

# Genes Regulated by Learning in the Hippocampus

Tarek A. Leil,<sup>1</sup> Alex Ossadtchi,<sup>2</sup> Thomas E. Nichols,<sup>3</sup> Richard M. Leahy,<sup>2</sup> and Desmond J. Smith<sup>1\*</sup>

<sup>1</sup>Department of Molecular and Medical Pharmacology and Crump Institute for Molecular Imaging, UCLA School of Medicine, Los Angeles, California

<sup>2</sup>Signal and Image Processing Institute and Department of Electrical Engineering, University of Southern California, Los Angeles, California

<sup>3</sup>Department of Biostatistics, School of Public Health, University of Michigan, Ann Arbor, Michigan

The enduring changes in long-term memory probably depend on regulation of gene expression in the hippocampus. To seek genes regulated by learning, we used microarray technology to compare hippocampal gene expression in mice undergoing training in the Morris water maze and control mice forced to swim for the same period in the absence of a hidden platform. ANOVA was employed to prioritize genes for further study, and three genes were confirmed by real-time PCR as being regulated during learning. One of the genes was the  $\alpha$  subunit of the platelet-derived growth factor receptor (*Pdgfra*); another showed homology to DnaJ and cAMP response element-binding protein 2 (*CREB2*); and a third was novel. These genes may provide useful insights into the molecular mechanisms of hippocampal learning.

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The hippocampus is essential for creation of long-term memories. In humans, this was most famously shown by patient H.M., who displayed severe anterograde amnesia after bilateral removal of the hippocampus (Scoville and Milner, 2000). Experiments in animals support these findings, and, in rodents, an intact hippocampus is required for successful spatial learning on the Morris water maze task (Moser et al., 1993; Cho et al., 1999). Functional imaging studies in both humans (Maguire et al., 1997) and mice (Bontempi et al., 1999) indicate that the hippocampus is activated during recall. Interestingly, in mice, it appears that the hippocampus is strongly activated during recall of recently learned events but that this activation shifts to other areas of the brain, particularly the cortex, as memories become more firmly consolidated (Bontempi et al., 1999).

The molecular mechanisms of memory formation have been illuminated by biochemical, pharmacological, and genetic studies. Regulation of neurotransmitter systems, synaptic strength, and downstream signaling events have all been implicated. Biochemistry and pharmacology were first used to demonstrate a role in learning and

memory of cAMP-coupled downstream signaling pathways (Mayford and Kandel, 1999). Classical or “forward” genetic screens in *Drosophila* have confirmed these findings (Davis, 1996; Belvin and Yin, 1997; Dubnau and Tully, 1998) as well as implicating integrin-based signaling (Grotewiel et al., 1998). In humans and mice, classical genetic approaches in the form of complex trait mapping have been used in an effort to find loci involved in cognitive ability (Plomin, 2001). Although this strategy is becoming increasingly feasible with the completion of the human and mouse genome projects, the obstacles confronting complex trait analysis remain daunting. Reverse genetic approaches, exemplified by genetically engineered mice, have made important contributions to the identification of molecular pathways for learning and memory (Chen and Tonegawa, 1997; Mayford et al., 1997). Although the role of redundant genes can be difficult to define using this technology, the behavioral role of homozygous inviable knockout genes can now be studied using tissue Cre-lox methodology (Tsien et al., 1996).

An additional approach to identifying relevant signaling mechanisms has emerged with the development of DNA microarray technology (Schena et al., 1995). This method identifies pathways by seeking genes regulated by environmental and genetic perturbations. Here, we describe the use of a 9,000 gene cDNA microarray to compare hippocampal gene expression differences between mice actively learning the position of a hidden platform in the Morris water maze compared with control mice constrained to swim for the same average time span in the

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\*Correspondence to: Desmond J. Smith, Department of Molecular and Medical Pharmacology, UCLA School of Medicine, 23-120 CHS, Box 951735, Los Angeles, CA 90095-1735. E-mail: dsmith@mednet.ucla.edu

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absence of a platform. The resulting expression data were investigated using an analysis of variance (ANOVA) framework to identify those genes that appeared to be regulated by the learning process, and the regulation of a number of these genes was confirmed using real-time polymerase chain reaction (PCR).

## MATERIALS AND METHODS

### Mice

The strain B6129PF1/J was employed for these experiments, because previous studies had shown that this F1 hybrid strain has excellent learning and memory (Owen et al., 1997; Leil et al., 2002). All mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were adult males 8–12 weeks old. It should be noted that, unlike the F2 hybrids, the F1 hybrids of a particular strain are genetically identical. All mice were housed two to four per cage on a 12 hr light/dark cycle, with food and water given *ad libitum*. All studies were approved by the Chancellor's Animal Research Committee of the UCLA Office for Protection of Research Subjects.

### Morris Water Maze

The Morris water maze tests were performed as described elsewhere (Smith et al., 1997; Leil et al., 2002), but with modifications designed for the purposes of this study. Naive mice were tested for their ability to locate an unmarked, submerged platform over five blocks (the hidden platform test). Three training blocks were administered on day 1 and two on day 2. Two hours after the conclusion of the fifth block, hippocampal RNA was harvested. Control mice were allowed to swim in the pool for the same average time in each block as the trained mice, but with the hidden platform removed.

### Overall Design

Three separate cohorts of mice were employed, each consisting of control and experimental animals. The first cohort was used to perform two microarray hybridizations. In the first hybridization, pooled hippocampal RNA from the trained mice was labeled with Cy3 and from the control mice with Cy5. These labeled samples were then cohybridized to a 9,000 gene microarray (Leil et al., 2002). In the second hybridization, a dye-flip strategy was used in which hippocampal RNA from the trained mice was labeled with Cy5 and the control mice with Cy3. The second cohort of mice was used to perform two additional hybridizations (standard and dye flip), giving a total of four microarray hybridizations. The third cohort was used for real-time PCR.

### Microarrays

Hippocampal RNA extraction, labeling of cDNA, and microarray hybridization were performed as described elsewhere (Leil et al., 2002). Mice were anesthetized with halothane and euthanized, and the brains were removed and placed on ice. Hippocampi (fresh) were then dissected from the brain by peeling them away from the ventral aspect. The hippocampi were placed in Trizol reagent (Gibco BRL, Gaithersburg, MD) and homogenized by passaging several times through a 26<sub>5/8</sub>-G needle. Subsequent steps for RNA isolation were performed following the manufacturer's instructions. This protocol resulted

in intact RNA as judged by gel electrophoresis and reverse transcription (RT)-PCR. In addition, all labeled cDNA samples in the microarray hybridizations exceeded signal-to-noise quality control thresholds of 2.5-fold above background for each gene in both the Cy3 and the Cy5 channels (Brown et al., 2002a, b). Moreover, labeled first-strand cDNA was prepared immediately after total RNA extraction, minimizing degradation. The average hippocampal mass was 15 mg, and each hippocampus provided 20–30  $\mu\text{g}$  total RNA. RNA abundance was assessed using fluorometry. To obtain sufficient RNA for the labeling reaction, it was necessary to pool hippocampi ( $n \geq 7$ ), which also decreased experimental noise resulting from variations between different mice. Variation was further minimized by virtue of the genetic homogeneity of the F1 hybrid strain employed. For selected genes, estimates of interindividual variance were provided by the real-time RT-PCR (below).

Synthesis of Cy dye-labeled first-strand cDNA was by RT using 100  $\mu\text{g}$  total RNA in a 40  $\mu\text{l}$  reaction volume. Final concentrations of reagents during cDNA synthesis were 1 $\times$  First Strand Buffer (Gibco BRL), 10 mM dithiothreitol (DTT), 50 nM dATP/dGTP/dTTP, 20 nM unlabeled dCTP, 100 nM FluorLink-dCTP (either Cy3 or Cy5 labeled; Amersham, Arlington Heights, IL), 1 U  $\mu\text{l}^{-1}$  RNasin, 12.5 U  $\mu\text{l}^{-1}$  Superscript II RT, and 1  $\mu\text{g}$  oligo-(dT)<sub>12–18</sub> (Gibco BRL). The reactions were incubated at 42°C for 1 hr, and 400 U Superscript II RT were added and incubation was continued for another 1 hr. The reaction was stopped using 55 mM EDTA, and RNA was removed by incubating in 0.2 N sodium hydroxide at 65°C for 1 hr. The reaction was then neutralized by addition of 25  $\mu\text{l}$  1 M Tris-HCl (pH 7.5). Cy5- and Cy3-labeled probes were combined and purified and concentrated by washing several times using a MicroCon 30 concentrator (Amicon, Danvers, MA).

The microarray data were processed using two types of normalization procedures. First, spatial trends existing in the data resulting from chip printing were removed by nonlinear transformation of the data sets. The second normalization procedure was designed to compensate for differences in the labeling and chemical properties of the Cy3 and Cy5 dyes, by aligning the histograms of the dye signals both within and between chips. These normalization procedures also helped to compensate for any minor variations in the amount of RNA employed in the labeling reactions as well as in labeling efficiency.

### Real-Time RT-PCR

Real-time quantitative RT-PCR employed TaqMan technology on an ABI Prism 7900HT Sequence Detection system (PE; Applied Biosystems, Foster City, CA; Gibson et al., 1996; Heid et al., 1996; Freeman et al., 1999; Leil et al., 2002). Taqman One-Step RT-PCR mix (Applied Biosystems) was employed, following the manufacturer's instructions. After RT, the amount of cDNA was quantitated using fluorometry, and 1 ng cDNA was employed in each real-time assay. The oligonucleotide sequences used for the primers and Taqman reporters are shown in Table I. The 5' reporter dye was FAM and the 3' quencher TAMRA. The amplification primers were at 200 nM and the reporter at 100 nM. A passive reference dye (ROX) provided an internal standard for normalization of FAM fluorescence, correcting for fluctuations resulting from volume changes. For relative quantitation, a standard curve was constructed for each primer and reporter set, using naive B6129PF1/J hip-

TABLE I. Primer and reporter sequences for real time RT-PCR

Gene	Forward Primer	Reverse Primer	Reporter
W11926	AGGCAACCAGAGTACCAGTGATG	CCGGGTCTGAGGTGTTGAAA	TGTCGTGAGCGAGCCACCCCT
AA272810	GTTGCAGGAGATGGAAAAAGAAG	GTTTCCCTACTACTCAGTGCCATT	CTTGGCGTGATGAGGGTTTCT
Pdgfra	ATATGATCTTTCTGTGTTAACTTGCT	CACTGCTTGGCAGAGCTACCT	CTCAGAGGGAGGAAACGGGGATG
AA237922	AATACTTCACCCAACCTAGAAAAAGG	GGTGACTGTCCCTGAAGTTAAGCT	ACGATCCACTTAAAGTGCTACTGTGTGCC
AA222477	AGACGGTGAGCAGCGAGAAA	CACGTCGGCGATCACAGA	CCGGCCACCAACCTCAACT

poampus total RNA. A ribosomal RNA control primer and reporter set (Applied Biosystems) was used for normalization purposes. The possibility of genomic DNA contamination was excluded by the use of no-reverse-transcriptase controls in combination with the ribosomal primers. All real-time PCRs were performed in duplicate using RNA from individual mice to give an average value for each animal. There were eight mice in each of the groups, experimental and control, giving an estimate of standard error for the selected genes.

## RESULTS

### Morris Water Maze

For this study, we selected the F1 hybrid strain B6129PF1/J, which had previously been shown to perform well in the Morris water maze test (Owen et al., 1997; Leil et al., 2002). The experimental group consisted of mice actively learning the position of a hidden platform in the water maze, and control mice were allowed to swim for the same average length of time in each block as the experimental group in the absence of a platform. Mice were analyzed 2 hr after the fifth block of training. This time point was employed because experimental mice show significant learning of the task at the fifth block and are approximately midway to block nine, usually considered to be asymptotic for the task. The control group was designed so that the mice experienced levels of physical activity similar to those of the actively trained group. Figure 1 shows the results of the water maze training for one of the three groups of trained mice.

### Microarray Analysis and Real-Time PCR

Four microarray hybridizations were performed. The first two hybridizations used one group of trained and control mice in conjunction with dye flipping. The second two experiments used an independent group of trained and control mice and also employed dye flipping. The data were subjected to normalization and log ratio transformation as described elsewhere (Brown et al., 2002a, b; Leil et al., 2002). An ANOVA model employing a balanced-loop design was used to analyze the data (Kerr et al., 2000; Kerr and Churchill, 2001a, b). The model is:

$$r_{ijk} = \mu + A_i + D_j + V_k + \epsilon_{i,j,k} \quad (1)$$

where  $r_{ijk}$  = normalized log gene expression ratio;  $\mu$  = grand mean;  $A_i$  = array effect;  $i = \{1,2,3,4\}$ ;  $D_j$  = dye effect;  $j = \{1,2\}$ ;  $V_k$  = variety effect, i.e., trained vs. control;  $k = \{1,2\}$ ; and  $\epsilon_{i,j,k}$  = error.

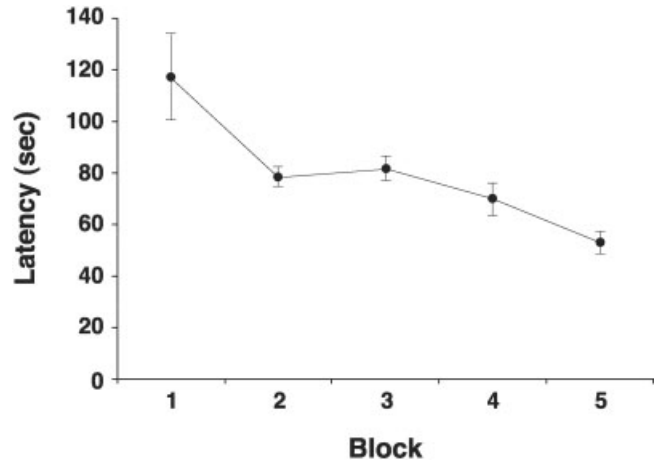


Fig. 1. Morris water maze. Hidden platform test across five blocks of training for the B6129PF1/J mouse strain (one training group,  $n = 8$ ). There was a significant decrease in latency to find the platform across blocks ( $F_{[4,35]} = 7.39$ ,  $P = 0.0002$ ).

Note that, in this model, the dye-variety interaction has been collapsed with the error term, estimated with 2 degrees of freedom. The design and aliasing matrices resulting from the model are shown in Figure 2A, and graphs of the F statistics for each of the effects are shown in Figure 2B. For a single comparison, the F value corresponding to a 5% significance level is 19. If a Bonferroni correction is used, which takes into account the 8,293 distinct genes on the microarray, the corresponding F value for a 5% significance level is 180,000. For the variety effect (control vs. training), none of the genes has an F statistic that reaches this threshold, the highest F value being about 14,000. However, the Bonferroni correction is known to be conservative, especially when there may be dependencies between the tests. Hence, not having any genes with F values greater than 180,000 is not inconsistent with some genes truly being modulated by learning.

Consequently, we used the F statistic as a screening tool to prioritize genes for further study. The top five genes were investigated using real-time PCR, and three proved significant (Fig. 3). Two were ESTs, W11926 and AA272810, and one was a known gene, platelet-derived growth factor receptor  $\alpha$  subunit (Pdgfra). The two genes not confirmed by real-time PCR were anonymous ESTs (AA237922 and AA222477).

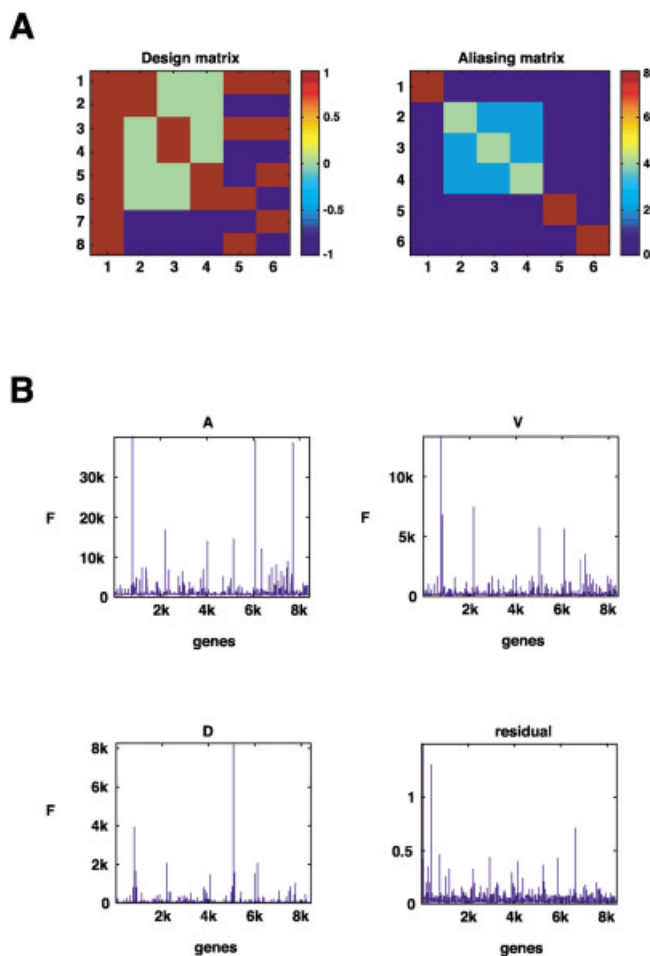


Fig. 2. ANOVA. **A**: Design and information (aliasing) matrices. Design matrix. The rows of the matrix represent the four microarray experiments, separated into Cy5 and Cy3 channels. The columns of the matrix correspond to the factors of the ANOVA (see equation 1). Column 1 represents the grand mean, columns 2–4 the array effect, column 5 the dye effect, and column 6 the variety effect (training vs. swimming control). Red = 1, green = 0, blue = -1. Information or aliasing matrix. The aliasing matrix is block diagonal, so the proposed model corresponds to a balanced orthogonal design, allowing for unconfounded estimates of the factors. Dark blue = 0, light blue = 2, green = 4, red = 8. **B**: F statistics for the effects A (array), V (variety, trained vs. controls), D (dye), and residual error.

## DISCUSSION

Several approaches have been employed in the search for genes involved in learning and memory. Genetic mapping strategies involve the fewest assumptions and therefore have the greatest prospects for surprise. However, such strategies are time consuming and laborious. In contrast, microarrays provide an opportunity for relatively rapid identification of genes for complex mammalian phenotypes. This technology has been employed with special advantage in the analysis of unicellular systems (Debouck and Goodfellow, 1999; Khan et al., 1999; Manger and Relman, 2000), but, so far, relatively few investigations have used microarrays to associate the behavior of higher organisms with gene expression.

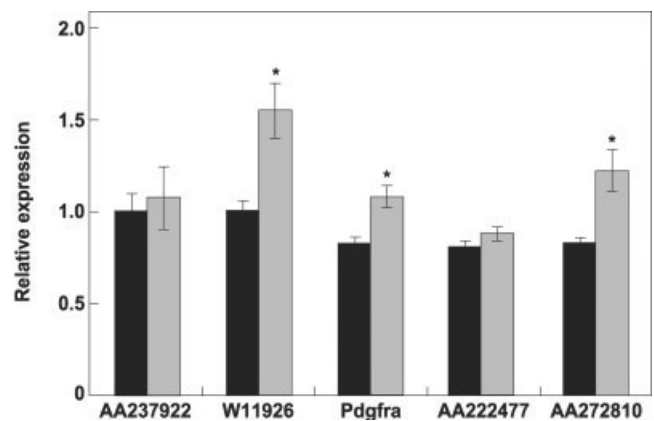


Fig. 3. Real-time PCR. Gray bars, trained mice; black bars, controls. Mean  $\pm$  SEM. \* $P < 0.05$ , two-tailed  $t$ -test.

The mouse is an especially favorable system for studies of complex traits, including learning and memory, because of its low interindividual genetic and environmental variability. Similar analyses are much more difficult in outbred organisms, such as humans.

In this study, a 9,000 gene microarray was used to look at differences in gene expression in the hippocampus of control mice undergoing a sham swimming task and mice undergoing active training on the hidden platform task of the Morris water maze. An F1 hybrid mouse strain was employed, because such strains have previously been shown to display excellent learning and memory (Owen et al., 1997; Leil et al., 2002). Although it is difficult to design an exact control group, it is probable that the sham swimming group represents an appropriate control for the actively learning mice. The two groups of animals presumably experienced similar levels of fear and anxiety, motor activation, stress, and novelty. The most significant difference between the two groups is likely the presence or absence of active learning.

Appropriate quality-control measures are important for microarray experiments. We performed microarray analyses using two independent cohorts of trained and control mice. For each cohort, two microarray hybridizations were performed, standard and dyes flipped, to give a total of four hybridizations. Artifacts can skew the data from microarray experiments. Factors such as incomplete washing, dust contamination, unevenness in slide surface properties, and variability in spot sizes can contribute to the experimental error (Sherlock, 2000). To minimize these influences, the data were subjected to signal processing by means of nonlinear normalization between slides.

One approach for determining differential expression in microarray experiments is to employ a twofold cutoff criterion. However, this does not account for inherent differences in the quality of data between slide hybridizations. In this study, an ANOVA model was employed. This strategy has the advantage that all the likely contributions to variance are defined ahead of the analysis. The main effects employed in the model were due to arrays, dyes, and variety (trained vs. control). Under the severe criterion imposed by a Bonferroni

correction for multiple hypothesis testing, the variety effect did not reach the strict thresholds for significance. Therefore, we used the F statistics as a tool for prioritizing genes for further analysis using real-time PCR. The five genes with the highest F statistics under the variety (trained vs. control) effect were chosen for real-time PCR using a third, independent cohort of mice. Among these five genes, three were confirmed by real-time PCR as showing significant regulation resulting from training. There are a number of possible reasons why the remaining two genes were not confirmed by real-time PCR. One is that changes in gene expression in the hippocampus resulting from learning and memory may be modest in magnitude. In addition, the hippocampus is a highly complex and nonhomogeneous region of the brain, and it is likely that genes will be regulated in only a subset of cells. These factors might contribute to the recovery of false positives when using the relatively noisy technology of microarrays.

Studies in unicellular organisms have shown that single genetic perturbations can result in expression changes that extend far downstream of the initial disturbance (Brown and Botstein, 1999). This phenomenon improves the prospects of using expression differences to understand the genetics of complex traits in mammals (Pugliese et al., 1997; Vafiadis et al., 1997; Malhotra et al., 1998; Aitman et al., 1999; Timtchenko et al., 1999; O'Connor et al., 1999; Smithies et al., 2000). Thus, even though the microarrays employed in this investigation did not cover the entire mouse genome, it was still possible to identify potential candidate genes for learning and memory.

Two of the three genes confirmed by real-time PCR were ESTs. One of these, W11926, was an EST of unknown function. The other EST, AA272810, showed two blocks of significant homology [89% identity over 65 amino acids and 77% identity over 38 amino acids; UniGene (<http://www.ncbi.nlm.nih.gov/UniGene>)] to a mouse DnaJ homolog, a protein folding chaperone. There was also weaker but still significant homology (45.2% similarity over 329 amino acids, including the two regions described above) to a *Xenopus laevis* cAMP response element-binding protein 2 [CREB2, or activating transcription factor 2 (ATF2); De Cesare and Sassone-Corsi, 2000], as judged using the Smith-Waterman alignment algorithm [EMBOSS (<http://www.ebi.ac.uk/emboss/align/>)]. CREB2 is a transcription factor that may function as a memory suppressor (Bartsch et al., 1995; Abel et al., 1998), and the CREB2 homolog identified in this study might possibly be induced as part of a homeostatic mechanism to ensure nonirretrievable consolidation of learning.

The third gene confirmed by real-time PCR is the  $\alpha$  subunit of the platelet-derived growth factor receptor (Pdgfra). This receptor is a tyrosine kinase that can homodimerize or heterodimerize with its cognate  $\beta$ -receptor upon binding of the ligand, platelet-derived growth factor. Mice homozygous for a knockout of the Pdgfra gene are nonviable and exhibit neural tube defects, in addition to increased apoptosis in migratory pathways followed by neural crest cells (Soriano, 1997). Heterozygous knockouts are apparently normal. It may be of value, based on the present investigation, to examine the heterozygous Pdgfra knockout mice for learning and memory phenotypes.

Loci for learning and memory in mice have been identified using complex trait mapping in combination with context- and cue-dependent fear conditioning (Caldarone et al., 1997; Wehner et al., 1997). None of the genes uncovered in the present study appears to localize to the mapped regions. This discrepancy may be due to a number of factors, including the use of a different learning and memory task and distinct mouse strains.

Recently, gene chip technology has been used to identify genes regulated in the cortex of mice subjected to an enriched environment compared with impoverished controls (Rampon et al., 2000). There was no overlap with the genes discovered in our study. This probably is due to a number of factors, including different genes on the array, mouse strains employed, and brain regions studied. Furthermore, it might be expected that a novel environment would invoke many diverse behavioral responses in addition to learning and memory, including fear and anxiety and increased motor movement. In another recent study, microarrays were employed to examine the hippocampi of rats that had undergone training in a multiunit T maze compared with yoked controls (Luo et al., 2001). Again, there was no overlap between the findings of that investigation and those of the present study. In the multiunit T maze learning paradigm, an aversive stimulus, electrical foot shock, is employed, and this stimulus was applied in equal duration and frequency to both the trained and the control groups. However, there were other differences between the two groups, principally in the amount of locomotor activity, which was essentially zero for the controls. In the experimental paradigm employed in the present study, a greater precision of matching of experimental and control groups might be expected, although it is impossible to control absolutely for all differences between the training and the swimming control groups. However, the more targeted approach employed here should yield a better circumscribed group of regulated genes, optimizing the potential for discovery of good candidate genes for learning and memory.

In conclusion, we employed microarray technology to identify genes regulated during the learning process in the hippocampus. We investigated a brain region intimately involved in learning and memory and also employed closely matched experimental and control groups. Thus, it is likely that the uncovered genes are involved in learning and memory, although their involvement in other behavioral processes cannot be excluded. In the future, knockout and transgenic strategies can be used to identify the exact role of the uncovered genes.

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