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ORIGINAL PAPER

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Ultrastructural localisation of intramuscular expression of BDNF mRNA by silver-gold intensified non-radioactive in situ hybridisation

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Abstract A non-radioactive in situ hybridisation method is described for the detection of low intramuscular levels of brain-derived neurotrophic factor (BDNF) mRNA at the electron microscope level. Application of high-grade silver-gold intensification of the diaminobenzidine end product of in situ hybridisation revealed that in adult rat muscle the constitutive expression of muscular BDNF is confined to the myofibres; satellite cells, Schwann cells, endothelial cells, fibroblasts or axons do not appear to contribute to BDNF production in normal muscle. Although muscular BDNF is a neurotrophic factor for innervating motoneurons and supposedly released only at the motor endplates, the production of BDNF mRNA appears to occur along the entire length of the myofibres and is not confined to nuclei in the postsynaptic regions.

Keywords BDNF mRNA · In situ hybridisation · Non-radioactive · Silver-gold intensification · Electron microscopy · Soleus muscle · Rat

Introduction

In situ hybridisation (ISH) has become a routine procedure to detect and localise the expression of mRNA in a wide variety of tissues. Initially, in order to be able to detect the hybridisation product, radioactive-labelled nucleic acids were incorporated in the mRNA probes and their intracellular presence was revealed with the use of autoradiography (see, for example, Scott Young 1986). A disadvantage of the autoradiographic ISH procedure was the low morphological resolution, because of the indirect image of the labelling in the form of silver grains in the overlying photographic emulsion. About a decade ago, biotin- or digoxigenin-labelled probes replaced the radioactive-labelled probes and immunological detection substituted photographic development (Guitteny et al. 1988; Morris et al. 1990). Clear advantages of the non-radioactive ISH procedure are an optimal signal-to-noise ratio and a high histological resolution. Presently, for the immunological detection of biotin or digoxigenin, fluorescent or enzymatic (for example, phosphatase or peroxidase) labels are used. High-grade silver-gold intensification of the diaminobenzidine end product in peroxidase histochemistry has been applied to improve the detection level of the ISH reaction product for light microscopy (Przepiorka and Myerson 1986; Mullink et al. 1992).

Electron microscopic (EM) ISH is required to determine the precise (sub)cellular site of expression of very low levels of mRNA, such as, for instance, the expression of mRNA encoding for brain-derived neurotrophic factor (BDNF) in muscle tissue. BDNF is a member of the neurotrophins, a family of polypeptide neurotrophic factors. Besides two other members of this family, neurotrophin-3 and neurotrophin-4, BDNF has been shown to support motoneuron survival during embryonic development and, after birth, to protect motoneurons from degeneration after nerve lesion (Sendtner et al. 1992; Yan et al. 1992, 1993; Henderson et al. 1993a, b; Hughes et al. 1993; Koliatsos et al. 1993). High levels of these neurotrophins are present during embryonic development in the main targets of the motoneurons, the muscles (Griesbeck et al. 1995). Northern blot analyses have shown that the expression of BDNF mRNA in rat postnatal muscles steeply declines to an extremely low level of less than 0.5 pg/µg in adult muscles (Griesbeck et al. 1995). Upregulation of the adult muscular BDNF mRNA level has been observed after nerve injury (Funakoshi et al. 1993; Koliatsos et al. 1993; Griesbeck et al. 1995; Zhang et al. 2000) and in diabetic rats (Fernyhough et al.

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1996; Copray et al. 2000). With standard EM ISH, it appeared to be difficult to detect (even the upregulated levels of) BDNF mRNA in adult muscles and to determine the muscular cell types (muscle fibres, satellite cells, Schwann cells, fibroblasts, endothelial cells) involved (Griesbeck et al. 1995). It remained unclear whether muscle fibres express BDNF mRNA uniformly and if so whether all nuclei in these large multinuclear cells contribute equally to this expression; is BDNF mRNA expression, for instance, maybe restricted to subsynaptic nuclei, as described for instance for the production of acetylcholine receptor (AChR) subunits (Merlie and Sanes 1985; Fontaine et al. 1988; Goldman and Staple 1989)?

The present study describes the detection and subcellular localisation of low levels of BDNF mRNA in muscle with a new silver-gold intensified non-radioactive ISH procedure for electron microscopy.

Materials and methods

Animals and tissue preparation

Wistar rats (weighing about 327±42 g) were used in this study. Animals were anaesthetised with a mixture of halothane/oxygen/nitrogen and perfused transcardially through the ascending aorta with 75 ml phosphate-buffered saline (PBS) containing 1% sodium nitrite, followed by 500 ml 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PBS, pH 7.4. Soleus muscles were dissected and subsequently immersed in the perfusion fixative for 1 h at 4°C. Serial 100-µm sections, taken along the entire length of the muscle, were cut on a vibratome (Oxford) and collected in PBS until further processing.

Preparation of BDNF probe

A plasmid containing a 1,127-bp cDNA fragment encoding rat BDNF was kindly provided by George D. Yancopoulos (Regeneron Pharmaceuticals, Tarrytown, New York, USA). To generate antisense and sense digoxigenin-labelled probes for non-radioactive ISH, the plasmid was linearised with BAM H1 and CLA1, respectively, and transcribed in vitro in the presence of the appropriate RNA polymerase and digoxigenin-conjugated UTP as recommended by the manufacturer (Boeringher, Mannheim, Germany)

In situ hybridisation

ISH was performed on free-floating (100 µm) vibratome sections. The vibratome sections were hybridised with digoxigenin-labelled antisense RNA probes. In order to retain optimal morphological integrity, proteolytic digestion of the section with proteinase K was omitted. The vibratome sections were rinsed in PBS and permeabilised by incubation in 0.02% Triton X-100 in PBS for 2 h at 4°C. Subsequently, the sections were rinsed in PBS, followed by a 2×5 min rinse with 2×SSC (1×SSC: 150 mM sodium chloride, 15 mM sodium citrate). The free-floating sections were prehybridised at 60°C in a solution containing 50% formamide, 0.3 M NaCl, 10 mM TRIS (pH 8.0), 1 mM EDTA, 0.05% tRNA, 1×Denhardt's [0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin (BSA), 0.02% Ficoll] and 10% dextran sulphate. After prehybridisation for 1 h, the digoxigenin-labelled probe (sense or antisense) was added to the solution with a final concentration of 1–5 ng/µl. The vibratome sections were then hybridised overnight in Eppendorf tubes in a water-bath at 60°C. The next day, the sections were treated with ribonuclease A (10 µg/ml) for 30 min at 37°C and washed in 0.1×SSC at 65°C.

The immunological detection of the digoxigenin-labelled RNA–RNA complex was preceded by a preincubation at room temperature in PBS containing 5% BSA for 30 min. Thereafter, sections were incubated overnight at 4°C with sheep anti-digoxigenin (1:200; Boehringer Mannheim) in PBS containing 1% BSA. Endogenous peroxidase was blocked using 0.3% H_2O_2 in PBS for 30 min, followed by thorough rinsing with PBS. Section were then incubated at room temperature with biotinylated rabbit anti-sheep for 2 h (1:300; Rockland), followed by a 1-h incubation with HRP-conjugated streptavidin (Amersham), diluted 1:300 in PBS-BSA. The peroxidase-labelled immunocomplexes were revealed by incubating the sections for 10 min in 0.05 M TBS containing 0.4 mg/ml 3,3′-diaminobenzidine (DAB; Sigma) and 0.01% H_2O_2, resulting in a brown precipitate. The sections were then given a final rinse and stored in TBS.

Gold-silver-substituted peroxidase (GSSP) intensification

Intensification of the final reaction products was effectuated using the GSSP method which is a modification of the procedure described by (Gallyas et al. 1982). This method aims to enhance the intensity of the HRP-DAB reaction product. After completing the DAB reaction, the vibratome sections were rinsed twice (15 min each) in 2% sodium acetate solution. After decolourising for 2.5–3.5 h in 10% thioglycolic acid (Fluka), the vibratome sections were immersed for 8 min in a freshly prepared physical developer containing 2.5% sodium carbonate, 0.1% ammonium nitrate, 0.1% silver nitrate, 0.5% phosphosilico-tungstic acid and 0.9% paraformaldehyde. Following a brief wash for 2 min with 1% acetic acid, the deposited silver particles were replaced by gold by immersing the vibratome sections in 0.02% gold chloride for 8 min at room temperature and finally fixed with 3% sodium thiosulphate (2×10 min). Following intensification, the brown DAB immunoreaction product turned black.

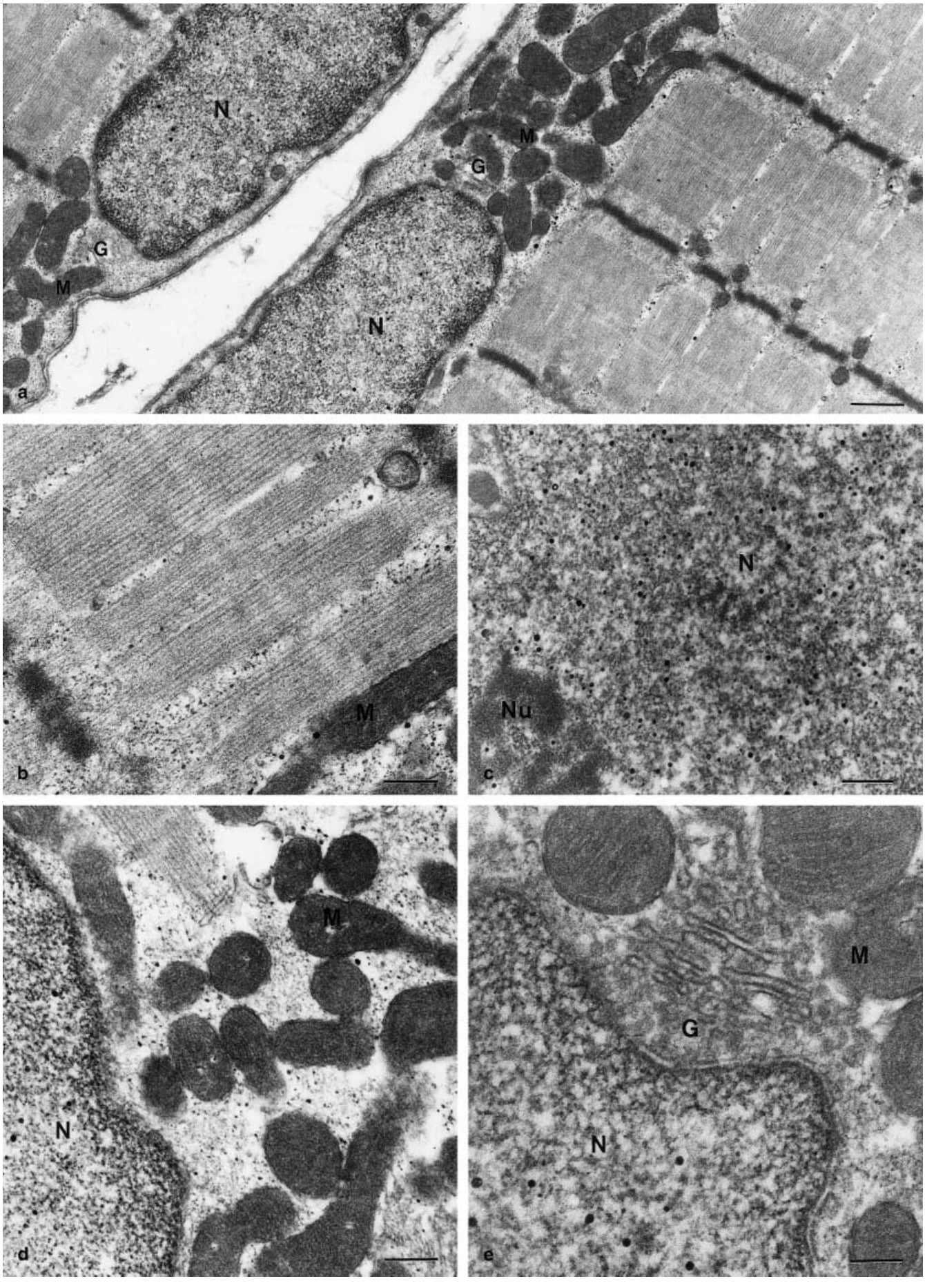
Controls

Negative ISH control procedures on parallel sections included: (a) hybridisation of sections with sense RNA probe of similar specific activity, (b) hybridisation of sections with omission of RNA probes and (c) immunological detection of the ISH product with omission of the primary antibody step.

Electron microscopy

The GSSP-treated vibratome sections were rinsed briefly in 0.1 M sodium cacodylate buffer, pH 7.6, and postfixed for 1 h in 1% osmium tetroxide (w/v) dissolved in the same buffer solution. The sections were then dehydrated through graded concentrations of ethanol and propylene oxide, and subsequently flat embedded in Epon 812. Before cutting ultrathin (60 nm) sections, semithin (1 µm) sections were obtained to check the presence of relevant areas. Moreover, since we found that a fairly even diffusion of the

Fig. 1a–e Electron micrographs of rat soleus muscle fibres after ▶ in situ hybridisation (ISH) for brain-derived neurotrophic factor (BDNF) mRNA using the silver/gold postintensification procedure. The reaction product consists of small metallic grain particles (diameter 8–10 nm), dispersed in the cytoplasm around and between the bundles of myofibrils (**a**, **b**), in the nucleus (**c**) and occasionally attached to the endoplasmic reticulum; no such gold grain particles could be detected in mitochondria (**d**), Golgi complexes (G in **e**) or other cell organelles. *N* Nucleus, *Nu* nucleolus, *M* mitochondrion, *G* Golgi complex. *Scale bars* **a** 0.54 µm; **b** 0.21 µm; **c** 0.33 µm; **d** 0.30 µm; **e** 0.16 µm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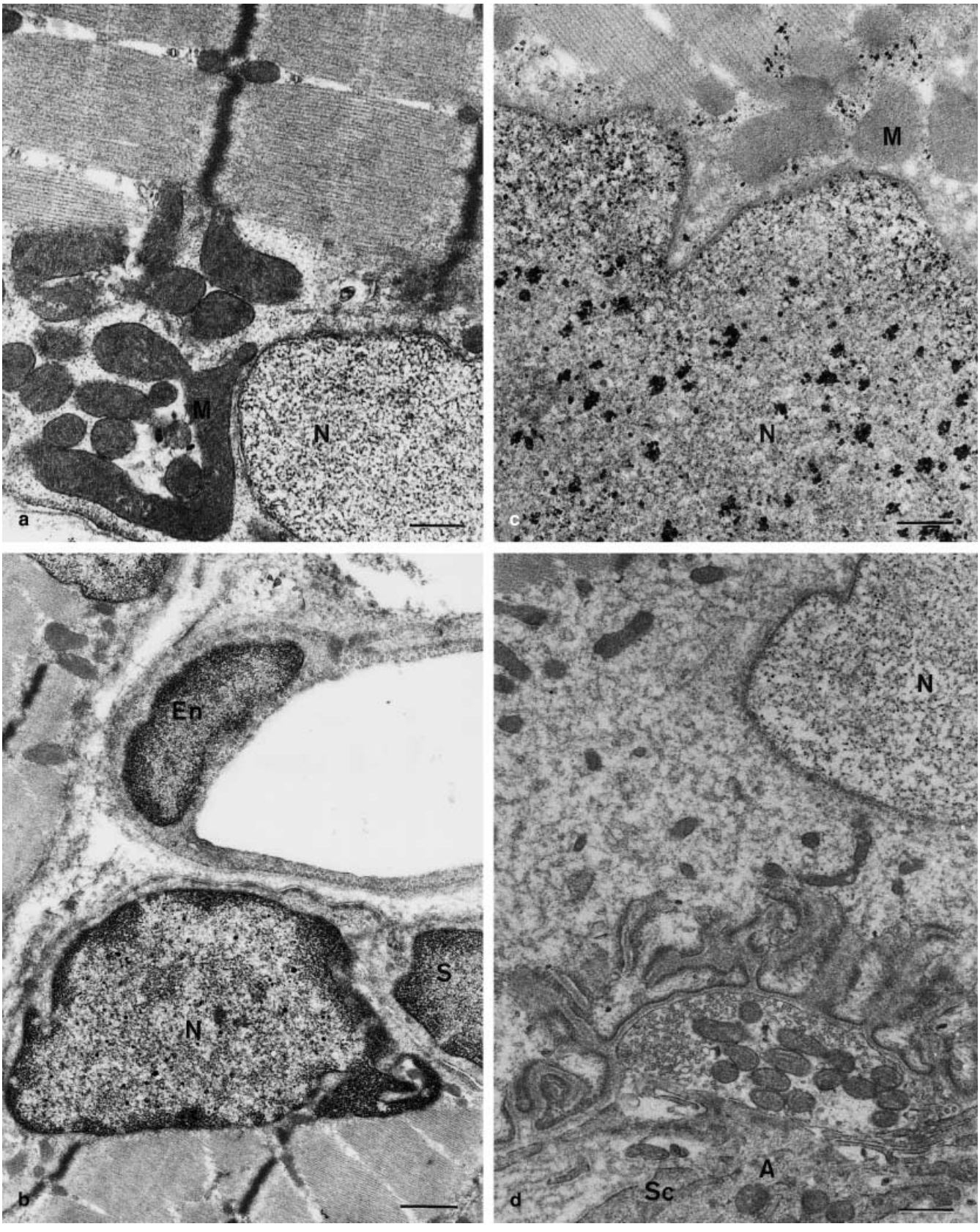
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various labelling components was restricted only to the first superficial 15 µm of the vibratome sections, ultrathin sections for analysis were only taken from within the superficial 10 µm area of the vibratome section. The ultrathin sections were examined with a Philips CM-100 electron microscope after counterstaining with uranyl acetate and lead citrate.

Results

GSSP intensification enables EM detection of BDNF mRNA in muscle tissue

Whereas non-radioactive ISH without the GSSP intensification procedure did not result in a visible ISH reaction product in the electron micrographs of muscle fibres from m. soleus, application of the intensification procedure led to a clear hybridisation product indicating the presence and distribution of BDNF mRNA in muscle fibres (Figs. 1, 2). The reaction product consisted of small metallic grain particles (diameter 8–10 nm) dispersed in the cytoplasm around and between the bundles of myofibrils (Fig. 1a, b), in the nucleus (Fig. 1c) and occasionally attached to the endoplasmic reticulum; no such gold grain particles could be detected in mitochondria (Fig. 1d), Golgi complexes (Fig. 1e) or other cell organelles. The negative control, i.e. ISH with the sense probe including the intensification procedure, did not result in labelling (Fig. 2a). Besides the myofibres, no other muscular cell types appear to express BDNF mRNA; with GSSP-enhanced non-radioactive ISH we were unable to detect BDNF mRNA in Schwann cells, satellite cells, fibroblasts, endothelial cells or neuronal axons in the rat m. soleus (Fig. 2b).

A crucial step in the application of the GSSP intensification procedure is the controlled exposure to the physical developer. Although pretreatment in 10% thioglycolic acid suppresses argyrophilia of the tissue (Gallyas 1982), overexposure in the developer will lead to aspecific silver deposition at sites of non-specific, background DAB polymers. Prolonged uncontrolled exposure in the developer might lead to a very intense precipitation product after gold replacement (Fig. 2c) and consequently facilitate detection, however, it can easily lead to obscuration of the cellular ultrastructure.

◀ **Fig. 2a–d** Electron micrographs of rat soleus muscle fibres after ISH for BDNF mRNA using the silver/gold postintensification procedure. The negative control, i.e. ISH with the sense probe including the intensification procedure did not result in labelling (**a**). Expression of BDNF mRNA is confined to the muscle fibre (**b**); the endothelial cell and the satellite cell (partly visible at the right) do not express BDNF mRNA. Overexposure in the developer leads to the formation of large clusters of silver deposition frequently at sites of non-specific, background 3,3′-diaminobenzidine polymers, as can be seen in the myofibre nucleus depicted in **c**. Granular reaction product representing BDNF mRNA can also be detected in nuclei at subsynaptic sites (**d**). However, in contrast to distal myofibre sites (Fig. 1a–e), the cytoplasm in the subsynaptic nuclear region is almost entirely devoid of the BDNF mRNA signal; no reaction product can be seen in the axon or the Schwann cell (partly visible at the bottom in **d**). *N* Nucleus, *M* mitochondrion, *S* satellite cell, *En* endothelial cell, *A* axon, *Sc* Schwann cell. *Scale bars* **a** 0.43 µm; **b** 0.54 µm; **c** 0.35 µm; **d** 0.46 µm

Subcellular localisation of BDNF mRNA in muscle fibres

In view of its function as a retrograde neurotrophic maintenance factor for adult motoneurons, we investigated with our GSSP-enhanced non-radioactive ISH procedure whether the production of BDNF mRNA was confined to nuclei situated at the motor endplate. We took sections of the muscle fibre at the neuromuscular junction and at various regions distant from that. Distal nuclei clearly exhibited a strong BDNF mRNA ISH signal (Fig. 1d). As expected, subsynaptic nuclei showed a high level of BDNF mRNA (Fig. 2d). Remarkably, however, in contrast to the nuclear region of distal nuclei, the cytoplasm around subsynaptic nuclei was almost devoid of BDNF mRNA, suggesting a different regulation of BDNF production in the subsynaptic region in comparison to the rest of the myofibre.

Discussion

Our adaptation of the GSSP intensification procedure for EM non-radioactive ISH clearly enables the detection and subcellular localization of extremely low intramuscular levels of mRNA, which are not detectable in standard EM ISH. The ultrastructural detection of low levels of mRNA entails a compromise between the necessity to maintain acceptable cellular morphology and the need for membrane permeabilising steps to insure free access of the hybridisation probes. We have tested various concentrations and exposure times of proteinase K, but it appeared that omission of proteolytic digestion in our EM non-radioactive ISH procedure did not prevent hybridisation and resulted in optimal morphological integrity. A very important step in the application of the GSSP intensification procedure is the controlled exposure (no longer than 8 min) to the physical developer. Although pretreatment in 10% thioglycolic acid suppresses argyrophilia of tissue (Gallyas 1982), overexposure in the developer can lead to aspecific silver deposition at sites of non-specific, background DAB polymers. Prolonged uncontrolled exposure in the developer can lead to a very intense precipitation product obscuring the cellular ultrastructure after the silver replacement by gold.

Our ultrastructural ISH analyses show that in normal adult muscle, low levels of BDNF mRNA are constitutively expressed only in muscle fibres, and not in other muscular cell types. So, in normal muscles, satellite cells and Schwann cells appear to be quiescent as far as BDNF production is concerned. However, both cell types are induced to express BDNF mRNA during nerve or muscle regeneration processes. After axotomy, Schwann cells at both proximal and distal sites of the lesion start to produce BDNF; this BDNF is thought to play a role in the

regeneration and outgrowth of the injured axon (Meyer et al. 1992; Funakoshi et al. 1993; Ide 1996; Frostick et al. 1998; Zhang et al. 2000). As a reaction to muscular trauma with loss of muscle fibres, myofibre neogenesis starts with the activation of satellite cells; these activated satellite cells produce BDNF, which presumably is involved in the myofibre differentiation process. Recently, low levels of BDNF have been demonstrated in rat and human blood; RNA and protein analyses have indicated endothelial cells to be a major source of this circulating BDNF (Nakahashi et al. 2000). In our ISH experiments we were unable to detect BDNF production in endothelial cells in the muscular vasculature.

A single muscle fibre maintains its function, its structure and its large volume of cytoplasm by means of hundreds of nuclei distributed along its entire length. It has been questioned whether these nuclei interact, whether their products completely intermingle and whether a muscle fibre is constructed of a mosaic of domains, each dominated by a single nucleus (Hall and Ralston 1989; Pavlath et al. 1989; Ralston and Hall 1989b). It has become clear that most mRNA is confined to an area within approximately 100 µm of the nucleus that expresses it (Ralston and Hall 1992). Cytoplasmic or membrane proteins are free to diffuse and they can spread along the entire length of the muscle fibre; targeted proteins such as proteins associated with the Golgi apparatus or with the contractile apparatus remain localized near their nucleus of origin (Pavlath et al. 1989; Ralston and Hall 1989a). Production of most muscular proteins seems to occur homogeneously distributed over the entire length of fibre. However, the synthesis of proteins involved in the structure and function of the motor endplate, appears to be regulated more locally. The postsynaptic region constitutes <0.1% of the muscle fibre surface but contains >95% of the AChRs. It has been shown that the restricted distribution of the AChR arises in part because nuclei near the synapse express higher levels of receptor subunit mRNA than do other nuclei (Merlie and Sanes 1985; Fontaine et al. 1988; Goldman and Staple 1989); interaction with various products of other subsynaptic and more distant nuclei results in the formation of AChR clusters on the postsynaptic membrane in the motor endplate (Gordon et al. 1992). Extrinsic influences, in particular the extent and pattern of electrical activity in the innervating motoneuron, have been shown to play an important role in this regulation. BDNF is a target-derived neurotrophic factor for motoneurons. After binding to the trkB receptor at the presynaptic membrane, it is retrogradely transported to the motoneuron soma where it activates several signalling pathways involved in the functional maintenance of the motoneuron. The extremely small quantities required and the restricted release to the motor endplate have led to the suggestion that BDNF is produced only by subsynaptic nuclei. However, our ISH analysis shows that all nuclei in the muscle fibre appear to be involved. Remarkably, the amount of BDNF mRNA in the cytoplasmic domains of the subsynaptic nuclei is very low. It is as yet unclear whether this is due to restricted transport from the nucleus to the cytoplasm or to a different half-life time of cytosolic BDNF mRNA in this region. Apparently, the regulation of the production of BDNF in the postsynaptic region of the muscle fibres is different from the rest of the fibre. Presumably external influences, for example, extent and pattern of electrical stimulation, derived from the innervating motor axon may be involved.

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