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Research report

One-trial aversive learning induces late changes in hippocampal CaMKIIα, Homer 1a, Syntaxin 1a and ERK2 protein levels

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Abstract

Most studies regarding altered gene expression after learning are performed using multi-trial tasks, which do not allow a clear discrimination of memory acquisition, consolidation and retrieval. We screened for candidate memory-modulated genes in the hippocampus at 3 and 24 h after one-trial inhibitory avoidance (IA) training, using a cDNA array containing 1176 genes. While 33 genes were modulated by training (respect to shocked-only animals), most of them were upregulated (27 genes) and only 6 were downregulated. To confirm and extend these findings, we performed RT-PCRs and analyzed differences in protein levels in rat hippocampus using immunoblot assays. We found several proteins upregulated 24 h after training: extracellular signal-regulated kinase ERK2, Ca2+/calmodulin-dependent protein kinase II alpha (CaMKIIα), Syntaxin 1a, c-fos and Homer 1a. The total level of none of these proteins were found to be altered when measured 3-h post-training. Several of the mRNAs corresponding to the upregulated proteins were changed at 3 h but not 24 h. Additionally, a number of other candidates were identified for the first time as modulated by learning. The results presented here suggest that single-trial tasks can expose previously unseen differences in dynamic regulation of gene expression after behavioral manipulations, both at the transcriptional and translational levels, and reveal a diversity of gene products modulated by this task, allowing deeper understanding of the molecular basis of memory formation.

Theme: Neural basis of behavior
Topic: Learning and memory: physiology

Keywords: Gene expression; Long-term memory; cDNA array; Hippocampus; Rat

1. Introduction

Long lasting memory formation is thought to involve changes in gene expression and protein synthesis that mediate modifications in synaptic plasticity in selected but distributed neuronal populations [19,36,44].

The quest for the identification and characterization of genes that were specifically regulated or, in the best possible scenario, necessary for long-term memory (LTM) consolidation has been painfully slow and fragmentary. Some of the earlier pharmacologic and genetic experiments have provided significant guidelines shedding some light on the role of particular signaling cascades, transcription factors or cellular adhesion molecules in learning and memory (see Ref. [33] and references therein). However, these approaches can only account for a partial description of these phenomena, since in many cases they required a thoughtful a priori hypothesis that, for unforeseen players, was unlikely to arise.

Although there have been some recent efforts to evaluate gene expression changes with large-scale transcriptional profiling experiments (for review, see Ref. [30]), most of them have been performed using tasks that consisted in several training sessions (multi-trial tasks) [21,22,37,50,51].

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Single-trial training tasks have advantages over multi-trial tasks when planning to elucidate changes in gene expression, in view of the fact that they allow more accurate descriptions, avoiding re-training effects and poor temporal resolution of ensuing molecular events [35]. When trying to analyze a process as complex and interconnected as gene expression network regulation, it is useful to have a clear-cut discrimination of acquisition, consolidation and retrieval [1]. We believe this can be achieved by the use of a single-trial paradigm. Here, we describe the use of biochemical techniques and cDNA array technology to compare hippocampal gene expression differences after inhibitory avoidance (IA) learning, a single-trial, extensively studied task. The hippocampus is crucially involved in the consolidation and retrieval of many types of memories, including IA learning [34]; hence, we chose this structure as the focus for this study.

Using cDNA arrays to identify novel changes in gene expression, we report here alterations in mRNA levels of 33 genes in the hippocampus of IA-trained animals. To confirm these results, we validated significant changes by RT-PCR. Our study is complemented and extended by data showing changes in protein expression levels of several genes after IA training, many of which were known to be regulated essentially at the activity level. These results broaden our view of the learning-related changes that underlie plastic responses and memory formation mechanisms in the brain, and provide new candidate genes to investigate in the context of orchestrated expression patterns triggered after behavioral experiences.

2. Materials and methods

2.1. Subjects

Male adult Wistar rats (age, 2.5 months old; weight, 220–250 g) from our own breeding colony were used. The animals were housed in plastic cages, five to a cage, with water and food ad libitum, under a 12-h light/dark cycle (lights on at 7:00 AM) at a constant temperature of 23 °C. The experimental protocol for this study followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Animal Care and Use Committee of The University of Buenos Aires. All efforts were made to minimize the number of animals used and their suffering.

2.2. Behavioral procedures

The animals were trained in IA as described [9,31]. Briefly, the apparatus was a 50×25×25-cm acrylic box whose grid was a series of 1-mm caliber bronze bars spaced 1 cm apart. The left end of the floor was covered by a 7-cm-wide, 2.5-cm-high wood platform.

There were three experimental groups of animals, subjected to different behavioral procedures, as follows: (1) animals withdrawn from their home cages and sacrificed immediately (Naïve group, *N*), (2) rats placed on the mentioned platform and received a 0.4-mA, 4-s electric footshock immediately after they stepped down to the grid (IA trained group, T) and (3) rats placed directly on the floor of the training apparatus, where they were submitted to an identical footshock but were not able to make the stepping down-shock association (shocked group, S). The time spent in the apparatus closely matched to the animals that were trained. Both T and S animals were immediately removed from the training box after receiving the shock. Rats were sacrificed by decapitation at 3 or 24 h after each behavioral procedure, the brains quickly removed and the hippocampi dissected out.

The same apparatus was used for IA and contextual fear, with the exception of the aforementioned wood platform. Contextual fear conditioning was performed as previously described [56].

2.3. Total RNA extraction

At 3 or 24 h after IA training, total hippocampal RNA from naïve, shocked or trained individual animals was extracted as described [31], using a single-step method based on guanidine isothiocyanate–phenol–chlorophorm extraction and TRIzol reagent (Life Technologies, Rockville, MD) [17]. Afterwards, RNA was DNAse-treated and lack of genomic contamination was assessed through RT-PCR using a pair of β-actin primers which amplify PCR products of different lengths according to the presence or absence of intronic sequences [31].

2.4. cDNA array procedure

Gene expression was analyzed using the Atlas™ Rat 1.2 Array I cDNA expression array (Clontech), which consists of two sets, each with two identical nylon membranes, which contain cDNA fragments specific for 1176 genes plus nine housekeeping genes. A complete list of genes is available online at www.clontech.com/atlas/genelists/7854-1_Ra12.pdf. Each one of the three independent replications for the cDNA array experiments were performed using RNA pooled from three different animals. The procedure was carried out according to the manufacturer's instruction (Clontech, Palo Alto, CA, USA). In short, each probe was synthesized by reverse transcription of an identical amount (5 μg) of DNAse-treated RNA using the reagents provided and [α-^{32}P]dATP (3000 Ci/mmol), purchased from NEN Life Science Products (Boston MA, USA). The radioactively labeled cDNA was purified from the unincorporated nucleotides and small cDNA fragments by column chromatography. Pre-hybridization was performed with a solution of Express-Hyb (Clontech) pre-warmed at 68 °C and heat-denatured sheared salmon testes DNA. The membrane was

incubated with the pre-hybridization solution in a hybridization bottle for 30 min with continuous agitation at 68 °C. Prior to the hybridization step, the labeled probe was incubated with 5 μl C_ot-1 DNA in boiling water for 2 min and then on ice for an additional 2 min. The mixture was added to the prehybridization solution in the hybridization bottle. Hybridization was performed overnight with continuous agitation at 68 °C. After hybridization, four washes of 30 min each were performed with pre-warmed 2× SSC, 1% SDS, followed by one 30-min wash with 0.1× SSC, 0.5% SDS and a final 5 min wash with 2× SSC. The membranes were then exposed to bleached phosphor screens for 2–3 days. Image acquisition was performed using a phosphorimager STORM 840 system (Amersham Biosciences), followed by analysis with ArrayGauge v 1.2 (Fuji Film, Tokyo, Japan).

2.5. Analysis of array data

Gene expression levels in the samples were adjusted for the total density level of each membrane instead of making comparisons with the expression of the housekeeping genes included in the filters, which provides less consistent results. After orientation and alignment of the two array membranes, array background was subtracted, and intensity of the detected genes on each membrane was adjusted by the normalization coefficient, which was calculated based on intensity of total genes. Visual inspection of the spots was also performed in order to avoid stained hybridization signals. The ratio of intensity between two corresponding genes on each array membrane was calculated. A minimum signal intensity of 1000 arbitrary densitometric units in at least one of the two probes was required to define genes with a measurable level of gene expression. In addition, the required signal to background ratio for every spot should be >2.5 to be considered different from background. Only genes that showed an expression ratio >1.5 or <0.6 and displayed these values for at least two of the three replicate experiments were considered to be differentially expressed [24], and were included in the subsequent analysis.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described [31], with minor modifications. A 1-μg amount of total RNA was reverse-transcribed to synthesize single-strand cDNA. For quantitation of PCR products, we used the individual samples that were pooled for cDNA array probe preparation; therefore, three independent experiments were conducted for RT-PCR determinations. The cDNA synthesis was performed and subsequently, 1 μl of the RT reaction was subjected to PCR in order amplify fragments using the following primers and annealing temperatures: TrkB-sense (5′ -TGA CGC AGT CGC AGA TGC TG-3′), TrkB-antisense (5′ -TTT CCT GTA CAT GAT GCT CTC TGG-3′), 57 °C; Akt/PKB-sense (5′ -CTG GCC AGG CCC AAG CAC CG-3′), Akt/PKB-antisense (5′ -CGT TCA CTG TCC ACA CAC TC-3′), 60 °C; STX1A-sense (5′ -CTC AGT GAG ATC GAG ACC AG-3′), STX1A-antisense (5′ -ATG ATG CCC AGA ATC ACA CA-3′), 56 °C ; IGF2-sense (5′ -CAT CGT GGA AGA GTG CTG CT-3′), IGF2-antisense (5′ -GGA CAT CTC CGA AGA GGC TC-3′), 65 °C; sdc3-sense (5′ -AGT ACC CTC ACC ACC CAC TA-3′), sdc3-antisense (5′ -TAG ATG AGC AAC GTG ACC AG-3′), 60 °C; D1A-sense (5′ -CTT GGT GGC TGT CCT GGT CAT-3′), D1A-antisense (5′ -GGT CAT CTT CCT CTC ATA CTG-3′), 55 °C; β-actin sense primer: (5′ -ACC ACA GCT GAG AGG GAA ATC G-3′); β-actin-antisense primer (5′ -AGA GGT CTT TAC GGA TGT CAA CG-3′), 60 °C. Negative controls without RNA and with non-retrotranscribed RNA were included in all the experiments. The number of cycles performed was well within the exponential phase of the amplification process, and quantitative data between two cycle number were averaged for each sample and primer pair combination.

After PCR, the amplification products were separated by agarose gel electrophoresis, bands were visualized under UV light, digitalized and quantified with the software ImageQuant version 5.1 for Windows NT (Molecular Dynamics, Sunnyvale, CA). Gene expression levels were normalized to β-actin values.

2.7. Immunoblot assays

All the procedure was carried out at 4 °C as described [14]. After sacrifice at different times post-training, the brains were immediately removed, the hippocampi were dissected out, pooled and homogenized in ice-chilled buffer (20 mM Tris–HCl, pH 7.4, 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, 15 μg/ml leupeptin, 50 mM NaF and 1 mM sodium orthovanadate). The samples were stored at −70 °C until used. Three independent experiments were conducted.

To investigate whether behavioral procedures affects gene expression at the protein level, ERK1/2, syntaxin 1a, CaMKIIα, Homer, Akt, c-fos, S100 β and Casein kinase IIα (CK2α) Western blots were performed. Samples of whole homogenates (10–30 μg of protein) were subjected to SDS-PAGE (10% gels), and immunoblots were performed as described previously [14]. Proteins were transferred onto PVDF membranes (Millipore, Bedford, MA) in transfer buffer (25 mM Tris, 0.19 M glycine, 10% methanol) overnight at 40 V and then blocked with 10% skim milk in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. Membranes were incubated with the following antibodies: anti-ERK1 and ERK2 (1:3000; Cell Signaling Technology, Beverly, MA), anti-syntaxin 1a (1:8000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CaMKIIα (1:1000; Santa Cruz Biotechnology), anti-Homer (1:1000; Santa Cruz Biotechnology), anti-Akt (1:2000; Cell Signaling Technology), anti c-fos

(1:1000; Santa Cruz Biotechnology), anti-S100 β (1:4000; Sigma, St. Louis, MO) anti-Casein-kinase IIα (1:1000; Santa Cruz Biotechnology), anti-β-actin (1:1000; Santa Cruz Biotechnology). After washing with TBST (four times, 15 min each), the blots were incubated for 1 h at room temperature with goat anti-rabbit or rabbit anti-goat IgG coupled to horseradish peroxidase (Bio-rad, 1:3000) in blocking buffer and then washed again in TBST. Following incubation with enhanced chemiluminescent substrate (ECL, Pierce, Rockford, IL), membranes were exposed to film, and the film was developed. Densitometric analysis of the films was performed by using an MCID Image Analysis System (version 5.02, Imaging Research, St. Catharines, Ontario, Canada). Western blots were developed to be linear in the range used for densitometry.

2.8. Data analysis

For RT-PCR and immunoblot experiments, statistical analysis was performed by non-paired Student's *t*-test, used when two independent groups were compared. In the IA task, a ceiling of 180 s was imposed on the test session values. Therefore, the use of nonparametric statistic is required. We used Kruskal–Wallis test for LTM test sessions, followed by Dunn's multiple comparison test. Comparisons in contextual fear among groups were performed by one-way ANOVA. For gene array analysis, means and S.E.M. of three hybridizations were calculated. For the comparisons of gene expression between two groups, Student's *t*-test ($p<0.01$) was performed on candidate genes that exhibited expression levels outside cut-off values.

3. Results

In order to identify genes whose expression levels are regulated by a single-trial learning task, we used an IA training procedure, an aversive and fear-motivated learning. So far, this task has been the most studied in terms of the formal biochemical requirements in the hippocampus for memory formation [1,34,44,57]. It is important to stress that shocked animals submitted to a test session 24 h later show no evidence of avoidance response (i.e. rats do not learn the IA task) (Fig. 1). In addition, rats submitted only to one (or up to three) mild footshock/s like the one/s used here do not exhibit contextual fear conditioning, as indicated by the low amount of time spent in the compartment were they previously received the shock/s (No shock, 22.0±6.6% total time; 1 shock, 19.2±2.4% total time; 2 shocks, 20.8±5.8% total time; 3 shocks, 28.3±6.0% total time; $n=10$, $p=0.6363$, ANOVA). Moreover, no sign of freezing was evident in any of the shocked groups (not shown). These results indicate that the exposure to a mild footshock by its own is not enough to acquire the stepping down-shock association and/or a contextual fear. However, we cannot rule out the possibility that some form of memory (not behaviorally expressed) could be consolidated.

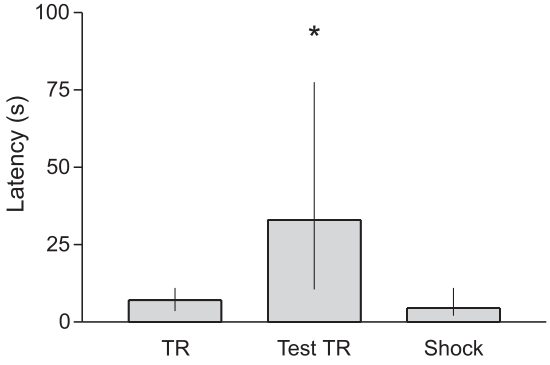

Fig. 1. Rats subjected to IA training show long-term memory while those exposed only to footshock do not acquire the task. Exposure to shock is not sufficient to acquire the stepping down-shock association. Medians (interquartile range) of latencies to step down from the platform of the IA box during training (TR), in the test session 24 h after training (test TR) or shock are shown. Test TR is significantly different from the other two groups, $*p<0.05$, Dunn's multiple comparison test after Kruskal–Wallis test, $n=7–8$ per group.

3.1. IA learning-induced changes in hippocampal mRNA levels

We analyzed changes in gene expression in hippocampal RNA from rats trained (T), shocked (S) or naïve (N) at two different time points (3 or 24 h after the behavioral procedure), using an Atlas cDNA expression array (Clontech). We selected the 3-h time point because at that time hippocampal transcription and translation are required for IA memory formation [31]. It must be pointed out that, despite the fact that we also described a macromolecular synthesis requirement around the time of IA training [31], most single-gene studies were carried out at early time points, and there is less information regarding what occurs at longer periods of time [14,26,29,67]. From the total 1176 genes, the S vs. N comparison revealed 23 (10 upregulated and 13 downregulated) candidate genes, but only 6 of them met the criteria established to define as differentially expressed in Materials and methods. This small proportion of changes confirmed previous findings from our laboratories using this task, regarding the fact that the total level of several proteins does not change in the shocked compared to the naïve group, while it is increased in the trained group; these proteins include PKCβI, αPKAc, ERK1/2, CaMKIIα, Elk-1 and NR1 [13–15,33,34,48]. Therefore, the shocked group was chosen as the control, which was subjected to a similar stressor but was unable to learn the avoidance task (Fig. 1).

Fig. 2 shows representative phosphorimages of hybridization signals from trained and shocked groups (Fig. 2A), and enlarged signals from selected spots (Fig. 2B). The Atlas Rat 1.2 Array includes 1176 cDNAs, nine housekeeping control cDNAs, and negative controls on a single

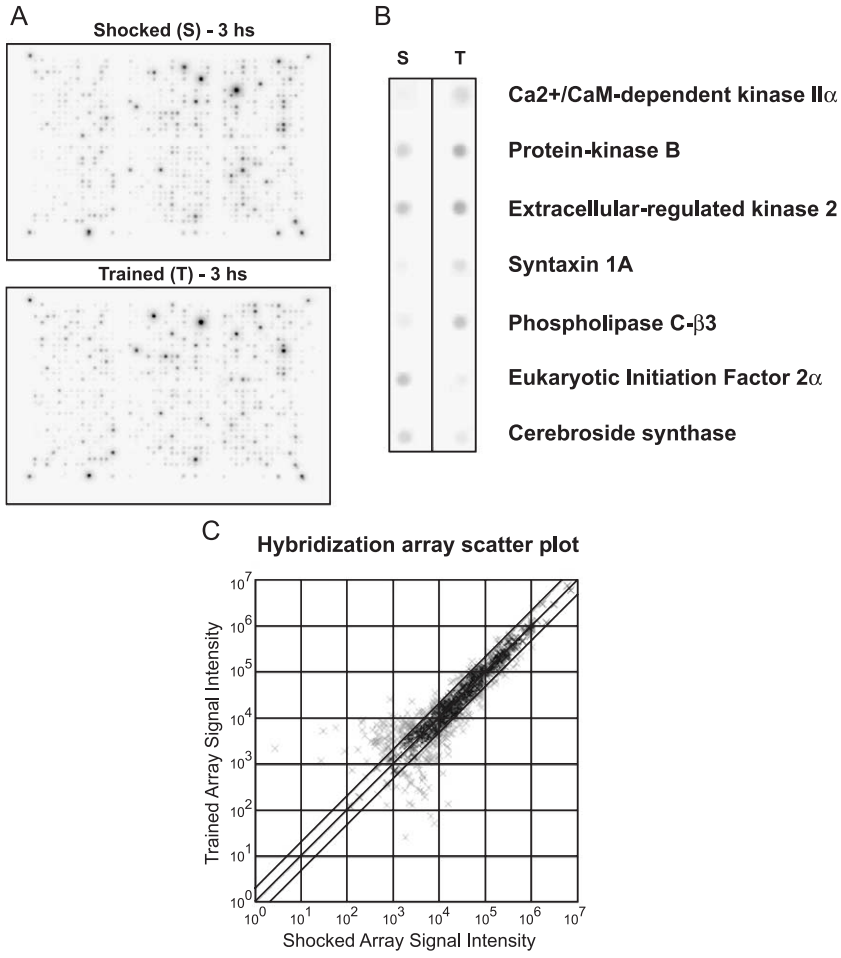

Fig. 2. cDNA array analysis of one-trial IA task in rat hippocampus. (A) Representative cDNA arrays showing differential gene expression profiles from Shocked (control) or Trained rats (n=3 independent experiments), using total RNA isolated from the hippocampus (n=3 per experiment). In this image, we show the hybridization patterns for the 3-h time point. (B) Selected spots have been enlarged to illustrate the observed differences in array analysis more clearly. Several of these examples correspond to genes whose protein products increased after avoidance learning (see Fig. 4). S, shocked group; T, trained group. (C) Hybridization array scatter plot. Array data is plotted as a function of the Trained array adjusted signal (y-axis) versus Shocked array adjusted signal (x-axis). The middle diagonal line is drawn through unity (Trained/Shocked signal ratio =1) and the outer diagonals represent cut-off values of expression ratios =0.6 and 1.5. Genes lying outside these boundaries are colored in grey, while genes not changed by treatment are depicted in black.

nylon membrane. The data were analyzed (see Materials and methods) and represented graphically in a logarithmic scatter plot (a representative plot of one experiment is shown in Fig. 2C). The results are expressed as changes in gene expression level, and genes were divided into five major functional categories as described in Table 1.

Gene expression profiling reveals that 767 (65.2%) of the 1176 genes screened were expressed significantly above background, while 33 (2.81%) of the 1176 were detected as significantly altered by training respect to shock. These can be subdivided in two ways: (a) in total, 27 genes (2.3%) are upregulated and 6 (0.51%) are specifically downregulated by training; (b) regarding the time course of these changes, 26 genes (2.2%) are modulated when analyzed 3-h post-training and only 7 (0.60%) 24 h after training (see Table 1). Our results are consistent with the percentage of regulated genes in experiments involving specific manipulations, around 0.1–2% of monitored genes changing by a factor $\geq$1.8 [41], and also with the proportion of genes affected in different learning tasks (see Discussion).

One-trial IA training increased the expression of several genes involved in signal transduction, cell metabolism, membrane receptors, channels and transporters, including CK2α, CK2β, CaMKIIα, ERK2, serine phospholipid-specific phospholipase A, 3-beta hydroxy-5-ene steroid dehydrogenase type III, metabotropic glutamate receptor 7 (mGluR7), insulin-like growth factor II (IGF2), syndecan 3 (sdc3), syntaxin 1a (STX1A), ATP-sensitive inwardly rectifying K+ channel KIR6.1, tyrosine kinase receptor B (TRKB) and structure-specific recognition protein 1 (SSRP1) (Table 1). On the other hand, it also reduced the expression of a smaller subset of genes, which include

Table 1
Genes showing differential expression in hippocampus after IA learning

Gene product	Accession #	Change (h)		Expression ratio (trained/shocked)
		3	24	
Synaptic transmission and cell signaling				
Casein kinase II alpha subunit (CK2 alpha)	L15618	▲	–	1.9±0.1
Casein kinase II beta subunit (CK2 beta)	L15619	▲	–	2.3±0.4
Protein kinase II, α subunit, calcium/calmodulin dependent (CaMKIIα)	J02942	▲	–	2.2±0.3
c-akt proto-oncogene; protein kinase B (PKB)	D30040	▲	–	1.6±0.2
Purkinje cells-specific protein tyrosine phosphatase (CBPTP)	D64050	▲	–	1.9±0.4
Extracellular signal-regulated kinase 2 (ERK2)	M64300	▲	–	1.6±0.1
Phospholipase C beta 3 (PLC-β3)	M99567	▲	–	2.5±0.7
Somatostatin receptor 5 (SSTR5)	L04535	▲	–	3.0±0.8
Metabotropic glutamate receptor 7 (mGLUR7)	D16817	▲	–	1.4±0.1
ATP-sensitive inward rectifier potassium J 8 (KCNJ8)	D42145	▲	–	1.9±0.3
Syntaxin 1A (STX1A)	M95734	▲	–	1.6±0.1
Dopamine receptor D1A (D1A)	M35077	–	▲	1.9±0.1
erbB4 proto-oncogene (HER4)/neuregulin receptor	U52531	–	▲	2.6±0.6
Tyrosine kinase receptor B (TRKB)	M55291	–	▲	2.1±0.4
Metabolism				
3-Beta hydroxy-5-ene steroid dehydrogenase type III (3-beta-HSD III)	M67465	▲	–	2.8±0.9
Lipid droplet-associated proteins A/B, perilipin A/B (PERIA/PERIB)	L26043	▲	–	1.7±0.1
Serine phospholipid-specific phospholipase A (PS-PLA1)	D88666	▲	–	2.5±0.7
Cytosolic thymidine kinase (TK1)	M22642	▲	–	3.5±0.9
Ceramide UDP-galactosyltransferase (cerebroside synthase)	U07683	▼	–	0.5±0.1
Ubiquitous mitochondrial creatine kinase (U-MTCK; CKMT1)	X59737	–	▲	2.2±0.4
Transcriptional and translational regulation				
Inhibitor of DNA binding 1 (ID1)	D10862	▲	–	2.2±0.5
Eukaryotic translation initiation factor 2 alpha subunit (eIF2-alpha)	J02646s	▼	–	0.4±0.1
Survival of motor neuron protein (rSMN)	U75369	▼	–	0.5±0.1
RL/IF-1	X63594	▼	–	0.3±0.1
Structure-specific recognition protein 1 (SSRP1)	L08814	–	▲	2.1±0.1
Cell–cell communication and cell–matrix interactions				
Insulin-like growth factor II (IGF2)	M13969	▲	–	3.6±1.1
Neuromedin K/Neurokinin B	M16410	▲	–	1.6±0.1
Syndecan 3	U52825	▲	–	1.6±0.1
Others				
Heat shock 70-kDa protein (HSP70)	Z27118	▲	–	4.7±1.6
ATPase hydrogen-potassium alpha 2a subunit	M90398	▲	–	2.0±0.3
Na/K-ATPase beta 3 subunit	D84450	▼	–	0.4±0.1
Brain digoxin carrier protein (BDCP)	U88036	–	▲	2.7±0.7
B-cell translocation gene 1; anti-proliferative factor (BTG 1)	L26268	–	▼	0.2±0.1

The genes are organized according to major functional categories, based on Atlas arrays classifications, and were analyzed using the normalized Trained intensity/Shocked intensity ratio for each gene. Significant changes are shown in the table ($p<0.01$, Student's t-test), and the expression ratio±S.E.M. is described, together with the gene Accession number (GenBank), direction (▲, upregulation; ▼, downregulation), and time of change (hours after behavioral procedure).

transcriptional and translational regulators, tumor suppressors and stress response proteins such as eukaryotic translation initiation factor 2 alpha subunit (eIF2α), survival of motor neuron protein (SMN), ceramide UDP-galactosyltransferase and BTG1 anti-proliferative factor (Table 1).

3.2. Validation of cDNA array results using RT-PCR

Given that changes in gene expression after behavioral training are generally of small magnitude [21,37,42,50], we addressed the problem of false positive values. Several of the candidate genes were randomly selected for further analysis, covering proteins that were involved in different aspects of cell function. Semiquantitative RT-PCR analysis of individual RNA samples showed expression in agreement with the array data. Although cDNA array analysis and semiquantitative RT-PCR have different precision and sensitivity ranges, differential expression ratios generated by the two methods were concordant in displaying the regulation for most of the genes tested. RT-PCR analysis confirmed the differential expression of STX1A, Sdc3,

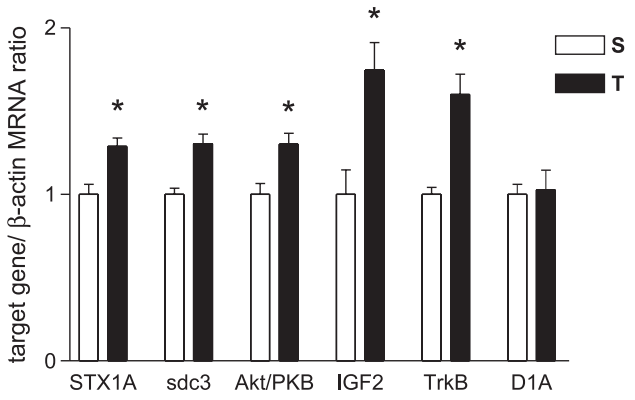

Fig. 3. Differential expression of selected candidate IA-modulated genes as determined by semiquantitative RT-PCR. The analysis was performed using mRNA extracted from individual hippocampi 3 h (for STX1A, sdc3, Akt, IGF2) or 24 h (for TrkB and D1A) after the behavioral procedure. The graph shows relative differences in mean densitometric units of corrected mRNA expression between experimental groups, Shocked (S, open bars) or Trained (T, closed bars) animals. Data are expressed as mean±SEM of gene/β-actin mRNA ratio (arbitrary units), n=5 per group, *p<0.05 with respect to Shocked group, Student's t-test.

Akt, IGF2 and TrkB mRNA levels normalized to β-actin (Fig. 3), although dopamine receptor 1A (D1A) transcript was not confirmed by this method. These results corroborate that the majority of genes tested were confirmed by an independent analysis.

3.3. Selected hippocampal proteins change their level after IA training

Although most microarrays studies perform additional validation techniques to confirm changes in mRNA levels, only a few of them make an analysis of what is the physiological outcome of that modification for the protein product, and generally in a single-gene basis [62]. Using immunoblot assays, we studied the levels of specific candidate proteins in hippocampal protein extracts from trained or shocked rats, 24 h after the behavioral procedure. We chose several proteins known to be involved in synaptic plasticity or memory formation, both by previous literature and our own experimental data [6,7,20,28,34,61]. These include proteins coded by mRNAs whose levels were found to be changed in the cDNA array analysis, and others such as the immediate-early genes (IEG) c-fos and Homer 1a (the latter not present in the cDNA array). Information regarding protein levels of IEGs at post-training intervals longer than classically studied is currently missing. Confirmation through immunoblotting assures that increases or decreases at the mRNA level are reflected in protein steady-state level. We found a 65% increase (t=4.416, df=13, p=0.0007) in Homer-1a immunoreactivity in trained animals compared to shocked controls (Fig. 4B). Syntaxin 1a immunoreactive protein was upregulated 31% (t=2.423, df=24, p=0.023)

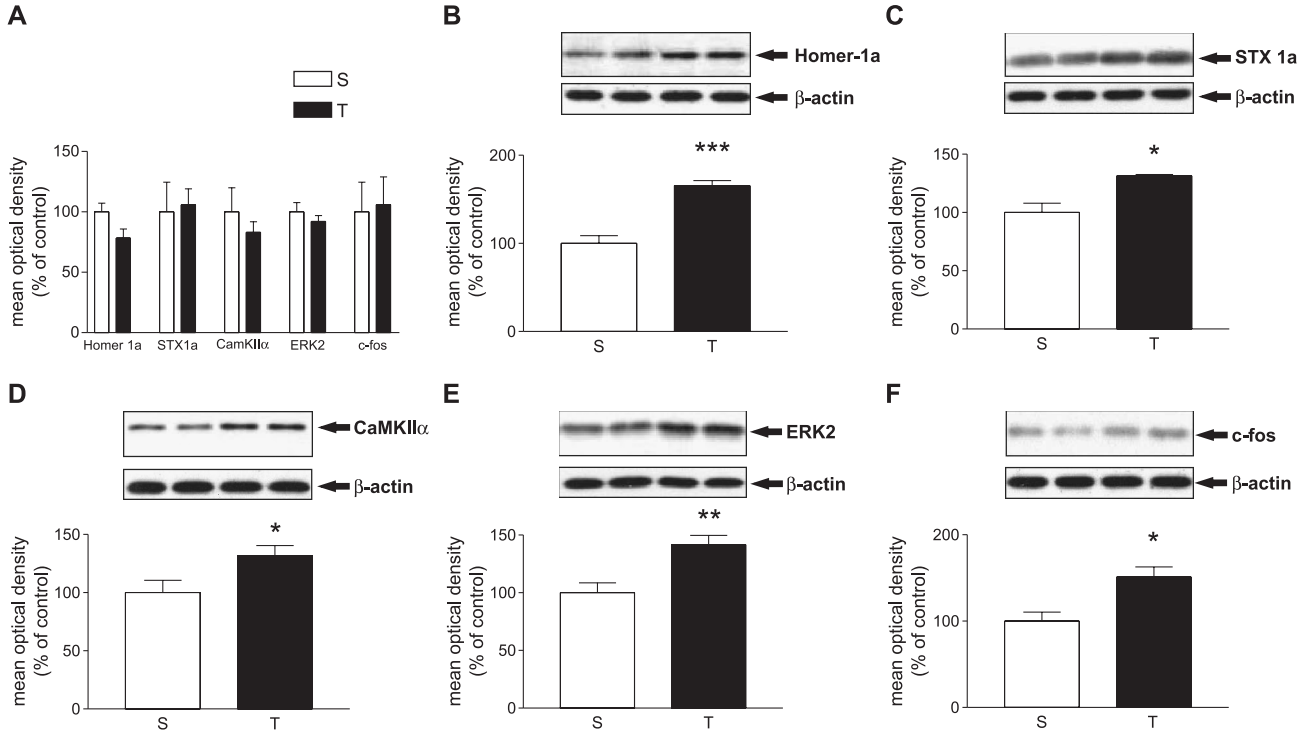

Fig. 4. Analysis of hippocampal levels of selected proteins from Trained (T) vs. Shocked (S) rats. Hippocampal protein levels were measured using extracts belonging to animals sacrificed 3 h (A) or 24 h (B–F) after both experimental procedures. 10–30 μg of whole protein homogenates from each animal was subjected to SDS-PAGE, blotted and probed with protein-specific antibodies. Data are expressed as percentage of Shocked (control) group. Plotted are mean±SEM of OD units from each group. Actin content was analyzed as control. (B) Homer-1a; (C) Syntaxin 1a; (D) CaMKIIα; (E) ERK2; (F) c-fos. Representative immunoblots for two S animals (left lanes) and two T animals (right lanes) are shown on top of each bar graph corresponding to each particular protein (top) and β-actin controls (bottom). Asterisks indicate a significant difference (Student's t-test); *p<0.05, **p<0.01, ***p<0.001, n=8–13 per group.

(Fig. 4C). In addition, CaMKIIα immunoreactivity increased by 32% (t=2.308, df=21, p=0.031) (Fig. 4D), ERK2 (also called MAPK p42) immunoreactivity was increased by 42% (t=3.556, df=15, p=0.003) (Fig. 4E) and c-fos immunoreactivity was increased by 48% (t=3.253, df=8, p=0.012) (Fig. 4F). An additional set of proteins was also tested: immunoreactivity for protein kinase B (Akt/PKB), showed increases in protein levels that did not reached statistical significance (137.2±15.9% Trained vs. 100±10.6% Shocked; t=1.945, df.=8, p=0,0877), while casein kinase II alpha subunit (CK2α) and the calcium-binding protein S100 β displayed nonsignificant changes in protein by immunoblot analysis (data not shown). Remarkably, the same proteins were tested 3 h after training, and none of them presented changes in immunoreactivity between groups (Fig. 4A).

Some of the genes previously shown to be involved in memory formation were only linked to modulation of activity (i. e. phosphorylation) rather than to changes in total protein levels. Our results show that a variety of protein products, with different structures and functions, are upregulated in the hippocampus 24 h after IA training.

4. Discussion

The combination of cDNA arrays with classical biochemical methods allowed us to identify several genes as specifically modulated by the IA training procedure. We performed a transcriptional profile 3 and 24 h after the training session and examined protein levels at the same time points. In this study, we used a ≈1200 gene cDNA array to investigate the differences in hippocampal gene expression of control rats just exposed to a footshock and of rats exposed to a step-down foot-shock association (IA training).

The proportion of genes whose expression levels are modified during LTM consolidation of this task is similar to related reports. Behaviorally stimulated rats showed a proportion of differentially expressed genes of 2.7% after acute stimulation [32]. Rats exposed to 200 trials of eyeblink trace conditioning and sacrificed 24 h after the end of training exhibited 3.8% of the genes changed [21]; rats showed regulated expression of approximately 3% of the total number of genes examined after spatial discrimination learning [51]; finally, recent microarray studies of rats subjected to avoidance [18] or contextual fear conditioning [38] revealed that the number of memory-related genes was around 3% and 0.6% of genes screened, respectively. However, it is critical to take into account that the number of genes spotted into the nylon cDNA array represent only a small fraction of the ≈30,000 genes of the rat genome. Additionally, sometimes region-restricted changes in mRNA and/or protein levels may suffer a dilution effect when total hippocampal tissue is used. However, this should not obscure the fact that this approach is expected to provide information regarding widespread modifications in gene expression within this structure during learning and memory.

Our survey displays similarities with other comparable transcriptional profiling studies. These include changes in the expression of specific gene families, such as protein kinases and phosphatases, membrane receptors, transcriptional and translational regulators, metabolic enzymes, among others [18,21,32,37,38,42,50,51]. Our results show such similarities that—together with the confirmatory techniques—provide further validation that this approach identifies gene regulation related to memory consolidation, in addition to the discovery of novel potential learning-associated genes.

4.1. Changes in cell signalling proteins

This was the largest group of regulated genes which includes several protein kinases, phosphatases and phospholipase C-β3. Interestingly, most members of this category of transcripts were upregulated 3 h after training (Table 1). Hippocampal CaMKIIα protein was found to be elevated at 24 h (Fig. 4C), but neither 3 nor 18 h after training (unpublished observations). The involvement of CaMKIIα activity in memory and synaptic plasticity is well established (see Refs. [16,40]), and there are some examples of experience-dependent or long-term potentiation (LTP)-induced increase of total CaMKIIα levels [59,65].

Another major component of the memory consolidation cascade is the activation (through phosphorylation) of ERK 1/2 [6,8,12,14,63]. However, until the present study there was virtually no evidence of an increase of total ERK mRNA and/or protein after plasticity or learning. Moreover, the selective effect on ERK2 (but not ERK1) mRNA and protein is consistent with the finding that ERK2 phosphorylation was selectively augmented in dorsal CA1 and amygdala after water maze spatial learning [2] and particularly with the finding that ERK1$^{-/-}$ mice show no differences with control wild-type littermates in the acquisition or retention of either contextual or cue fear conditioning [54]. Our findings reveal that ERK2 mRNA is increased 3 h after training and ERK2 protein is increased at 18 (not shown) and 24 h post-training (Fig. 4E). No changes in hippocampal ERK2 protein were observed at the 3 h time point, while ERK1 protein was neither changed at 3 nor 24 h.

Protein kinase CK2 is a ubiquitous and pleiotropic seryl/threonyl protein kinase, with a wide variety of substrates and functions [46]. It has been proposed that, apart from its involvement in general cellular processes, CK2 might be associated with memory formation (see Ref. [11] and references therein). Both α (catalytic) and β (regulatory/targeting/docking) subunits transcripts are upregulated 3 h after training, which may be of relevance due to the non-canonical properties of CK2, namely its high constitutive activity and lack of an acute mode of regulation. However, we could not find a significant increase in CK2α protein levels measured by immunoblot assays at 3 or 24 h post-

training, although preliminary experiments suggest an increase in CK2α protein measured 18 h after training (unpublished observations).

A remarkably consistent finding is the increase in PKB mRNA after IA learning, confirmed through RT-PCR and partially by immunoblot assays (see Results). PKB mRNA was also found to be increased in the hippocampus of rats subjected to spatial discrimination learning [51]. Phosphorylation of this serine/threonine protein kinase by phosphatidylinositol 3-kinase is involved in LTP expression and memory formation and extinction [7,39], and a novel function described for this protein is the regulation of synaptic strength [64]. Although earlier reports identified syntaxin 1B as a plasticity-induced mRNA [53], the fact that STX1A upregulation was confirmed through RT-PCR and immunoblots demonstrate that this isoform, a key component of the transmitter release machinery, is also induced after behavioural experiences.

4.2. Changes in membrane receptors

The differentially expressed genes that code for cell membrane receptors were all upregulated after training (Table 1). Neurotrophin receptor TRKB is a high affinity brain-derived neurotrophic factor (BDNF) receptor, a trophic factor positively implicated in many forms of plasticity and learning in vertebrates [60]. Forebrain-restricted, TRKB knockout mice show increasingly impaired learning behavior in some hippocampus-dependent tasks [47], suggesting a role for BDNF/TRKB receptor signaling in complex learning. Additionally, there are reports that also describe an increase in TRKB mRNA after different kinds of learning (i.e. Ref. [26]). The neuregulin receptor ErbB4 was also induced 24 h after training. ErbB4 is a tyrosine kinase receptor that interacts with membrane-associated guanylate kinases and colocalizes with NMDA receptors; thus, activity-dependent activation of ErbB4 receptor by neuregulins may regulate synaptic plasticity by recruiting tyrosine kinases that regulate NMDA receptor function [25]. mGluR7 is also upregulated 3 h post-training; it is highly expressed at Schaffer collateral-CA1 synapses, and mGluR7 knockout mice display impaired conditioned fear responses, whose acquisition and expression is dependent on the dorsal hippocampus (Ref. [43] and references therein). These results imply that mGluR7s are necessary to form this kind of association (i.e. between taste and an aversive stimulus). Additionally, the somatostatin receptor 5 (SST5) is strongly upregulated 3 h after training, and may be implicated in somatostatin-mediated potentiation of hippocampal NMDA receptor function through activation of inositol-1,4,5-trisphosphate receptors and protein kinase C [49].

4.3. Regulation of IEG expression

Homer proteins are important post-synaptic membrane proteins involved in several processes including locomotor activity and behavioral plasticity, axonal pathfinding, agonist-independent mGluR activity, trafficking of type I mGluRs and regulation of the coupling of mGluRs to calcium and potassium channels (for review, see Ref. [58]). We analyzed protein levels of the IEG member of this family, Homer-1a, at different times after training. Hippocampal Homer-1a protein is markedly increased at 24 h (Fig. 4B), but not at 3 (Fig. 4A) or 18 h post-training (unpublished observations). However, the relevance of this unique splice variant in the modulation of synaptic transmission and dendritic spine morphogenesis makes this finding significant to understand the physiological basis of learning-related plastic changes. Recent findings regarding the in vitro effect of Homer-1a over-expression raise the issue of the role of this protein in the regulation of synaptic structure and function [58]. Sala et al. [52] have recently proposed that, since Homer-1a expression is induced by synaptic activity and also inhibits synaptic transmission and dendritic spine morphogenesis, it may operate in a negative feedback loop that ultimately resets the global activity in that neuron to a normal level. It would be of great interest to explore if the late increase of this IEG after IA training provides the grounds for an inhibitory role for Homer-1a also functioning in vivo. Another IEG that we found increased at the protein (but not transcript) level is c-fos (Fig. 4F); it is intriguing that genes known to be regulated as IEGs (such as c-fos or Homer 1a) have their protein levels increased as late as 24 h post-training, while their mRNAs are upregulated very rapidly after behavioral manipulations [3,10,61]. An appealing view (alternative to that of their classical plasticity function), is that IEGs might act to increase memory storage for later events, facilitate consolidation of experiences yet to come [27].

4.4. Other genes regulated

Transcriptional profiling also revealed a number of IA-regulated genes that were not obviously involved in plastic changes or previously related to behavioral manipulations. eIF2α phosphorylation is known to inhibit eIF2B and subsequently decreases translation, but regulation of the total amount of its mRNA has never been associated with changes associated with memory consolidation (Table 1). It has been recently reported that several protein kinases involved in synaptic plasticity (including ERK) can regulate eIF2α phosphorylation in the hippocampus [4]. One could speculate that an increased level of total eIF2α protein might allow a more dynamic regulation of the translational response necessary for LTM formation. The role of IGF2 is more intriguing; despite IGF1 involvement in cognitive performance in humans, no clear behavioral phenotype has been linked to IGF2 function, although it has been extensively implicated in embryonic development [5].

Beyond the specific control of each gene through regulatory elements in their promoters, modulating the access to DNA packaged into chromatin is critical for

fine-tuning gene expression. SSRP1 is an ATP-dependent chromatin-remodelling enzyme and does not activate transcription, but is postulated to facilitate DNA-binding and enhance the transcriptional activity of other factors (i.e. Ref. [66]). The increase in SSRP1 expression is the first evidence of an involvement of this type of enzymes in learning, since the role of this protein in behavioral responses has not been previously studied.

4.5. Concluding remarks

The idea of local protein synthesis in dendrites has received increasing experimental support (see Ref. [55]), and although subject to abundant speculation, one of its most convincing functions would be to simplify the task of targeting newly synthesized proteins to the activated synapses, the sites that requires them to induce plasticity. In addition, the findings mentioned above regarding modulation of the mRNA localization, transcriptional and translational machinery are in accordance with a recent report that shows the requirement of the pumilio/staufen family for LTM formation in *Drosophila* [22]. Another important point was raised 10 years ago, when it was suggested the pioneering idea that "enduring changes in synaptic efficacy or memory formation may initially require phosphorylation of select proteins, followed by changes in gene expression of the very same proteins that initially participate in the post-translational modification" [45]. Indeed, this seems to be the case for many of the proteins found in the present work to be changed 24 h after training, whose total expression levels were not previously reported to be altered by learning.

The finding that all the protein products we examined were upregulated 24 h but not 3 h after training, and that their respective mRNAs (as assessed by the DNA array) behaved the opposite way (upregulated at 3 but not 24 h), suggests at least three possible scenarios that might explain these results. The first is that those transcripts that are synthesized early on (3 h) are kept translationally repressed until later, where they are translated afterwards into the proteins they code for. This regulation may include the mRNA translocation from the soma to the appropriate dendrites. The findings by Dubnau et al. [22] reporting that both the transcriptional repressor pumilio and the RNA-binding protein staufen (involved in RNA transport to dendrites) are upregulated at 0 and 6 h after spaced training in Drosophila, and specially that these two proteins are required for normal LTM, indicate that the role of mRNA transport, targeting and repression in LTM formation may be more widespread than previously thought. The second hypothesis involves the existence of one or more additional peaks of mRNA synthesis after the period analyzed (0–9 h) [31] which would account for the peak of newly produced protein at 24 h. However, preliminary experiments using intrahippocampal infusions of transcriptional inhibitors did not reveal a transcriptional requirement between 9 and 24 h (unpublished observations). A third possibility that could not be ruled out is the potential role of decreased protein turnover in trained rats, which could be related to the recently described activity-regulated change in half-life of synaptic proteins [23].

In conclusion, our findings show a modulation in the expression of several key genes involved in memory processing, and uncover others that were not associated with memory formation. The transcriptional analysis and protein assessment presented here suggests that many results obtained basically at the mRNA level should be cautiously evaluated when correlating transcriptional to translational responses. We are currently undertaking studies attempting to dissect the role of several of these candidates in the mechanisms of memory formation.

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