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Late memory-related genes in the hippocampus revealed by RNA fingerprinting

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ABSTRACT **Although long-term memory is thought to require a cellular program of gene expression and increased protein synthesis, the identity of proteins critical for associative memory is largely unknown. We used RNA fingerprinting to identify candidate memory-related genes (MRGs), which were up-regulated in the hippocampus of water maze-trained rats, a brain area that is critically involved in spatial learning. Two of the original 10 candidate genes implicated by RNA fingerprinting, the rat homolog of the ryanodine receptor type-2 and glutamate dehydrogenase (EC 1.4.1.3), were further investigated by Northern blot analysis, reverse transcription–PCR, and *in situ* hybridization and confirmed as MRGs with distinct temporal and regional expression. Successive RNA screening as illustrated here may help to reveal a spectrum of MRGs as they appear in distinct domains of memory storage.**

Identifying the mechanisms responsible for memory formation and consolidation has long been a goal of behavioral neuroscience. Many experiments over the past few decades have demonstrated that inhibitors of transcription or translation interfere with long-term memory formation, indicating the requirement of *de novo* gene expression (1–4). Despite the importance of this finding, little is known about the identity and specificity of the required proteins. Changes in early inducible genes, for example, are known to occur not only during learning and memory, but also during a broad range of behaviors, including motor activity and sensory discrimination (5–10). Changes in the expression of late effector genes, such as those encoding BiP and calreticulin, have been described during long-term sensitization in *Aplysia* but not in associative memory (11, 12). To our knowledge, no changes in late effector genes have been previously demonstrated during associative memory.

To identify memory-related genes (MRGs) we have used a new and sensitive approach, RNA fingerprinting by arbitrarily primed PCR (13, 14), to compare gene expression in control swimming rats with water maze-trained rats. The Morris water maze is a learning paradigm in which a rodent learns to locate a submerged island in a large pool by creating a spatial map using extra-pool cues (15–17). This learning ability represents a complex faculty involving input from different senses including visual, olfactory, auditory, and somatosensory information (18–20). The hippocampus has been shown to be a brain locus for spatial memory (21). Pyramidal cells in the rat hippocampus discharge selectively at specific locations of a spatial environment (22, 23) and maintain their receptive field when the relevant cues are removed (24) or when the light is turned off (25). Lesions of the hippocampus result in impaired acquisition of tasks that depend on spatial strategies (26–28) and spatial memory impairment parallels the magnitude of dorsal hippocampal lesions (29).

MATERIALS AND METHODS

Water Maze Learning. Male Wistar rats, 60–90 days old (200–300 g) were housed individually in plastic cages with *ad libitum* access to food and water, constant temperature (23°C), and a 12-hr light/12-hr dark cycle. All tests were carried out in the light phase and were in accordance to National Institutes of Health guidelines. The rats were trained in a large swimming pool, 1.5 m in diameter and 0.6 m high, containing water at 26 ± 1°C. The pool was in the center of a room containing various salient cues (table, door, computers, etc.). A 10 cm^2 transparent square platform was hidden in a constant place in the pool with its top surface submerged 1 cm below the water level. To reduce stress in the experimental day, the first day was dedicated to swimming training in the absence of an island. Each rat was placed in the pool for 2 min and then returned to its home cage. During the next day one-half of the rats were placed again in the pool for a 2.5 min swimming session, and these rats were used as controls. The other half were given four consecutive trials to locate the platform, each trial lasting up to 2 min. In case the rat did not reach the platform in time, it was guided to the platform. Rats were required to spend 30 sec of an intertrial interval on the platform. The rats' escape latency was measured using a stopwatch. The rats were sacrificed 2, 6, 12, and 24 hr after training using a standard small animal guillotine. The rat brains were dissected on ice and the hippocampi quick frozen on dry ice. To verify that indeed the rats that were used learned the spatial location of the island, a comparable set of nine rats were trained to find the island, and 6 hr later they were tested on a quadrant analysis test. The island was removed and the search strategy of the rats was monitored for 1 min to measure if the rats tend to search in the quadrant where the island was previously located.

RNA Fingerprinting. Hippocampal RNA was extracted with the RNA Isolator (Genosys, The Woodlands, TX) and fingerprinted by the Delta RNA fingerprinting kit (CLONTECH).

Abbreviations: MRG, memory-related genes; GDH, glutamate dehydrogenase; RYR1, -2, -3, ryanodine receptor types 1–3; PGK1, phosphoglycerate kinase 1; RT, reverse transcription; DG, dentate gyrus; CA, cornu ammonis.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. U95147 (MRG-1), U95148 (MRG-2), U95149 (MRG-3), U95150 (MRG-4), U95151 (MRG-5), U95152 (MRG-6), U95153 (MRG-7), U95154 (MRG-8), U95155 (MRG-9), U95156 (MRG-10), U95157 (RYR2)].

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Briefly, first-strand cDNA was synthesized using 2 μg of total RNA from control and water maze-trained rats as a template, oligo(dT) as a primer, and Moloney murine leukemia virus reverse transcriptase (MoMLV-RT). Two dilutions of each cDNA template (corresponding to 5 and 20 ng of reverse transcribed RNA) were used for the PCR fingerprinting. In addition to the template, each PCR reaction contained 50 μM dNTPs, 1 μM primers, 50 nM [α-^{33}P]dATP (1,000–3,000 Ci/mmol; Amersham), and 1× Advantage Klen*Taq* polymerase and reaction mixes (CLONTECH). PCR primers used were a pairwise combination of arbitrary "P" (P1–P3) and oligo(dT) "T" (T1–T3) primers (see reference manual for oligonucleotide sequences). Thermal cycling was performed using a DNA Thermal Cycler 480 (Perkin–Elmer) and the following program: 1 cycle of 94°C for 5 min, 40°C for 5 min, and 68°C for 5 min; 2 cycles of 94°C for 2 min, 40°C for 5 min, and 68°C for 5 min; 22 cycles of 94°C for 1 min, 60°C for 1 min, and 68°C for 2 min. PCR products were electrophoresed on a 8% acrylamide/8 M urea gel and run in 0.1 M Tris·borate/2 mM EDTA buffer (pH 8.3) (1× TBE). The gels were dried under vacuum and exposed to BIOMAX-MR x-ray films (Eastman Kodak). The presence of genomic DNA contamination was negligible as assessed using total RNA as template. In a typical RNA fingerprint, about 80–100 bands were evident in each amplification. Differentially expressed cDNAs were eluted from the gel, reamplified, blunt-ended with *Pfu* DNA polymerase (Stratagene), subcloned into the *Srf*I cloning site of pCR–Script Amp SK(+) cloning vector (Stratagene), and sequenced by the chain-termination method using T3 and T7 primers with Sequenase version 2.0 T7 DNA polymerase (United States Biochemical). Nucleotide sequences were subjected to FASTA searches for sequence homologies.

Ryanodine Receptor Type-2 (RYR2) cDNA Cloning. Using RNA fingerprinting, we originally isolated MRG-1, a 155-bp cDNA fragment that was 95% identical to bases 2032–2186 of the mouse RYR2 (30), but also shared some homology (82–83%) with the RYR1 and RYR3. To unequivocally determine the identity of MRG-1, we isolated an overlapping 1.6-kb cDNA clone by the 5′ rapid amplification of cDNA ends technique. Briefly, first-strand cDNA was synthesized from poly(A) hippocampal RNA using the gene-specific primer MRG-R (complementary to residues 122–142 of MRG-1) and MoMLV-RT. After first strand synthesis, the original template was destroyed with RNase H and an anchor sequence (oligo-dC tail) was added to the 3′ end of the cDNA using terminal deoxynucleotidyl transferase. Amplification of target cDNA with MRG-R and an oligo-dG(18) primer resulted in a 1.6 kb DNA product, which was blunt-ended, subcloned into the *Srf*I cloning site of pCR–Script Amp SK(+) cloning vector (Stratagene), and sequenced.

Northern Blot Analysis. Hippocampal RNA (20 μg) from control and water maze-trained rats was denatured, electrophoresed on a 1% agarose/formaldehyde gel, and capillarity transferred to Hybond-N+ membranes (Amersham). The probes for RYR2 and glutamate dehydrogenase (GDH) mRNAs were MRG-1 and MRG-2, respectively. The RNA blots were hybridized overnight at 42°C in a buffer (6× SSC/5× Denhardt's solution/200 μg/ml denatured salmon sperm/50% formamide) containing the probes (10^7 cpm/ml) radiolabeled to a specific activity of 10^9 cpm/μg with [α-^{32}P]dATP (6,000 Ci/mmol; 1 Ci = 37 GBq; Amersham) and a random primed labeling kit (Boehringer Mannheim). At the end of hybridization, blots were washed twice with a solution 1× SSC/0.1% SDS for 20 min at 42°C and once with a solution 0.1× SSC/0.1% SDS for 15 min at 50°C, before being exposed to BIOMAX-MR x-ray film (Eastman Kodak for autoradiography. After hybridization with the RYR2 and GDH probes, the blots were rehybridized with the probe for phosphoglycerate kinase 1 (PGK1) (31) to quantify and simultaneously correct for variations in the amount of RNA loaded on the gel.

Reverse Transcription (RT)–PCR. Forward (F) and reverse (R) primers used to identify RYR2 and GDH mRNAs by RT-PCR were as follows: RYR-F, 5′-CATCGGTGATGAAATTGAAGA-3′; RYR-R, 5′-AGCATCAATGATCAAACCTTG-3′; GDH-F, 5′-ACAGCAGAGTTCCAGGACAG-3′; and GDH-R, GTCTATGTGAAGGTCACGCC-3′. Expected amplification products for the RYR2 and GDH were 130 and 212 bp, respectively. To control for the integrity of RNA and for differences attributable to errors in experimental manipulation from tube to tube, primers for rat PGK1 were included in the PCR reactions and generated a 183-bp PCR product. Oligonucleotide sequences were as follows: PGK-F, 5′-AGGTGCTCAACAACATGGAG-3′; PGK-R, 5′-TACCAGAGGCCACAGTAGCT-3′. Hippocampal RNA was treated with RNase-free DNase to remove any residual genomic DNA and reverse transcribed to single-stranded cDNAs using reverse primers and MoMLV-RT (GIBCO/BRL). To the cDNA reaction was added a PCR master mix to yield the following final concentrations: 1 μM of specific primers, 200 μM dNTPs, 100 nM [α-^{32}P]dATP (6,000 Ci/mmol; Amersham), 2.5 units of *Taq* DNA polymerase, and *Taq* buffer (Perkin–Elmer) containing 1.5 mM $MgCl_2$. PCR (25 cycles of 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min) was performed on a DNA Thermal Cycler 480 (Perkin–Elmer). Amplification products arising from RT-PCR were electrophoresed on a 2% agarose gel, visualized by ethidium bromide staining, excised from the gel, and counted for radioactivity. The use of RT-PCR was validated on the basis of size and the sequence of the amplification products (data not shown). In a preliminary series of experiments, the RT-PCR protocols used were optimized to obtain high sensitivity and specificity. Under these conditions, the efficiencies of amplifications were in the linear range. The amount of radioactivity of RYR 2 and GDH mRNAs were divided by those of PGK1 and expressed in arbitrary units.

***In Situ* Hybridization.** The brains were removed and frozen at −70°C. Coronal sections of 12 μm thickness were cut on a cryostat, thaw mounted onto gelatinized slides, and stored at −70°C until hybridization. The sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3), acetylated in 0.1 M triethanolamine hydrochloride containing 0.25% acetic anhydride, dehydrated in serial alcohol solutions, dilipidated in chloroform, partially rehydrated, and air dried. Two 48-mer antisense oligonucleotides were designed to detect transcripts encoding GDH, 5′-AAGATGCTTCCTTCATAGACCTTGGCTTTGGGGAAGCCCAGAATTGAT-3′ and RYR2, 5′-AAAGCATCAATGATCAAACCTTGTATGATAGCCAAAAGGATGACAATC-3′; sense oligonucleotides complementary to these were used as control. Probes were 3′-end labeled with [^{35}S]dATP using terminal deoxyribonucleotidyl transferase (Boehringer Mannheim). The sections were hybridized overnight at 37°C in a buffer (4× SSC/50% formamide/10% dextran sulfate/250 μg yeast tRNA/1× Denhardt's solution) containing the probe ($2–4 \times 10^7$ dpm/ml). Sections were washed four times in 1×X SSC for 15 min at 55°C, and then twice in 1× SSC for 1 hr at room temperature. Sections were rinsed in distilled water and dried. The hybridized sections were exposed to film (hyperfilm βmax; Amersham) for 2–3 weeks for the generation of autoradiograms. Hybridization with the sense oligonucleotide probes led to no detectable signal. Evaluation of hybridization signals for GDH and RYR2 were obtained by using a computer-assisted image analysis system and the National Institutes of Health IMAGE 1.49 software (Wayne Rasband, National Institutes of Health, Bethesda). Mean densities of silver particles (average gray level of the pixels within the given area) in the dentate gyrus (DG), cornu ammonis (CA) 1, and CA3 regions of the hippocampus were measured and expressed in arbitrary units.

RESULTS AND DISCUSSION

To relate mRNA induction to a learning task we trained the rats for four consecutive trials to locate a submerged island in the water maze. The rats completed the task within 4.2 ± 0.18 min (n = 36) and their latency time to find the island was reduced from 73.1 ± 6.4 sec to 18.4 ± 2.3 sec (Fig. 1*A*), indicating that indeed the rats learned the task. To reduce stress, we acclimated and pre-trained all the rats for 2 min 1 day before training in the water pool in the absence of an island.

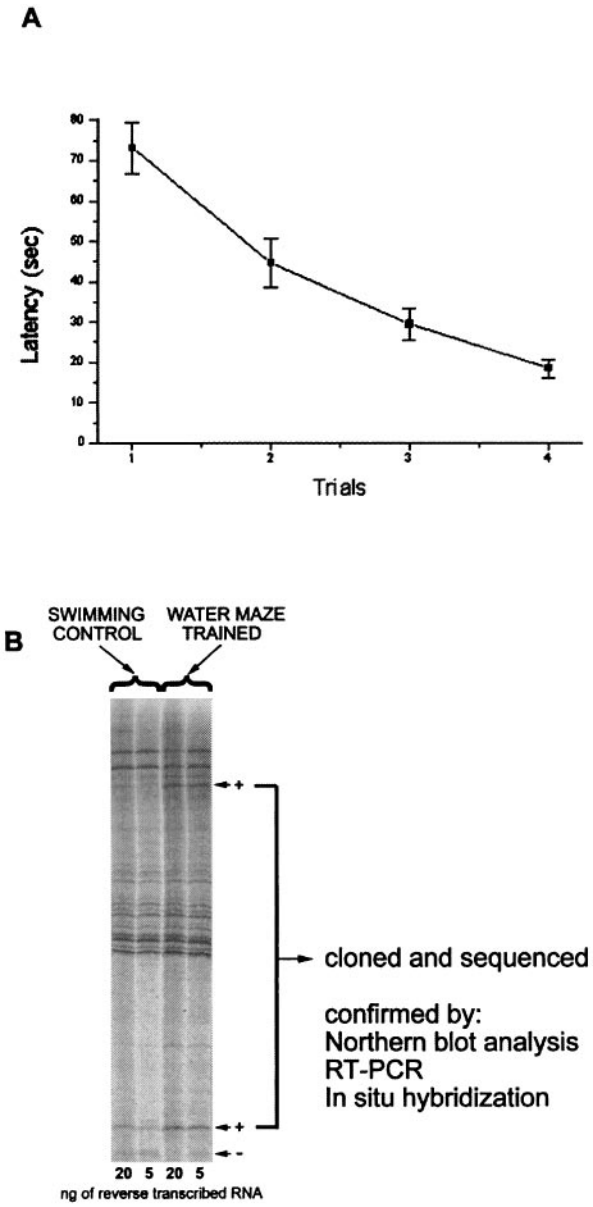

FIG. 1. MRG cDNA cloning experiments on water maze-trained vs. control rats. (*A*) Escape latencies of rats swimming to a submerged platform in the water maze. Rats were trained for four consecutive times to locate the submerged platform. The values are mean ± SEM (n = 36). The shorter latency time is statistically significant (ANOVA with repeated measures $F_{3,102} = 21.95$, $P < 0.0001$; the trend is linear). (*B*) Example of RNA fingerprinting. Two dilutions (20, 5 ng) of reverse-transcribed hippocampal RNA from control swimming and water maze-trained rats were PCR fingerprinted using a pairwise combination of arbitrary primers. PCR products were electrophoresed on a sequencing gel and visualized by autoradiography. Arrows mark three potential differentially expressed cDNAs. Clones that appeared up-regulated were sequenced and further analyzed by Northern blot analysis, RT-PCR, and *in situ* hybridization.

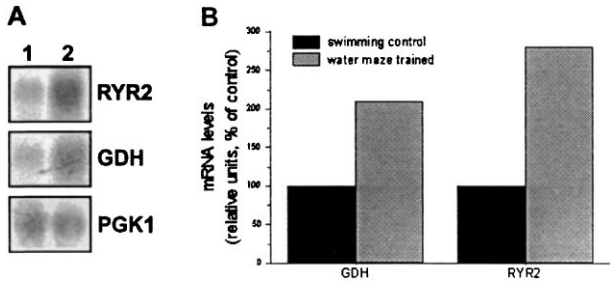

FIG. 2. Hippocampal changes in RYR2- and GDH-mRNA levels 6 hr after water maze training. (*A*) Northern blot analysis of RYR2 and GDH mRNAs in control swimming and water maze-trained rats. Hippocampal RNA from control swimming (lane 1) and water maze-trained rats (lane 2) was hybridized with the labeled probes for RYR2, GDH, and PGK1. (*B*) Relative mRNA levels of RYR2 and GDH. To quantify the amount of RYR2- and GDH-mRNA levels, the densitometry of their hybridization signal was divided by that of PGK1 obtained in the same sample and expressed in relative units.

Control rats were allowed to swim in the pool in the absence of the island for 2.5 min. To verify that indeed the rats that were trained in fact learned the spatial location of the island, a comparable group of rats was trained to find the island and 6 hr later tested on a quadrant analysis test. The trained rats swam significantly longer in the quadrant where the island was located (31.1 ± 2.0% of the total distance compared with 24.4 ± 1.1% and 23.3 ± 0.8% in the two adjacent quadrants and 21.2 ± 2% in the opposite quadrant, ANOVA $P < 0.01$).

Hippocampal RNA from control swimming rats and water maze-trained rats 6 hr after training was reverse transcribed and then fingerprinted using different pairs of arbitrary se-

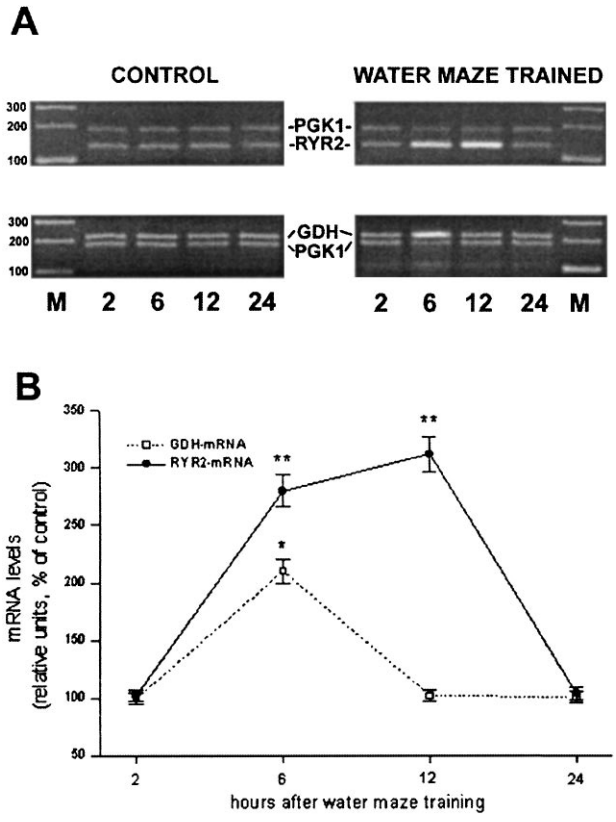

FIG. 3. Time-course changes of RYR2- and GDH-mRNA levels after water maze training. (*A*) RT-PCR analysis of RYR2 and GDH mRNAs in control swimming and water maze-trained rats at 2, 6, 12, and 24 hr after training. (*B*) Relative mRNA levels of RYR2 and GDH in control swimming and water maze-trained rats at 2, 6, 12, and 24 hr after training (n = 6; *, $P < 0.01$, **, $P < 0.001$).

lected primers (Fig. 1*B*). MRGs were operationally defined as those cDNAs that were induced, based on RNA fingerprinting, in the hippocampi of rats 6 hr after water maze training. Occasionally, clones that appeared to be down-regulated were observed (Fig. 1*B*), but they were not investigated further here. We isolated and sequenced 10 candidate MRG cDNAs. MRG-1 was a 155-bp cDNA fragment that was 95% identical to bases 2032–2186 of the mouse RYR2 (30), but also shared some homology (82–83%) with the RYR1 and RYR3. To unequivocally identify MRG-1 as the rat homolog of the RYR2, we isolated a 1.6-kb cDNA clone whose sequence (GenBank accession no. U95157) overlapped the first 142 bases of MRG-1 and was 95% homologous to the mouse RYR2 (bases 538-2173). MRG-2 was a 1039-bp cDNA fragment completely identical to residues 1350–2388 of the rat GDH (EC 1.4.1.3) previously described (32). The sequences of MRG-3 to MRG-10 share no significant homology with any known sequences.

The changes of GDH and RYR2 mRNA levels were further assessed by a combination of Northern blot analysis (Fig. 2) and RT-PCR (Fig. 3). GDH mRNA levels were increased by 110% 6 hr after water maze training and returned to normal values at 12 hr. RYR2 mRNA levels, instead, were increased at 6 hr (180%), remained elevated at 12 hr (210%), and returned to normal values 24 hr after the training. Levels of GDH and RYR2 mRNAs were then measured in different subdivisions of the hippocampus by *in situ* hybridization (Fig. 4). GDH mRNA was detected widely in the hippocampus, whereas RYR2 transcript was more abundant in the DG and CA3 region. Clear changes in the mRNA expression of each protein were consistently observed 6 hr after water maze training. All trained rats exhibited a significant increase in GDH and RYR2 mRNA levels in CA3 and DG areas. GDH mRNA was elevated more in CA1 (pyramidal layer and lacunosum molecolare) than in DG, whereas RYR2 mRNA increased predominantly in DG and CA3. Interestingly, changes of GDH but not RYR2 mRNA levels were also observed in other regions of the brain such as the laterodorsal nucleus of the thalamus and cingulate cortex (Fig. 4*A*).

The changes of different MRGs at selective times and hippocampal subfields may indicate distinct contributions to learning and memory. In the past, a number of hypotheses have been advanced regarding the role of *de novo* synthesized proteins in the hippocampus during learning and memory (1–4). Although our study did not directly assess the role of GDH and RYR2 in spatial memory, some hypotheses can be suggested based on previously identified functions. GDH is an enzyme, central to glutamate metabolism, which catalyzes the reversible conversion of α-ketoglutarate to glutamate (33, 34). Increased steady-state levels of GDH mRNA, therefore, may reflect an increased turnover of the excitatory neurotransmitter glutamate, which has been implicated in learning and memory (35, 36). RYR2, the major ryanodine receptor isoform in the brain, is an intracellular Ca^{2+} release channel that,

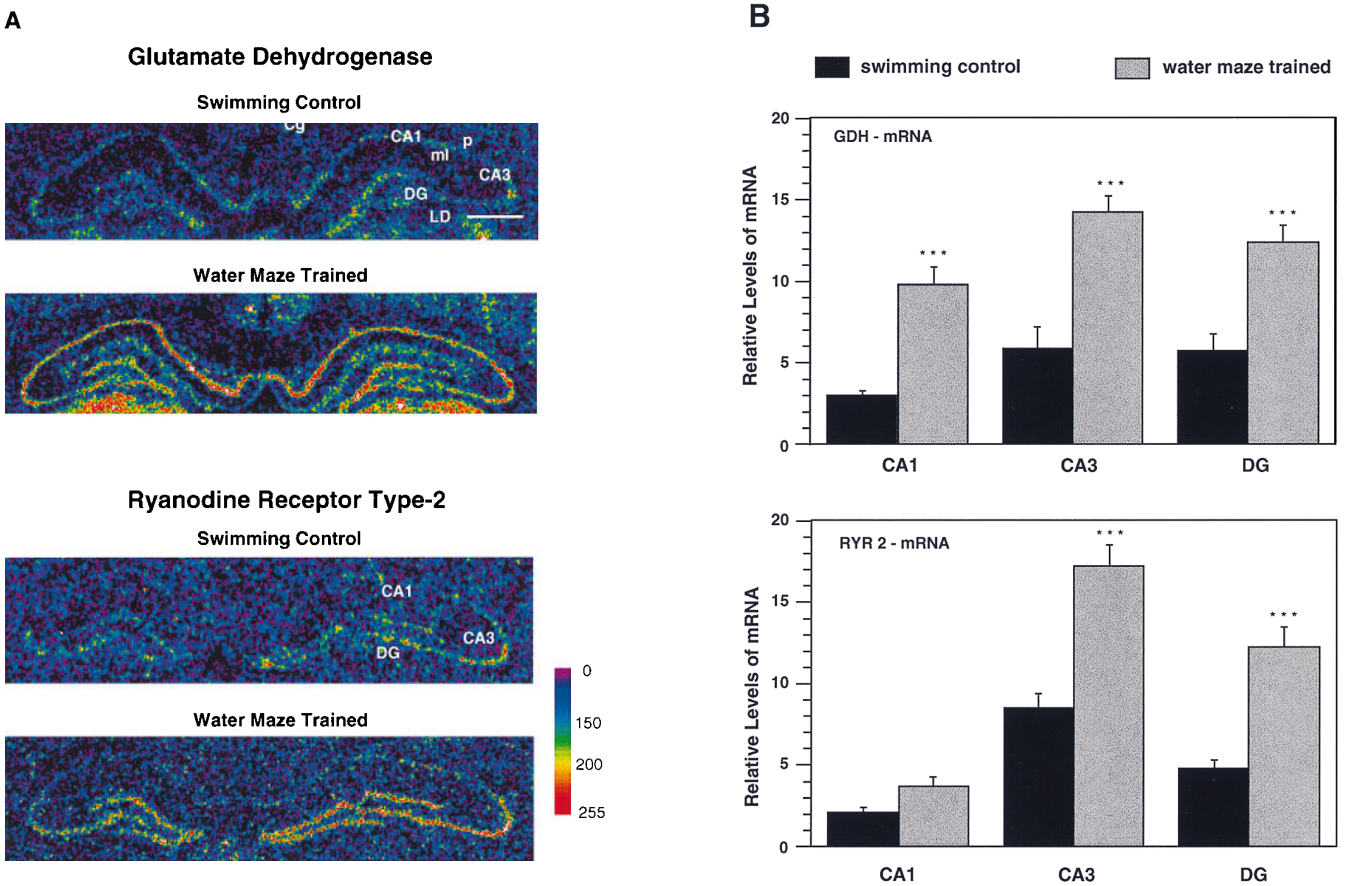

FIG. 4. Localization by *in situ* hybridization of RYR2 and GDH mRNAs in hippocampal subfields after water maze training. (*A*) Pseudocolor representation of RYR2- and GDH-mRNA expression in control swimming and water maze-trained rats. Six hours after training, a general increase of GDH mRNA is seen in the pyramidal layer (P) of CA areas and DG; increases also emerge in the lacunosum molecolare (ml) of CA1, cingulate cortex (Cg), and laterodorsal nucleus of thalamus (LD). RYR2-mRNA expression is low in CA1 and increased predominantly in CA3 and DG after water maze training. Color spectrum on the right side of the figure represents the pixel value of gray levels. (Scale bar = 800 μm.) (*B*) Relative mRNA levels of RYR2 and GDH in different hippocampal subfields of control swimming and water trained rats. Quantification of induction increase is achieved by comparison of pixel values of an area of interest in four sections from each of four pairs of rats. Changes in mRNA levels are expressed as the density ratio of trained-to-control animals. Values are mean ± SEM.

like IP_3 receptors, participates in the homeostasis of cytosolic calcium (37–41). Levels of intracellular calcium, in turn, have been implicated in changes of synaptic weight during associative (42, 43) and nonassociative memory (44, 45). The increased expression of the RYR2 demonstrated here (with three independent techniques) could provide an important means to further amplify learning-induced changes of $[Ca^{2+}]$ that arise from *N*-methyl-D-aspartate and voltage-dependent Ca^{2+}-influx and/or IP_3-mediated release due to metabotropic receptor activation.

It should be emphasized that in this study we screened a small fraction, perhaps less than 10%, of the genes that may be differentially expressed during long-term memory. Successive screening and confirmation with Northern blot analysis, RT-PCR, and *in situ* hybridization may uncover, therefore, a spectrum of MRGs as they appear in distinct temporal domains of memory storage.

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