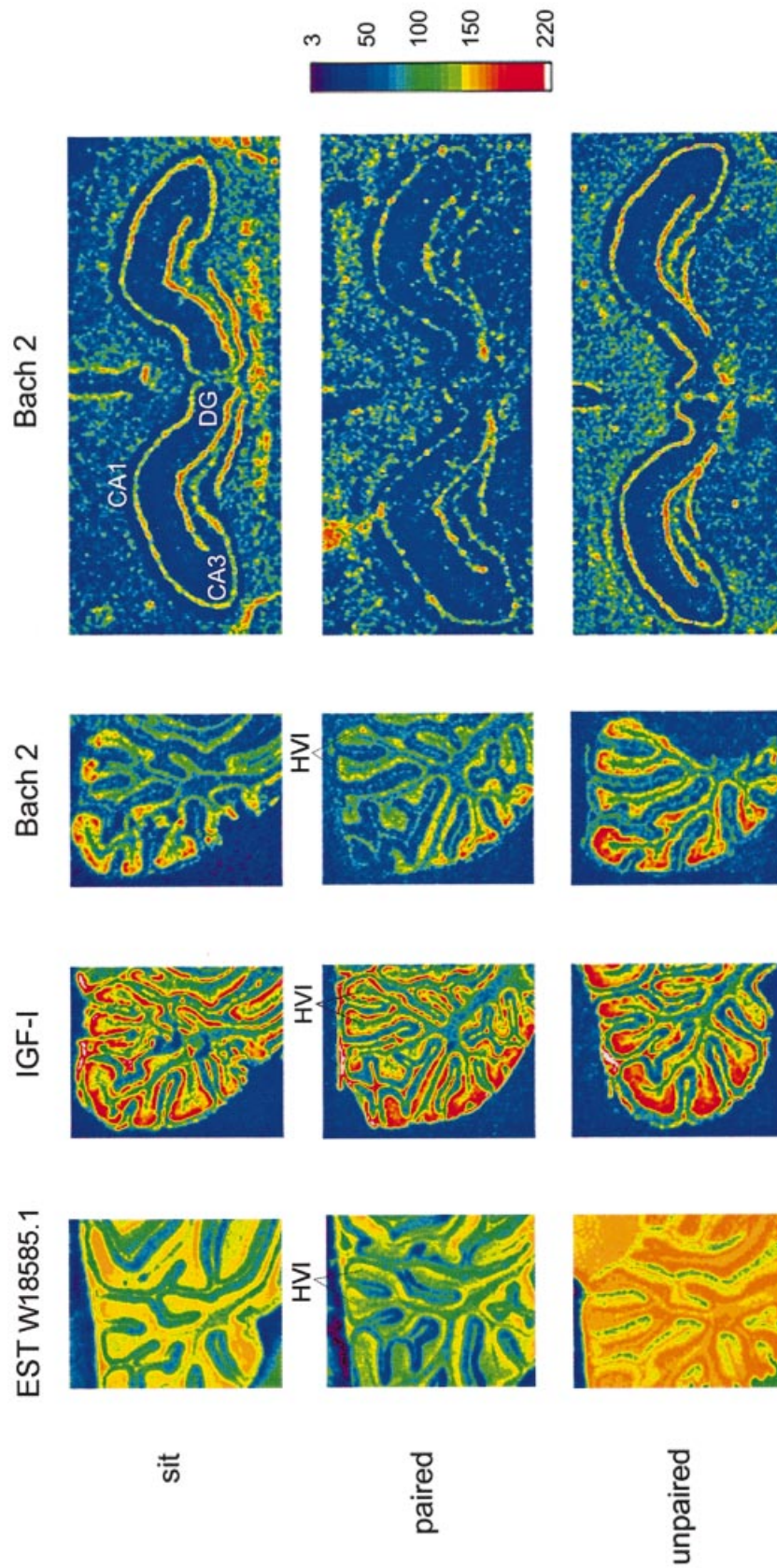
FIG. 2. Examples of *in situ* hybridization validation and localization of differential gene expression. Specific riboprobes labelled with [α-³⁵S] for EST W18585.1, IGF-I and Bach 2 mRNAs were hybridized with horizontal sections through the cerebellum and coronal sections through the forebrain of sit, paired and unpaired rabbits. Labelled mRNA signals were revealed with autoradiography. The pictures show the representative distribution of EST W18585.1, IGF-I and Bach 2 mRNAs. The colour spectrum on the right side of the figure represents the pixel value of the grey levels.

Among the group of differentially expressed genes involved in transcription regulation is 7SK, a small nuclear RNA involved in the control of transcription (Krause, 1996) and TR2, an orphan receptor belonging to the family of steroid/thyroid hormone receptors (Young *et al.*, 1998); the rest of the genes have functions related to transcription factors. One of these is similar to the CCAAT enhancer-binding protein (C/EBP) family of transcription factors that have been implicated in LTM consolidation after inhibitory avoidance learning (Taubenfeld *et al.*, 2001) and long-term facilitation, a synaptic mechanism which in *Aplysia* is thought to contribute to LTM (Alberini *et al.*, 1994). Interestingly, selectively enhanced contextual fear conditioning (24 h after training) has been shown in mice lacking the transcriptional regulator C/EBP delta, implicating some isoforms of this family of proteins in specific types of learning and memory as memory suppressor genes (Sterneck *et al.*, 1998). WBSCR11 is a putative transcription factor gene that is commonly deleted in Williams–Beuren syndrome, and could contribute to the spectrum of associated developmental symptoms which include mental retardation and profound impairment of visuospatial cognition (Osborne *et al.*, 1999). The bifunctional protein dimerization cofactor of transcription factor HNF1/pterin-4-α-carbinolamine dehydratase (DCoH/PCD) is both a dimerization cofactor of transcription factor HNF1 and a cytoplasmatic enzyme PCD involved in the regeneration of tetrahydrobiopterin, the cofactor for aromatic amino acid hydroxylases (Strandmann *et al.*, 1998). Bach 2 is a transcription factor expressed almost exclusively in neurons that forms heterodimers with MafK and could play an important role in coordinating transcription activation and repression (Oyake *et al.*, 1996). Pirin is a putative nuclear factor I-interacting protein (Wendler *et al.*, 1997). DRG11 is a paired homeodomain protein specifically expressed in sensory neurons and a subset of their CNS targets (Saito *et al.*, 1995).

The present data demonstrate differential gene expression at one time point in the long term retention of learning and memory and demonstrate the feasibility and utility of a cDNA microarray system as a means of dissecting the molecular mechanisms of associative memory. Despite the general expectation that genes are up regulated during learning (Davis & Squire, 1984; Cavallaro *et al.*, 1997; Stork & Welzl, 1999; Zhao *et al.*, 2000), our results provide an intriguing counterpoint to the current way of thinking. If changes of more than twofold were considered (Zweiger, 1999), a general downregulation of gene expression following learning was observed. That is not to say, however, that all genes are downregulated. We noted earlier that two genes (AA212893.1, AA175692.1) were upregulated in cerebellar lobule HVI following 3 days of classical conditioning. At levels below the relatively stringent twofold threshold used in the present experiment, a large number of genes were also upregulated (Fig. 1B and C).

How might downregulation of a number of genes play a role in memory storage? First, the downregulation may be an end point to a dynamic gene expression process recruited during the acquisition and retention of memory, and secondly, memory storage may require decreased expression of proteins that exert inhibitory constrains (Alberini *et al.*, 1994). These might be memory suppressor genes (Abel *et al.*, 1998; Cardin & Abel, 1999).

The majority of differentially expressed genes identified in the present experiment have no known function, and the microarray results represent only the first clue to the question of what these genes do. Further studies will be required to evaluate additional time points and better understand the role of differentially expressed gene networks during the pathophysiology of memory.

Acknowledgements

Abbreviations

C/EBP, CCAAT enhancer-binding protein; CS, conditioned stimulus; DCoH/PCD, dimerization cofactor of transcription factor HNF1/pterin-4-α-carbinol-amine dehydratase; IGF-I, insulin-like growth factor-I; LAR, leucocyte common antigen-related; LTM, long-term memory; RT-PCR, reverse transcription-polymerase chain reaction; STM, short-term memory; US, unconditioned stimulus.

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