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Fine-structural localization of proenkephalin mRNAs in the hypothalamic magnocellular dorsal nucleus of the guinea pig: a comparison of radioisotopic and enzymatic in situ hybridization methods at the light- and electron-microscopic levels

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Abstract. With the aim of localizing proenkephalin mRNAs in neurons of the hypothalamic magnocellular dorsal nucleus of the guinea pig, we compared the in situ hybridization signals obtained on Vibratome sections with a method employing either a biotinylated or a digoxigenin-labeled oligonucleotide detected by means of the alkaline phosphatase reaction. Since the hybridization approach using the biotinylated probe was more sensitive than the digoxigenin method, the ultrastructural localization of hybrids in neurons of the magnocellular dorsal nucleus was studied by the use of the former procedure, and was further compared with results of in situ hybridization using a ^{35}S-labeled probe. Biotin was detected via an amplified avidin-biotin-peroxidase complex. Radioactive hybrids were localized over extended cytoplasmic compartments rich in rough endoplasmic reticulum and also in nuclear indentations. The method based on biotinylated probe proved to be sensitive and provided high-resolution labeling in well-preserved specimens. Proenkephalin mRNAs were clearly localized within circumscribed cytoplasmic compartments. The immunoprecipitates were mainly observed within the rough endoplasmic reticulum, especially at the periphery of the cell. The reticulum was dominated by elongated parallel cisternae. The labeling also appeared in a paranuclear position, mainly in nuclear indentations. The labeling was found on the outer surface of the endoplasmic lamellae. The remainder of the reticulum was unlabeled. Neuronal processes were free of labeling.

Key words: Hypothalamus – Magnocellular dorsal nucleus – In situ hybridization – Proenkephalin mRNA – Ultrahistochemistry – Guinea pig

Introduction

Recent hybridization studies have opened the way for localizing neuropeptide mRNAs in the central nervous system at the ultrastructural level (Guitteny and Bloch 1989; Jirikowski et al. 1990; Trembleau et al. 1990, 1991; Le Guellec et al. 1992). This subject is of great interest since it has been postulated that the mRNAs are not necessarily homogeneously distributed in the cell body; it has also been shown that mRNAs occur in cell processes (Bloch et al. 1990; Jirikowski et al. 1990). Moreover, it seems that differences are observed according to cell type.

Neurons of the hypothalamic magnocellular dorsal nucleus (MDN) of the guinea pig are immunoreactive for met-enkephalin (Tramu et al. 1981) and contain proenkephalin mRNAs (Mitchell et al. 1992). The ultrastructural correlates of enkephalin immunoreactivity have been shown to be located mainly in neurosecretory granules (Beauvillain et al. 1982). The precise distribution of proenkephalin mRNAs in these neurons has not previously been investigated, and ultrastructural in situ-hybridization studies should provide evidence of the localization of these mRNAs.

In the literature, ultrastructural in situ-hybridization has been performed with radioactively labeled probes (Trembleau et al. 1988; Tong et al. 1989; Le Guellec et al. 1991) or with nonradioactive probes (Webster et al. 1987; Wolber et al. 1988; Jirikowski et al. 1990; Liposits et al. 1991; Le Guellec et al. 1992). These methods do not have the same resolution and sensitivity but they are valuable tools, each having advantages for investigating the distribution of neuropeptide mRNAs.

In an attempt to obtain information about the cellular distribution of proenkephalin mRNAs at the ultrastructural level in the MDN cells of the guinea pig, it was decided to use parallel radioactive and nonradioactive in situ-hybridization approaches. The radioisotopic procedure was based on a preembedding method using a ^{35}S-labeled probe. For the nonradioactive study, before starting the ultrastructural work, it had to be determined whether a biotin-labeled or digoxigenin-labeled oligonucleotide gave the best results in this model. This was evaluated at the light-microscopic level on tissues fixed in solutions with or without glutaraldehyde. The visual-

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ization of hybrids was obtained by means of the alkaline phosphatase reaction; these reagents are, to date, the most sensitive enzymatic substrates.

In view of the initial results, a peroxidase marker was later employed; this is known to be generally less sensitive but is needed for an observation at the ultrastructural level when a preembedding method is used.

Materials and methods

Tissue preparation

Adult male guinea pigs weighing 400 g (Cob Labo Lap, Yffiniac, France) were used. They were anesthetized with ketamine (20 mg/kg) and xylazine (0.2 ml/kg), and perfused intracardially with saline followed by 700 ml of a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, or 4% paraformaldehyde with 0.1% glutaraldehyde in the same buffer. Brains were dissected out and post-fixed for 1 h in the fixative used for the perfusion. They were cut on a Vibratome (Technical products International, St Louis, USA) in 60 µm-thick frontal sections. Sections containing the MDN at its maximum extent were collected in 0.1 M phosphate-buffered saline (PBS).

Probe

The probe was a 45-base oligodeoxynucleotide obtained from Eurogentec S.A. (Belgium) corresponding to amino acids 85–99 of bovine proenkephalin. It was labeled by addition of a tail of ^{35}S-dATP (1350 Ci/mmol; New England Nuclear, Boston, Mass.) as previously described (Mitchell et al. 1992), or by addition of biotin-11-dUTP (Boehringer-Mannheim, France) or digoxigenin-11-dUTP (Boehringer) as follows. Aliquots of 10 pmol or 100 pmol oligonucleotide were incubated, respectively, with 0.5 nmol biotin-11-dUTP, or 1 nmol digoxigenin-11-dUTP and 9 nmol dATP, and with 25, or 55 U terminal deoxynucleotidyl transferase (Amersham, France) for 1.5 h at 37° C. The reaction was stopped by the addition of 1 µl 200 mM EDTA, pH 8, and 1 µl yeast tRNA (5 µg/µl). The probe was precipitated for 30 min in 4 µl 4 M LiCl and 100 µl ethanol at −80° C. It was centrifuged for 45 min at 12000 rpm, dried, and re-suspended in hybridization buffer.

In situ hybridization

The Vibratome sections were prehybridized for 1 h in 50% deionized formamide, 4 × standard sodium citrate (1 × SSC = 150 mM NaCl/15 mM trisodium citrate, pH 7.0), 1 × Denhardt solution (0.02% bovine serum albumin/0.02% polyvinylpyrrolidone/0.02% Ficoll) at room temperature for hybridization with the ^{35}S-labeled probe (for sections obtained from animals fixed with 4% paraformaldehyde and 0.1% glutaraldehyde) and at 37° C for non-radioactive probes (for tissues fixed with 4% paraformaldehyde or 4% paraformaldehyde and 0.1% glutaraldehyde), under gentle agitation.

Sections were hybridized for 16 h at 30° C with the radioactive probe in the hybridization buffer (50% deionized formamide; 8 × SSC; 20 × sarcosyl; 1.2 M phosphate buffer; 100 × Denhardt solution) or at 37° C in 10 mM biotinylated probe or digoxigenin-labeled probe in the hybridization buffer (50% deionized formamide, 80 mM TRIS-HCl, pH 7.5, 0.6 M NaCl, 4 mM EDTA, 0.1% sodium pyrophosphate). Radioactive hybridized sections were rinsed in 4 × SSC for 1 h at room temperature and 1 h at 40° C, then in 0.1 × SSC for 1 h at 40° C. Nonradioactive sections were immersed three times in 2 × SSC for 30 min and 0.1 × SSC for 30 min at 37° C.

Detection of the radioactive hybridization signal

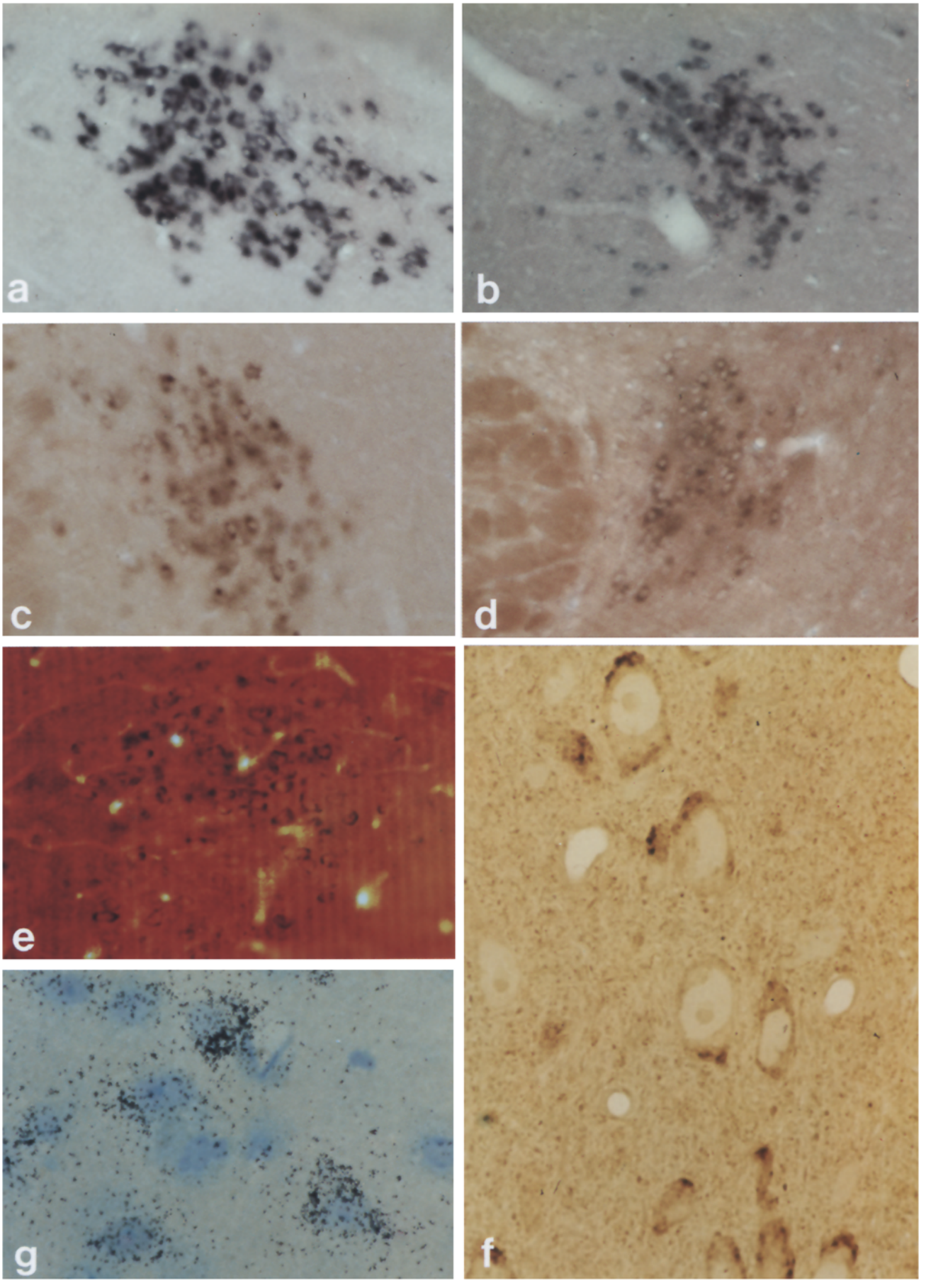
After hybridization, radioactive sections were osmicated with 1% osmium tetroxide in 1 × SSC buffer for 30 min. Sections were dehydrated in a graded series of methanols and propylene oxide, and finally embedded in Araldite. Semithin sections (1.5 µm thick) were dipped in Amersham LM emulsion. Ultrathin sections were collected on glass slides bearing a celloidin film, stained with uranyl acetate and lead citrate, slightly vaporized with carbon and dipped in an Amersham EM emulsion. After exposure (20–40 days for semithin sections and 7–9 weeks for ultrathin sections), sections were developed in Kodak D-19 and Microdol X (Kodak Paris), respectively.

Detection of the nonradioactive hybridization signal

In parallel studies, mRNA-biotinylated and mRNA-digoxigenin-labeled hybrids were detected with the use of alkaline phosphatase reagents according to the following protocols. A group of Vibratome sections hybridized with the biotinylated probe was incubated with streptavidin [1:100 in TRIS buffer saline (TBS)] for 30 min, followed by biotinylated alkaline phosphatase (1:100 in TBS) included in the Dako kit (Dakopatts, Germany) for 30 min. A second group of sections, hybridized with digoxigenin-labeled probe, were treated with a sheep anti-digoxigenin IgG tagged with alkaline phosphatase (diluted 1:5000 in 0.1 M TRIS buffer, pH 7.5, containing 0.15 M NaCl and 50 mM $MgCl_2$). After 3-fold rinses in the same buffer for 10 min, sections were rinsed for 10 min in 0.1 M TRIS buffer, pH 7.5, with 0.15 M NaCl and 100 mM $MgCl_2$, and in 0.1 M TRIS buffer, pH 9.5, with 0.015 M NaCl and 100 mM $MgCl_2$. The sections were stained in nitroblue tetrazolium (NBT: 0.34 mg/ml) with 5-bromo-4 indolyl phosphate (BCIP: 0.18 mg/ml) and the reaction product was monitored with a microscope. The reaction was stopped in PBS and sections were mounted in glycergel medium (Dako).

In a third experiment, Vibratome sections hybridized with the biotinylated probe were reacted with the amplified avidin-biotin-peroxidase complex (ABC, Vector Labs, Burlingame, California) according to a slightly modified method of Arai et al. (1988). Slices were incubated in ABC for 30 min in PBS. After two rinses in PBS, for 10 min, sections were incubated in PBS containing the biotinylated anti-avidin serum (1:100 in PBS for 12 h at 4° C, Vector labs). After rinsing in PBS 2 × 10 min, sections were again incubated in ABC for a further 30 min. For the detection of enzyme activity, sections were kept for 24 h at 4° C in a diaminobenzidine (DAB: 5 mg/10 ml TRIS) and 30% hydrogen peroxide solution

Fig. 1. Nonradioactive (**a–f**) and radioactive (**g**) in situ-hybridization labeling for proenkephalin mRNAs in the magnocellular dorsal nucleus of the guinea pig; Vibratome (**a–e**) and semithin (**f, g**) sections. **a, b** Biotinylated probe detected by the alkaline phosphatase procedure in tissue fixed with paraformaldehyde alone (**a**) or with paraformaldehyde + glutaraldehyde (**b**). **c, d** Digoxigenin end-labeled probe detected by alkaline phosphatase/anti-digoxigenin in tissue fixed with paraformaldehyde (**c**) or paraformaldehyde + glutaraldehyde (**d**). Note the lower intensity of the reaction in digoxigenin-treated tissue (**c, d**) in comparison with that in biotinylated tissue (**a, b**). The signal decreases when tissues are fixed with glutaraldehyde (**b, d**). **a–d** ×100. **e, f** In situ-hybridization using a biotinylated probe on (**e**) Vibratome and (**f**) semithin sections treated with the amplified avidin-biotin-peroxidase complex. **e** Note a circumscribed population of positive neurons in the MDN. **f** Immunoprecipitates are mostly found at the periphery of the cell. Note the resolution of the labeling. **e** ×150; **f** ×1000. **g** Numerous silver grains are scattered over the cytoplasm of MDN neurons and sometimes over the nucleus. Note the presence of aggregates in the cytoplasm and the fact that silver grains extend beyond the perikarya. ×700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(10 µl/10 ml TRIS). These sections were postfixed in 1% osmium tetroxide for 10 min and flat embedded in Araldite. Semithin (1.5 µm thick) and ultrathin sections were cut, the latter being counterstained with uranyl acetate and lead citrate.

Controls

The specificity of the probe sequence for the mRNA of MDN neurons of the guinea pig and the specificity of the radioactive hybridization reaction have previously been demonstrated (Mitchell et al. 1992). The specificity of the staining and the control of the background following treatment with biotinylated and digoxigenin-labeled probes were studied (1) by incubating some sections with a 20-fold excess of the proenkephalin probe simultaneously with the labeled probe, and (2) in tissues in which all steps of the detection process were performed without the probe. In addition, some control sections were treated with the "sense" oligonucleotide. Under these respective conditions, no signal was detected.

Results

Radioautographic demonstration of the proenkephalin hybridization signal

On frontal semithin sections cut from Vibratome slices, we observed a well-circumscribed population of ^{35}S-labeled neurons in the region of the MDN. Although tissues were fixed with a solution containing glutaraldehyde, the labeling was intense. It appeared as a preferential accumulation of silver grains over some cytoplasmic compartments in the perikarya of the magnocellular neurons (Fig. 1g). Occasionally, silver grains were found over the nucleus and extended beyond the cell soma.

In ultrathin sections, most of the silver grains were scattered over the neuronal cytoplasm. Grains were rarely observed over the nucleus, but they were numerous in the vicinity of the nuclear membrane, especially in the nuclear indentations (Fig. 2a). In the cytoplasm, the signal appeared predominantly in regions that were rich in rough endoplasmic reticulum (RER), particularly in the area where it formed parallel lamellae (Fig. 2b). In most cases, this type of RER was located at the periphery of the enkephalinergic cells.

Detection of the biotinylated or digoxigenin-labeled probe using alkaline phosphatase

Proenkephalin probes (biotinylated or digoxigenin-labeled) reacting with the respective alkaline phosphatase conjugates and the NBT-BCIP, displayed blue/purple (biotin) or red/brown (digoxigenin) deposits and were restricted to a circumscribed population of neurons in the region corresponding to the MDN (Fig. 1a–d). There was no background staining. Such deposits were not found in control sections hybridized without probe or with the "sense" probe. Only the cytoplasm was labeled; the nucleus did not carry any label.

The intensity of the hybridization signal was a function of the tag that was used (biotin or digoxigenin) for labeling of the probe. In tissues subjected to a similar fixation procedure, the intensity of the signal provided by the biotinylated probe (Fig. 1a, b) was higher than that exhibited by the digoxigenin-labeled probe (Fig. 1c, d). Thus, on Vibratome sections through corresponding rostrocaudal areas of the MDN, the positive cells that were hybridized using the biotinylated probe (Fig. 1a, b) were more numerous than those appearing after hybridization with the digoxigenin probe (Fig. 1c, d).

The intensity of the signal was also a function of the fixation procedure. The addition of 0.1% glutaraldehyde had a critical influence on the intensity of the signal obtained with the biotinylated (Fig. 1b) and the digoxigenin-labeled probe (Fig. 1d). The intensity of the labeling of MDN neurons obtained from brains fixed with glutaraldehyde and formaldehyde was weaker than that in tissue specimens fixed with formaldehyde alone. However, in most neurons hybridized with the biotinylated probe, the intensity of the signal was still strong and clearly visible (Fig. 1b). In contrast, when the digoxigenin-labeled probe was used after glutaraldehyde fixation, the staining was faint (Fig. 1d).

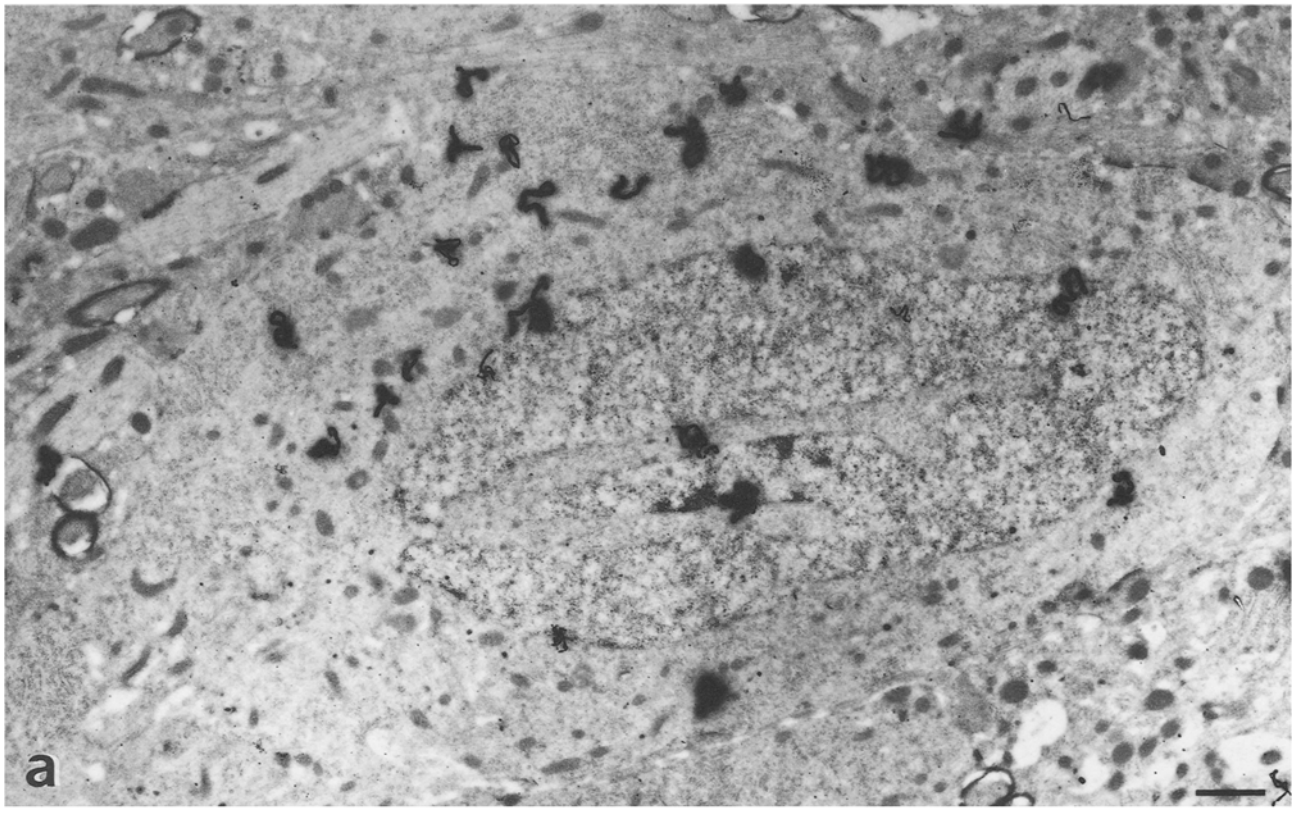
Thus, the preembedding procedure using peroxidase for ultrastructural detection was undertaken only with the biotinylated probe in paraformaldehyde- and glutaraldehyde-fixed tissues.

Ultrastructural detection of the biotinylated probe using the amplified avidin-biotin-peroxidase complex

On Vibratome sections, a well-outlined population of labeled neurons appeared in the MDN (Fig. 1e). The slices had darkened because of the osmification and the long incubation time in DAB; when the revelation procedure was carried out without the probe, the background displayed a similar reactivity. The positive neurons were examined in semithin sections obtained from the entire thickness of the Vibratome section, a result superior to conventional immunocytochemical detections. Positive cells displayed brown deposits in their cytoplasm (Fig. 1f). The reaction product was not uniformly distributed but was restricted to certain cytoplasmic areas. The background staining was weak.

At the ultrastructural level, the preservation of the tissue was assessed by examination of the surrounding tissue and was considered to be adequate. The immunoprecipitates were observed over the RER, most often at the periphery of the cell soma (Figs. 3, 4). The labeled RER showed arrays of flattened and parallel lamellae scattered over the cytoplasm. However, this type of parallel RER was only partly labeled (Fig. 4a). The labeling

Fig. 2a, b. Ultrastructural radioautographic detection of proenkephalin mRNAs by in situ-hybridization in the hypothalamic MDN of the guinea pig. Tissue fixed with 4% paraformaldehyde + 0.1% glutaraldehyde. Exposure time: 7 weeks. **a** Silver grains can be observed in some cytoplasmic areas of the cytoplasm of the neuron, close to the nuclear envelope, and within cytoplasmic indentations. **b** Most grains are observed over regions rich in parallel cisternae of the rough endoplasmic reticulum. *Bars*: 1 µm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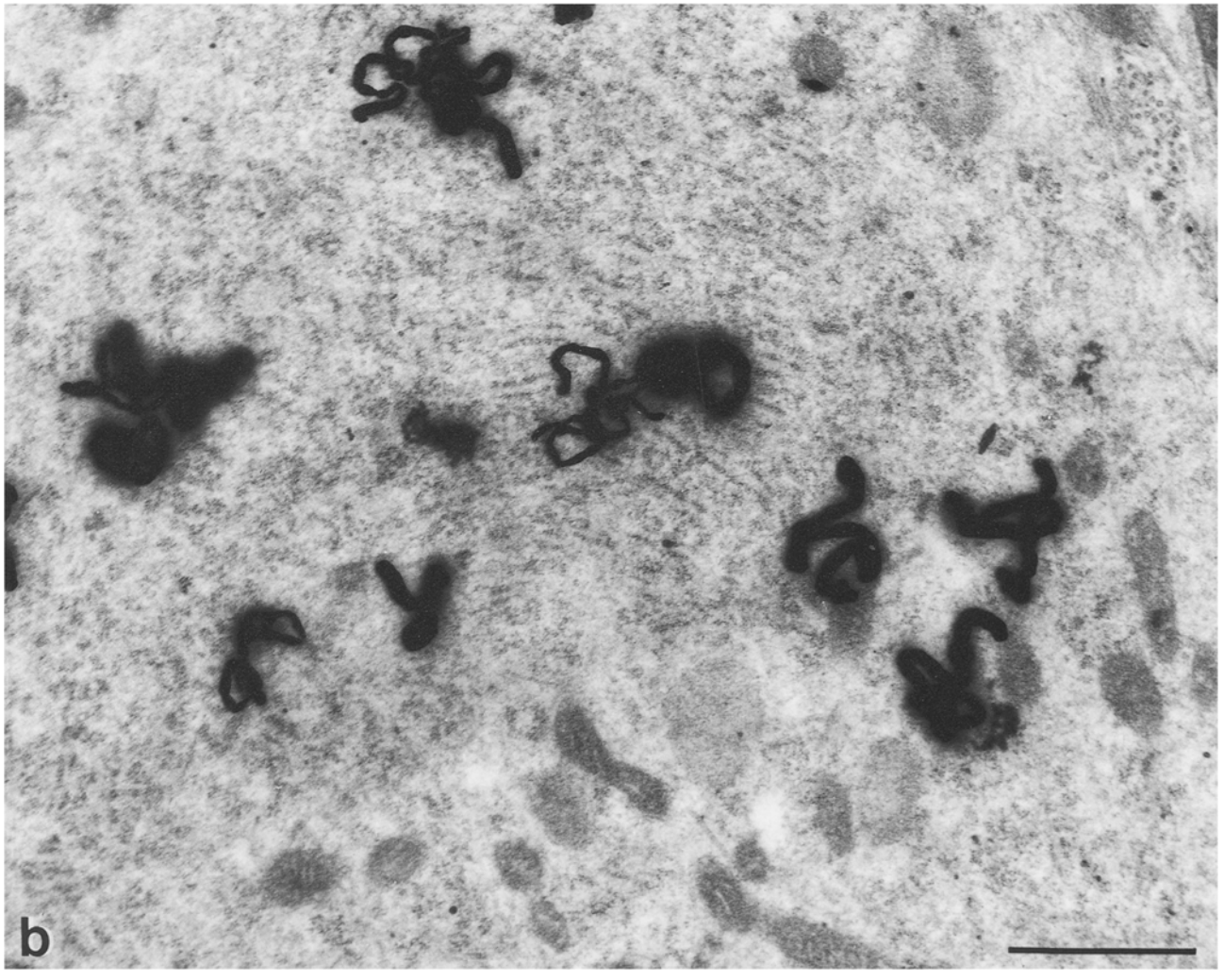
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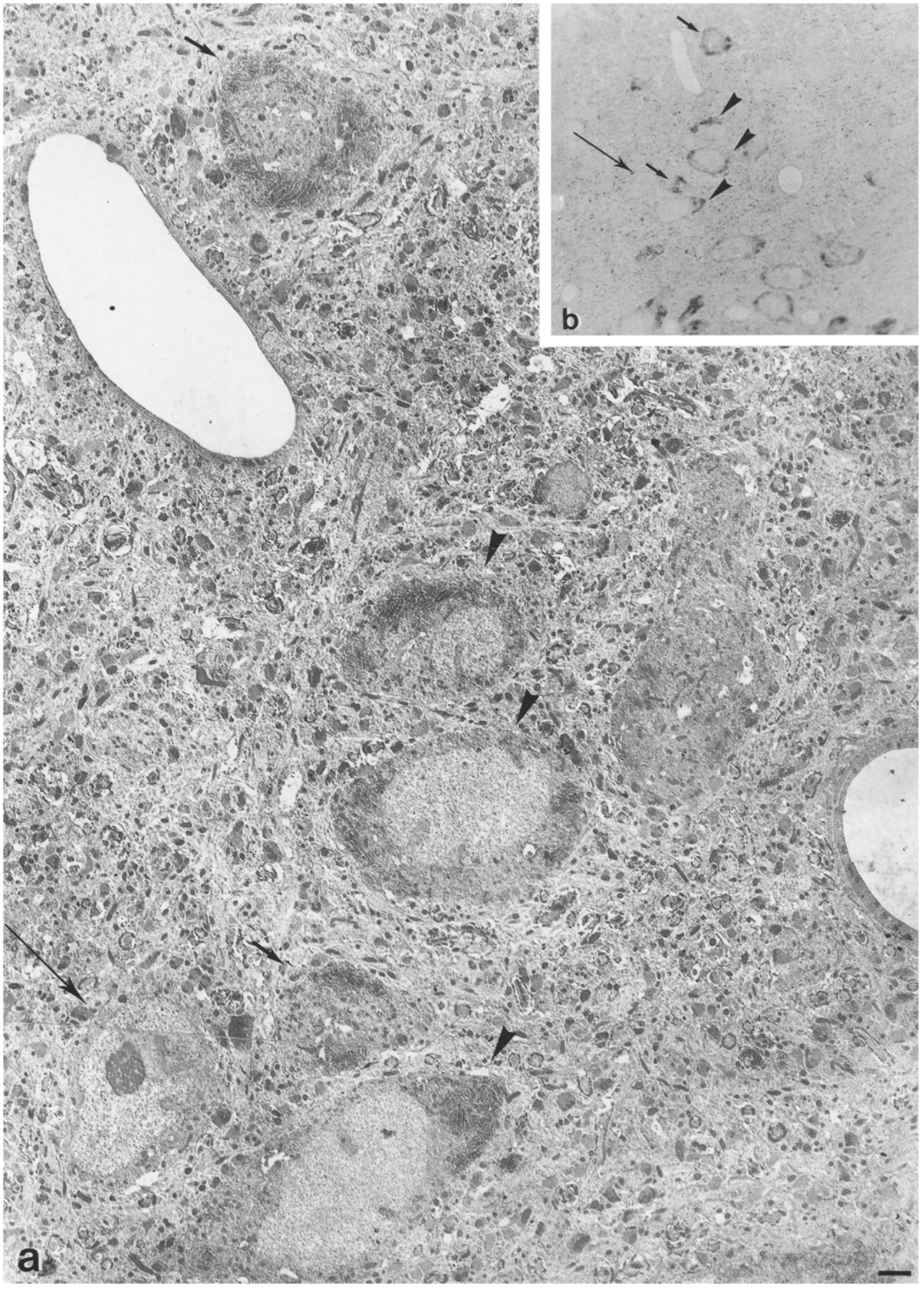

Fig. 3a, b. Adjacent semithin (**b**) and ultrathin (**a**) sections showing neurons of the MDN hybridized by means of biotinylated oligonucleotide and detected with the use of amplified avidin-biotin complex. Tissue fixed with 4% paraformaldehyde + 0.1% glutaraldehyde. The same perikarya (*arrowheads*) and their circumscribed sections (*arrows*) are visible in the semithin and the adjacent ultrathin sections. Note DAB products in the peripheral portions of MDN perikarya. The labeling is mostly linked to the rough endoplasmic reticulum. Note a neuron free of reaction product in the semithin and the adjacent ultrathin section (*long arrows*). *Bar*: 2 μm; **b** × 550

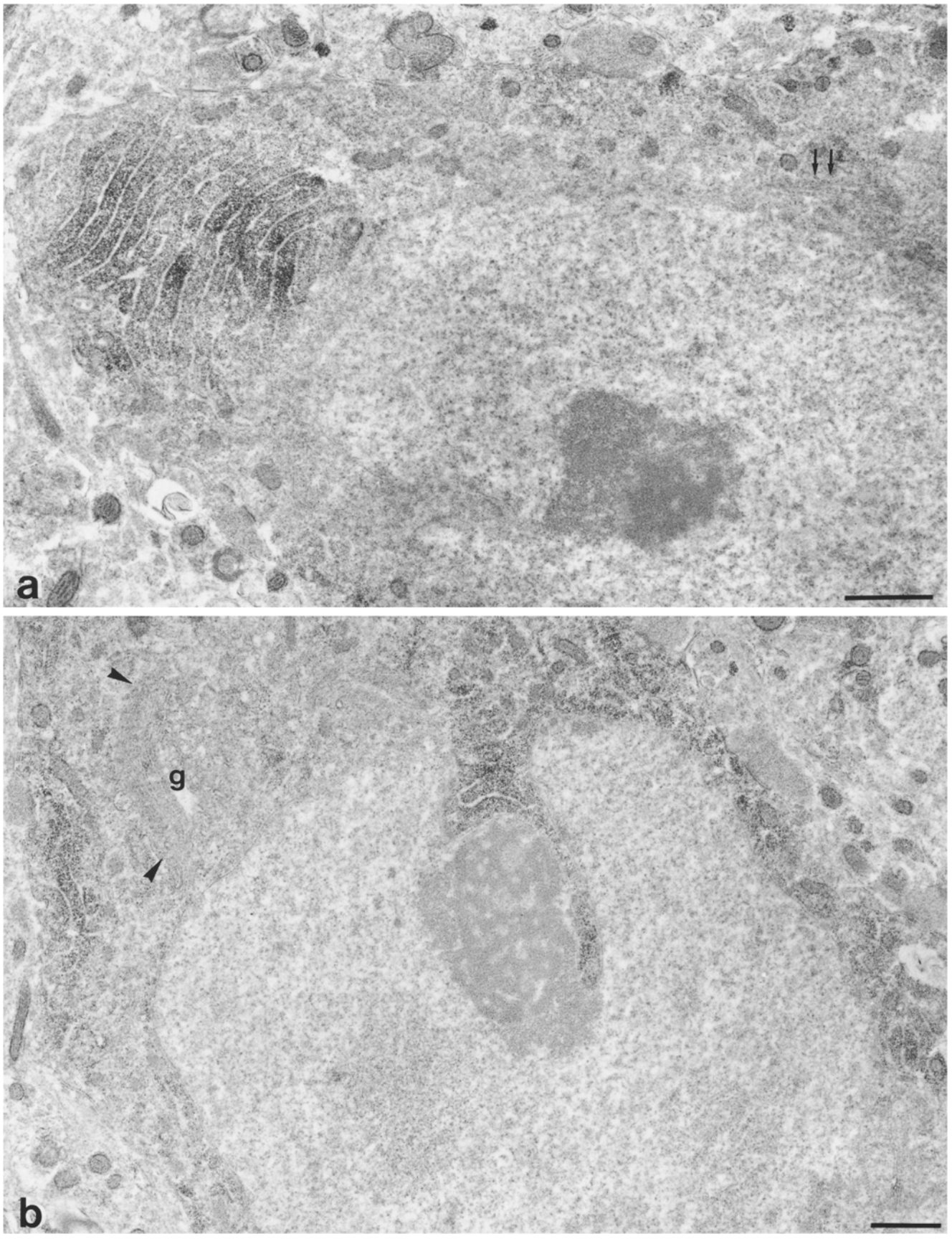

Fig. 4a, b. Ultrastructural localization of proenkephalin mRNAs using a biotinylated probe detected by amplification of the avidin-biotin complex. Tissue fixed as in Fig. 3. **a** DAB precipitates occur between the parallel cisternae of the rough endoplasmic reticulum (RER). The intensity of labeling is not homogeneous. Some regions of the RER do not display any labeling (*arrows*). **b** Precipitates are also seen in the RER located close to the nuclear membrane and within its indentations. Other organelles are negative, especially the Golgi apparatus (*g*). *Bars*: 1 μm

was also found in the RER adjacent to the nuclear envelope, especially within the cytoplasmic protrusions where the labeling was as intense as in the peripheral RER structures (Fig. 4b). Furthermore, immunoprecipitates were observed between the lamellae, i.e. outside the sacks (Fig. 4a, b); the intensity of this labeling was, however, heterogeneous (Fig. 4a). In addition, flattened sacks of RER, devoid of preferential arrangement, were not labeled (Fig. 4a). In close vicinity to the labeled cell bodies, some profiles of such neurons exhibited small electron-dense inclusions probably corresponding to RER (Fig. 4a–b). No labeling was observed in the Golgi area (Fig. 4b), on the secretory granules, and in the nucleus, but a higher electron density was occasionally seen on mitochondrial membranes. Neuronal processes were free of labeling.

Discussion

The present study addresses the problem of the localization of proenkephalin mRNAs at the electron-microscopic level and compares various methods of hybridization with emphasis on improved tissue preservation and good resolution of the signal. The radioactive hybridization method showed the signal preferentially in regions rich in RER and in the vicinity of the nuclear membrane. In parallel experiments, the biotinylated oligonucleotide method, rather than the digoxigenin procedure, was applied to investigate the ultrastructural localization of proenkephalin mRNAs. The use of the biotinylated oligonucleotide detected by the amplified ABC system led to an improvement in the resolution; the labeling was more precisely associated with certain lamellae of the RER. Here, we discuss these methods in terms of the specificity, tissue preservation, sensitivity, resolution, and subcellular localization of the proenkephalin gene.

The specificity of the bovine proenkephalin oligonucleotide and the radioautographic hybridization technique have been described in a previous report (Mitchell et al. 1992); the controls used in the present study are in accord with this protocol. For the nonradioactive methods, all the detection experiments have also been performed without the probe or using the "sense" oligonucleotide; these procedures never led to positive labeling or labeling of the background. The specificity of the hybridization reaction employing the biotinylated or the digoxigenin-labeled probe has thus been ascertained.

To date, the best fixative for in situ-hybridization is paraformaldehyde. In a previous light-microscopic study, after treatment of tissues preserved in this fixative with a radioactive probe, we have observed heavily labeled neurons in the MDN of the guinea pig (Mitchell et al. 1992). Even though it is well established that addition of glutaraldehyde decreases the level of radiolabel (Bloch et al. 1986; Le Guellec et al. 1991), we were able to obtain a good intensity of the hybridization signal when tissues were fixed with fixatives containing 0.1% glutaraldehyde. This result is noteworthy since the use of glutaraldehyde improves the preservation of the cell, facilitating the ultrastructural localization of mRNAs. However, the use of glutaraldehyde for nonradioactive hybridization methods is more problematic. To our knowledge, there is no example of a successful ultrastructural detection of neuropeptide mRNAs in tissues after fixation with glutaraldehyde and subsequent application of a nonradioactive preembedding method.

Although the fixative used for the radioactive and the nonradioactive ultrastructural procedures was identical (4% paraformaldehyde with 0.1% glutaraldehyde), the preservation of tissues treated with the biotinylated probe and the complementary reagents was inferior to that of material treated with the radiolabeled probe. The visualization of peroxidase activity by DAB may be responsible for the detrimental effect of the preservation. This phenomenon is well known in immunocytochemistry. However, despite this fact, glutaraldehyde leads to a noticeable improvement in preservation in comparison to paraformaldehyde.

The sensitivity of the reaction obtained with the biotinylated oligonucleotide is comparable to that with the radioactive probe. The concentration applied to tissue sections was similar for the radioactive and the biotinylated probes. Whereas the amount of the digoxigenin-labeled probe was 10 times higher than that currently used with the radioactive probe, the intensity of digoxigenin labeling is lower than that obtained with the radioactive and the biotinylated probes. The 3′-end labeling of the probe by digoxigenin-d-UTP may be less efficient than that by the ^{35}S-dATP and biotin-dUTP because of the large size of the steroid molecule. The results of Farquharson et al. (1990) disagree with our observations since the digoxigenin 3′-end labeling of the POMC oligonucleotide produced better results than the use of biotin. Indeed, few reports have compared these methods, and the quality of the reaction might depend also on the cell type. Taking into account our results, we have a strong preference for the biotinylated probe when investigating the ultrastructural localization of proenkephalin mRNAs. For this purpose, we advance the preembedding approach using peroxidase reagents because it allows the observation of the signal directly on Vibratome sections. In addition, the specificity of the subsequent ultrastructural labeling can be ascertained. Le Guellec et al. (1992) employed a postembedding approach with conjugated gold particles for the detection of the growth hormone mRNAs; this method has essentially been successful for the detection of virus genomes (Puvion-Dutilleul and Puvion 1989). Contrary to the assertations of a previous work (Trembleau et al. 1991), the use of detergent was unnecessary in our study. A critical point in our approach seems to be the color development the time of which is longer than usually assumed for immunocytochemical methods. Moreover, it is essential to use the amplified ABC system; the protocols utilizing the ABC system alone or the avidin peroxidase procedure will fail to label the MDN. This is in agreement with the observation of Le Guellec et al. (1992).

In the present study, the use of the radioactive probe has shown that proenkephalin mRNAs are localized in the cytoplasm of neurons in areas rich in RER and in

zones close to the nuclear envelope. There is a crucial limitation in the resolution capacity of this method since it depends on the presence of silver grains in the emulsion covering the cell body. Under these circumstances, we are unable to localize the precise source of the radiation, and there is no way to decide whether the silver grains located close to the nuclear envelope are intra- or extra-nuclear or marking the membrane itself. In our study, this restricted resolution does not seem to depend on the use of the sulphur isotope since in previous studies conducted with tritium, mRNAs were localized in wide cytoplasmic compartments (Trembleau et al. 1988, 1991). Indeed, the quality of the subcellular resolution obtained by us with the ^{35}S is surprising, taking into account that the scatter of silver grains in the emulsion around the source may exceed at least 10 times that of tritium or ^{125}I.

The application of peroxidase techniques greatly improves the resolution in comparison to the radioautographic method. In semithin sections, the DAB precipitates are localized in restricted portions of the cytoplasm. This location pattern has already been mentioned by Guitteny et al. (1989). In ultrathin sections, both the preservation and intensity of the labeling make the observation of mRNA distribution easier. Only certain lamellae of the RER display a positive reaction for proenkephalin mRNAs. Both in the peripheral cytoplasm and in the nuclear indentations, the DAB precipitates are regularly observed on the outer surface of the parallel saccules. This is in accord with the generally known location of cytoplasmic mRNAs. The labeled saccules are probably involved in the synthesis of met-enkephalin intended to be packaged into granules before being transported from the perikaryon to the nerve ending. The scattered saccules of RER are not labeled and do not contain proenkephalin mRNA hybrids. This type of RER probably takes part in the synthesis of proteins other than enkephalins. Thus, the variation in subcellular localization of mRNAs coding for various neuropeptides in the same neurons might be of great interest. More information is needed concerning the mRNAs coding for the neuropeptides in peptidergic neurons of the hypothalamus.

Inside the positive RER, the labeling is not distributed uniformly. Thus, the quantity of proteins synthesized may not be similar throughout the RER system. This observation appears to continue the recent results of Le Guellec et al. (1992) dealing with the ultrastructural detection of growth hormone mRNAs in the pituitary. Moreover, in the latter model, the labeling obtained using a postembedding protocol is essentially found in the form of aggregates of gold particles heterogeneously distributed in the RER of the growth-hormone-producing cells.

The labeling is mainly located in circumscribed areas of the RER; it was never observed on the Golgi apparatus, secretory granules or lysosomes. This pattern of proenkephalin mRNAs is in agreement with the generally known localization of the neuropeptide mRNAs. Concerning the secretory granules, only Jirikowski et al. (1990) have reported that oxytocin mRNAs are located in large granular secretory vesicles in the neurohypophysial axonal terminals, at least under some experimental manipulation. Our observation of the absence of signal of hybridization in the numerous lysosomes previously observed in MDN neurons (Mitchell et al. 1990) disagrees with recent results of Dirks et al. (1992). These authors observed, in peptidergic neurons of *Lymnaea stagnalis*, a lysosomal site of mRNAs for neurohormones; they hypothesized that these sequences might be taken up by lysosomes to be degraded. In neurons of the MDN, the membranes of some mitochondria appeared electron dense. It is likely that the reaction procedure may generate these images, since they are often observed when the preembedding immunocytochemical approach has been used. However, other alternatives cannot be ruled out.

We did not observe labeling in dendritic processes, which in principle are known to contain RER. This is not a general feature, since Bloch et al. (1990) have reported the presence of some neuropeptide (vasopressin, oxytocin, luteinizing hormone-releasing hormone) mRNAs in neuronal processes. In our study, labeled structures free of nuclei may rather correspond to circumscribed sections through perikarya or to somatic spines located in the vicinity of the labeled neuronal somata. Thus, proenkephalin mRNAs are probably not expressed beyond the level of neuronal perikarya, at least under normal conditions.

Our results and other reports indicate the absence of reactive material in the nucleus of neurons. Nuclear mRNAs are strongly linked to proteins and are rapidly transported toward the cytoplasm. However, we cannot assert that enkephalin mRNAs are completely absent from this compartment, since the hybridization signal may not be sensitive enough to detect nuclear mRNAs. The cytoplasmic mRNAs are ready to be translated to proteins and are probably more accessible to the oligonucleotide probe.

In conclusion, this study reports our experience regarding radioactive and nonradioactive hybridization at the ultrastructural level under improved conditions of tissue preservation. It also provides morphological information regarding the cellular localization of mRNAs coding for a specific neuropeptide.

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