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ARTICLE

Comparison of treatment regimens to sensitize in situ hybridization for low-abundance calmodulin transcripts in the white matter of the rat spinal cord

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ABSTRACT We compared two in situ hybridization protocols for the detection of low-abundance calmodulin (CaM) I mRNA populations in the lipid-rich white matter of the rat lumbar spinal cord. Digoxigenin-labeled, CaM I gene-specific antisense and sense cRNA probes were used in these experiments. Although microwave heating followed by chloroform and 0.1% triton X-100 treatment resulted in the specific labeling of several cells in the gray matter of the spinal cord with a low nonspecific signal, it did not detect any CaM I expressing cells in the white matter. The protocol involving a hybridization solution adjusted to a slightly alkaline pH (pH 8.0), however, resulted in the detection of a large number of CaM-expressing cells not only in the gray matter, but also in the white matter of the spinal cord, with a nonspecific hybridization signal that was essentially identical to that of the background, and it also retained a much better overall tissue quality as compared with the protocol involving microwave heating and triton X-100 treatment. Numerous medium-sized astrocyte-like cells and smaller cells resembling oligodendrocytes were detected that expressed CaM I mRNAs throughout the white matter of the lumbar spinal cord. Thus, in situ hybridization carried out at a slightly alkaline pH is a far superior method for the detection of low-abundance mRNA populations in lipid-rich regions of the central nervous system, such as the white matter areas, as compared with microwave heating combined with triton X-100 treatment.
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KEY WORDS

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In situ hybridization is widely used to study the spatiotemporal distribution of gene expression in nervous tissue. We are especially interested in the study of the distribution of calmodulin (CaM) mRNA populations in the rat brain under normal (Palfi et al. 1999) and experimental conditions (Palfi and Gulya 2001; Palfi et al. 2001; Vizi et al. 2000) by the use of in situ hybridization methods in which radioactive (Kortvely et al. 2002; Palfi et al. 1998, 1999) and digoxigenin (DIG)-labeled riboprobes (Kovacs and Gulya 2001, 2002) are used. The three CaM genes (CaM I, II and III) are widely expressed in the brain, where a widespread and differential area-specific distribution of the CaM mRNAs has been detected (Palfi et al. 1999). The expression patterns corresponding to the three CaM genes differed most considerably in the olfactory bulb, the cerebral and cerebellar cortices, the diagonal band, the suprachiasmatic and medial habenular nuclei, and the hippocampus. Moreover, the significantly higher CaM I and CaM III mRNA copy numbers than that of CaM II in the molecular layers of certain brain areas revealed a differential dendritic targeting of these mRNAs. CaM mRNA populations are abundant in the piriform cortex, the pyramidal and granular cell layers of the hippocampus, the amygdalohippocampal area, the nucleus of the lateral olfactory tract, the parafascicular thalamic nucleus, the superior colliculus, the pontine nuclei and the dorsal tegmental area. Low CaM mRNA populations are characteristic of the molecular (plexiform) layers of the olfactory bulb, the hippocampus and the cerebellum, and in general all white matter areas, including the anterior commissure, the cerebellar white matter, the corpus callosum, the internal capsule, the pyramidal tract and the ventral hippocampal commissure. Interestingly, there are no data on the CaM gene expression in the white matter of the spinal cord, possibly because of the very low amounts of CaM mRNAs in this area that are available for detection with conventional in situ hybridization methods, which possess only average sensitivity.

In our study, we explored the possibility that the lack of data regarding CaM gene expression in the white matter of the rat spinal cord is due to its generally very low expression level, and to the relative insensitivity of most current in situ hybridization protocols for low-abundance mRNA populations. Thus, the objective of the study was to localize CaM I mRNAs in the mature rat spinal cord white matter by using different in situ hybridization methods, involving either

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microwave heating combined with triton X-100 treatment or a slightly alkaline hybridization solution (pH 8.0) to increase the sensitivity, using CaM I gene-specific DIG-labeled antisense and sense cRNA probes in cryostat sections of the lumbar spinal cord of adult rats.

Materials and Methods

Experimental animals and tissue preparation

The experimental procedures were carried out in strict compliance with the European Communities Council Directive (86/609/EEC), and followed the Hungarian legislation requirements (XXVIII/1998 and 243/1998) regarding the care and use of laboratory animals. Adult (200-220 g) male Sprague-Dawley rats were maintained under standard housing conditions. The animals were killed by decapitation; their lumbar spinal cords were quickly removed, embedded in Cryomatrix embedding medium (Shandon Scientific Ltd., Pittsburgh, PA, USA) and frozen immediately at -70°C. Serial coronal cryostat sections (15 μm) were cut onto 3-aminopropyltriethoxysilane-coated glass slides, air-dried and stored at -70°C until further processing.

cRNA probes

For the preparation of DIG-labeled cRNA probes, in vitro RNA syntheses from the purified and linearized vectors were carried out to prepare antisense and sense CaM I cRNA probes, using a DIG RNA labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's protocol, as described previously (Kovacs and Gulya 2001, 2002). Briefly, genomic sequences of the 3'-nonhomolog regions of CaM I, II and III mRNAs were amplified by polymerase chain reactions (PCRs). PCRs were performed by employing EcoR I and BamH I restriction enzyme cleavage site-extended primers. The primer sequences complementary to rat genomic DNA were as follows: for CaM I, 5'-AGACCTACTTTCAACTACT, corresponding to the 30-48-bp sequence, and 5'-TGTAAAACTCATGTAGGGG, corresponding to the 236-254-bp sequence of exon 6 (Nojima and Sokabe 1987); for CaM II, 5'-ATTAGGACTCCATTCCTCC, corresponding to the 144-162-bp sequence (numbered 1929-1947), and 5'-CACAACTCCACACTTCAACAGC, corresponding to the 353-374-bp sequence (numbered 2138-2159) of exon 5 (Nojima 1989); and for CaM III, 5'-ATGATGACTGCGAAGTGAAG, corresponding to the 12-31-bp sequence (numbered 7058-7077) of exon 6, and 5'-CAGGAGGAAGGAGAAAGAGC, corresponding to the sequence 153-172-bp downstream to the stop codon (numbered 7228-7247; Nojima 1989). Standard PCRs were run for 35 cycles (Palfi et al. 1998), and the resulting PCR products were cloned into a pcDNA3 vector (Invitrogen Corp., Carlsbad, CA, USA) and sequenced (AB 373 DNA Sequencer, PE Applied Biosystems, Foster City, CA, USA) to confirm their identity. In vitro RNA syntheses from the purified and linearized vectors were carried out to prepare antisense and sense cRNA probes. The complementary probe sequences were 225 bp (CaM I), 231 bp (CaM II) and 157 bp (CaM III) long. Labeled probes were purified by size exclusion chromatography on a ProbeQuant G-50 Sephadex micro column (Pharmacia Biotech, Uppsala, Sweden).

In situ hybridization

In situ hybridization of DIG-labeled cRNA probes was carried out in accordance with our previously described protocols (Kovacs and Gulya 2001, 2002). To check the effectiveness of microwave heating combined with triton X-100 treatment either with or without chloroform treatment, selected coronal cryostat lumbar spinal cord sections were heated by microwave irradiation (5 + 4 + 4 min, to prevent boiling) in 0.01 M Na-citrate solution (pH 6.0). The sections were either rinsed in chloroform for 5 min at room temperature (RT) and fixed for 5 min in 2x SSC containing 4% formaldehyde or were fixed immediately, then washed twice in 2x SSC for 1 min and rinsed in 0.1 M triethanolamine containing 0.25% acetic anhydride for 5 min at RT. At the end of the prehybridization procedure, the sections that underwent microwave heating were washed with either 0.1% or 1.0% triton X-100 in 2x SSC for 3 min. The sections were next washed in 2x SSC for 5 min, dehydrated, air-dried and hybridized in 50 μl hybridization solution (50% formamide, 4x SSPE, 1x Denhardt's reagent, 10% dextran sulfate, 100 mM DTT, 0.1% SDS, 100 μg/ml salmon sperm DNA and 100 μg/ml yeast tRNA) containing 200 ng/ml DIG-labeled probe. Hybridization was performed under parafilm coverslips in a humidified chamber at 55°C for 24 h. The pH of the hybridization solution was adjusted to either neutral (pH 7.4) or slightly alkaline (pH 8.0). The sections were rinsed in 2x SSC at RT and at 55°C for 5 and 10 min, respectively, and then treated with RNase A (16 μg/ml) at 37°C for 30 min. The sections were washed in 2x SSC/50% formamide at 55°C for 2 x 10 min, and in 2x SSC at 55°C and at RT for 10 min and 5 min, respectively. After posthybridization, the sections were washed in buffer B1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 5 min, blocked in 5% heat-inactivated sheep serum in buffer B1 for 2 h and incubated in sheep anti-DIG-alkaline phosphatase conjugate (Boehringer Mannheim GmbH, Mannheim, Germany; 1:1000 dilution) in 5% sheep serum in buffer B1 at 4°C for 24 h. Sections were washed in buffer B1 for 3 x 5 min, and then in buffer B2 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 10 min, and were developed in buffer B2 containing 340 μg/ml nitro blue tetrazolium and 180 μg/ml 5-bromo-4-chloro-3-indolyl phosphate for 24 h under darkroom conditions. The color reaction was terminated by rinsing the sections in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA for 5 min at RT, and the

sections were then dehydrated and covered with glycerin/gelatin. Hybridized lumbar spinal cord sections were examined under a Leica DM LB microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany). Microscopic images (1600 x 1200 pixels, 8-bit gray scale) were captured with a Polaroid DMC 1 digital microscope camera (Polaroid, Cambridge, MA, USA) connected to a Power Macintosh computer (Apple Computer, Inc., Cupertino, CA, USA). Some of the sections were counterstained with toluidine blue or safranin.

Results

Microwave heating followed by triton X-100 treatment detected a number of CaM I mRNA-expressing cells in the gray matter of the lumbar spinal cord of the adult rat (Fig. 1A, C), irrespective of the chloroform treatment. Although most of the labeled cells were in the ventral horn, cells also expressed CaM I mRNA with lower frequency in the intermediate and dorsal horn. The concentration of triton X-100 used affected the number and intensity of the hybridization signal in the gray matter, as more intense labeling was apparent when the lower triton X-100 concentration was used (Fig. 1C). No specific hybridization signal was detected when the sense probe was applied at either triton X-100 concentration (Fig. 1B, D). However, no labeled cells were found in the white matter area of the spinal cord when the in situ hybridization protocols involving microwave heating and triton X-100 treatment were used to detect CaM I-expressing cells (Figs. 1A, C, and 2A, C).

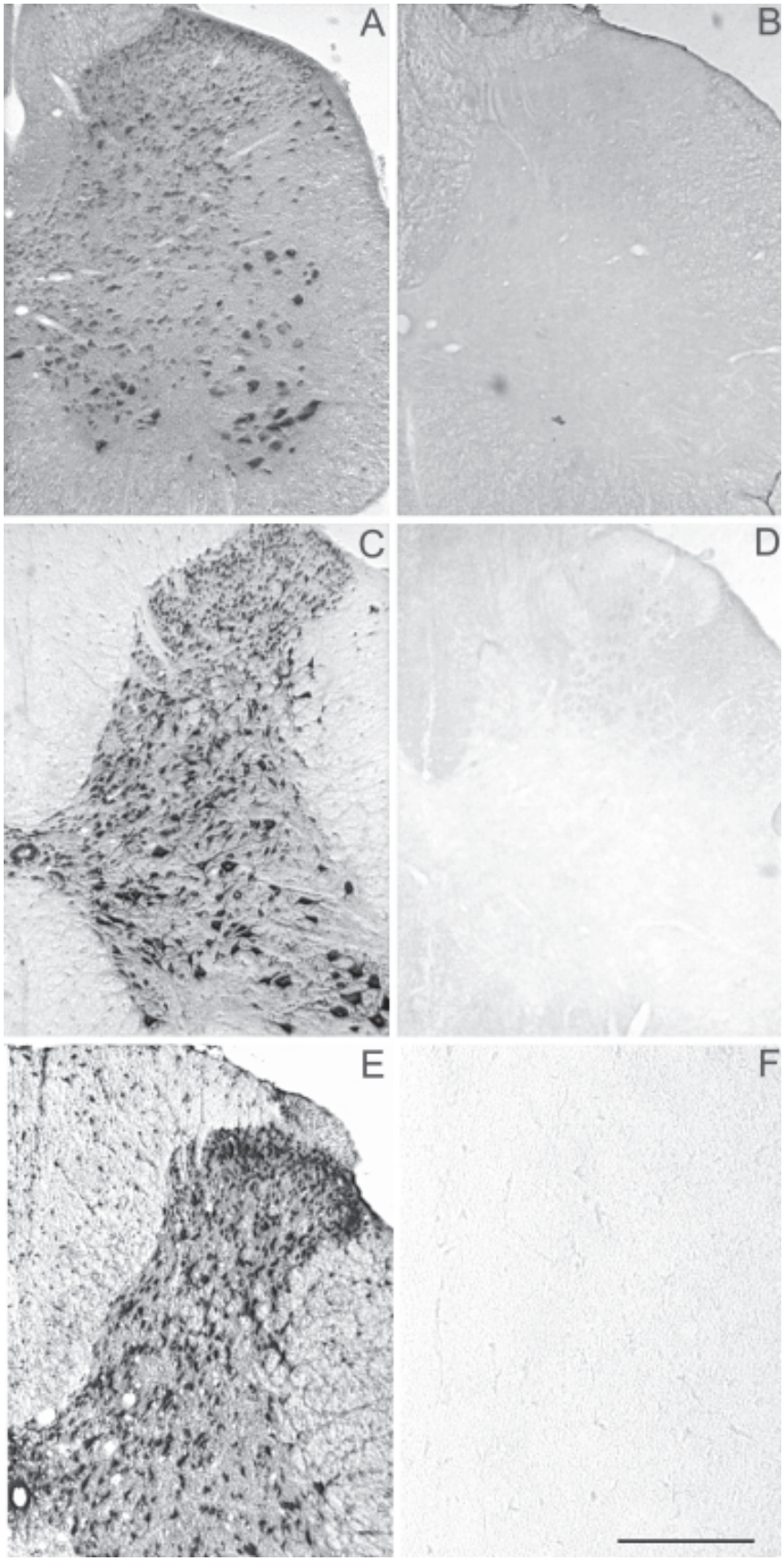
In a slightly alkaline hybridization solution, however, not only did the gray matter area of the spinal cord display numerous heavily labeled cells (Fig. 1E), but a large number of CaM I-expressing cells were also detected in the white matter area (Figs. 1E, and 2E). These cells were either medium-sized astrocyte-like cells, sometimes with short processes, or smaller cells resembling oligodendrocytes. The sense probe did not label any recognizable cellular elements, and resulted in a very faint staining (Figs. 1F, and 2F).

Discussion

White matter areas of the brain such as the anterior commissure, the corpus callosum, the internal capsule or the pyramidal tract contain very low amounts of CaM transcripts (Palfi et al. 1999), so the white matter of the spinal cord could likewise be very low in these mRNA populations. However, no data were available on the CaM-expressing cells in the white matter of the spinal cord by in situ hybridization, possibly because of its generally very low expression level and the relative insensitivity of most current in situ hybridization protocols for low-abundance mRNA populations. The high lipid content in myelin-rich regions of the nervous system could be a hindrance to the detection of mRNAs, and especially those populations that are in low abundance. Therefore, we speculated that the use of a more sensitive in situ hybridization method might successfully detect low-abundance CaM transcripts.

In recent years, several in situ hybridization techniques have been employed to increase the amount of detectable mRNAs or to sensitize the method for the detection of mRNA populations present in low abundance in the tissue. The amount of hybridizable mRNAs, for example, can be increased by either lipid removal or enzyme digestion with proteases, such as proteinase K (Relf et al. 2002), but enzymatic unmasking of the mRNA often occurs at the expense of tissue and cellular morphology (Lan et al. 1996). This has led to the extensive use of tissue heating methods such as microwave irradiation (Mitchell et al. 2001) or autoclaving (Eastwood and Harrison 1999; Oliver et al. 1997). Autoclaving proved to be superior to microwave heating because the latter method often results in local superheating of the tissue or evaporation of the buffer solution during irradiation in both immunocytochemical and in situ hybridization protocols (Norton et al. 1994; Oliver et al. 1999). While autoclaving can indeed increase the amount of hybridizable mRNA, especially in paraffin-embedded sections, other means of tissue preparation, such as the use of frozen sections, did not benefit from this procedure. A quantitative study confirmed that autoclaving is a suitable procedure that allows formalin-fixed, paraffin-embedded nervous tissue to be used for in situ hybridization, as the signal intensity was stronger (by 35–50%, in terms of nCi/g tissue equivalents) in autoclaved sections than in frozen sections (Eastwood and Harrison 1999).

Our in situ hybridization protocol at a slightly alkaline pH overcomes this problem and it is superior to other techniques in which tissue heating is combined with triton X-100 treatment. Basyuk et al. (2000) introduced alkaline fixation to in situ hybridization techniques in order to improve the sensitivity. They reported that alkaline formaldehyde (pH 9.5) dramatically increased (5-6-fold) the in situ hybridization signal with riboprobes for the detection of both low- and high-abundance mRNAs. Since alkaline fixation does not improve the retention of mRNA during in situ hybridization (Basyuk et al. 2000), but rather increases the accessibility of the target for the riboprobe, hybridization at alkaline pH is crucial for the increased sensitivity. Our protocol was successfully used for DIG-labeled CaM gene-specific riboprobes on both cryostat sections of the spinal cord (Kovacs and Gulya 2001, 2002) and paraffin sections of the retina (Kovacs and Gulya 2003), i.e. tissues with either very high or very low lipid content, but both with very low CaM mRNA abundance.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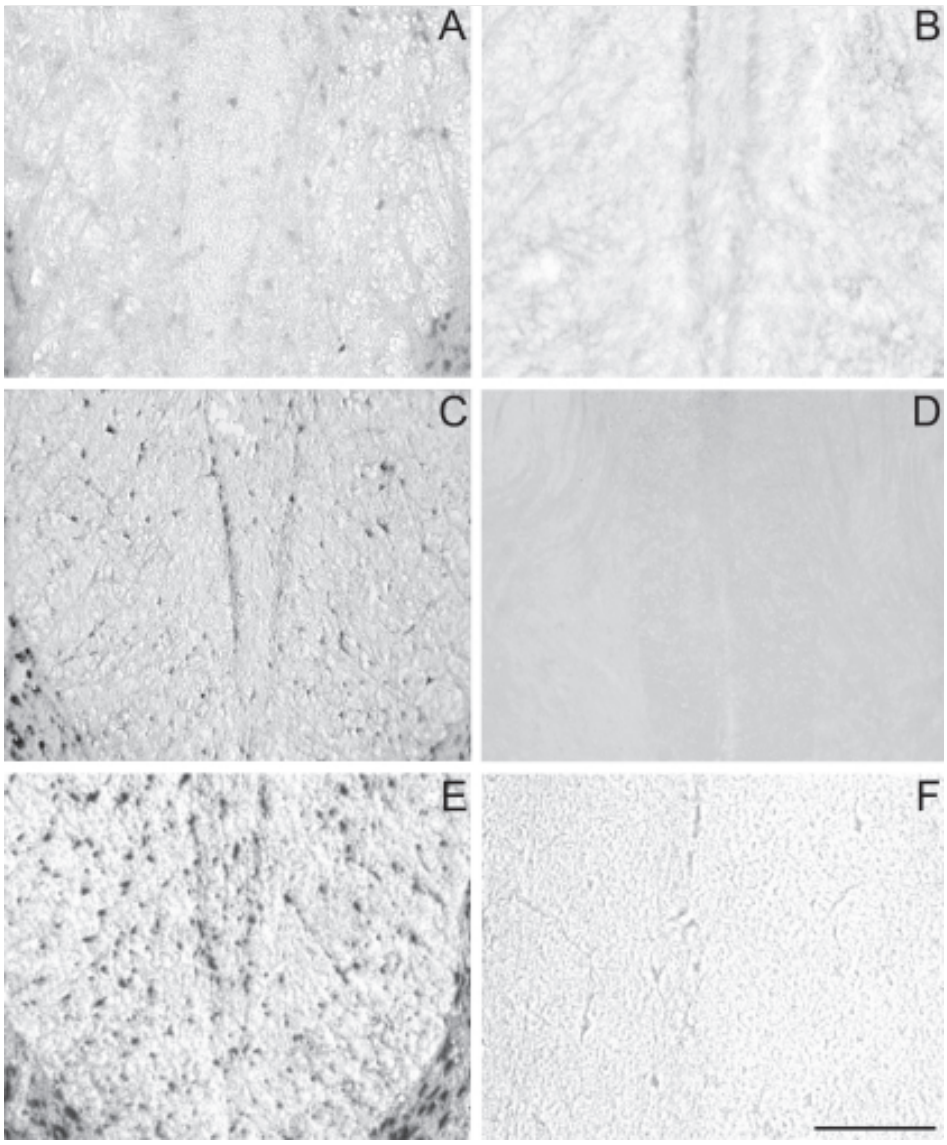

Figure 2. Hybridization of DIG-labeled antisense (A, C, E) and sense (B, D, F) cRNA probes specific for CaM I mRNAs in cryostat sections of the dorsal white matter of the adult rat lumbar spinal cord. The sections underwent the following treatments: A, B: microwave heating (5 + 4 + 4 min) before the immersion fixation, and 1.0% triton X-100/2x SSC treatment for 3 min at the end of prehybridization; C, D: microwave heating (5 + 4 + 4 min) before chloroform treatment and immersion fixation, and 0.1% triton X-100/2x SSC treatment for 3 min at the end of prehybridization; E, F: hybridization in a slightly alkaline solution (pH 8.0). Hybridization with the sense probes resulted in a very low signal in all cases (B, D, F). Microwave heating and triton X-100 treatment resulted in no labeled cells in the dorsal column (A). The highest number of labeled cells and the strongest signal in the dorsal column of the white matter could be detected after hybridization in a slightly alkaline solution (E). The CaM I-expressing cells, sometimes with their processes, could be easily visualized. Portions of the gray matter can be seen in the lower corners of the pictures in A, C, E. Scale: 200 μm.

Figure 1. Hybridization of DIG-labeled antisense (A, C, E) and sense (B, D, F) cRNA probes specific for CaM I mRNAs in cryostat sections of the adult rat lumbar spinal cord. The sections underwent the following treatments: A, B: microwave heating (5 + 4 + 4 min) before immersion fixation, and 1.0% triton X-100/2x SSC treatment for 3 min at the end of prehybridization; C, D: microwave heating (5 + 4 + 4 min) before chloroform treatment and immersion fixation, and 0.1% triton X-100/2x SSC treatment for 3 min at the end of prehybridization; E, F: hybridization in a slightly alkaline solution (pH 8.0). Microwave heating and treatment with 1.0% triton X-100 resulted in relatively high nonspecific labeling in both the gray and the white matter areas. CaM-expressing cells could be detected only in the gray matter. The neuropil displayed a relatively strong, diffuse signal. Microwave heating and treatment with 0.1% triton X-100 resulted in a better tissue quality with a lower nonspecific background. Occasionally, the cells in the gray matter exhibited their processes (C). No labeled cells could be seen in the white matter of the spinal cord (D). When the hybridization took place in a solution adjusted to a slightly alkaline pH, a large number of cells demonstrated CaM I expression not only in the gray matter, but also in the white matter of the spinal cord (E). The cells in the ventral horn were heavily labeled, while the cells in the intermediate and dorsal horn were less intensely labeled. Hybridization with the sense probe resulted in a very low signal (F). Scale: 500 μm.

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