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Upregulation of B-50/GAP-43 in Schwann cells at denervated motor endplates and in motoneurons after rat facial nerve crush

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Abstract

Crush or transection of peripheral nerves of the adult rat is accompanied by changes in protein expression, including the growth associated protein (GAP-43) B-50. Following peripheral nerve crush in rat enhanced B-50 immunoreactivity was observed in regenerating nerve fibres and in newly formed axon terminals. However, before reinnervation was apparent, an unexpected transient increase in B-50 immunoreactivity was observed at denervated motor endplates [J. Neurosci. 8 (1988) 1759]. This study was performed to clarify this observation. Four days following facial nerve crush B-50 immunoreactivity was detected by double immunofluorescence microscopy in S100-positive Schwann cells covering the denervated endplates. Using diluted polyclonal and monoclonal B-50 antibodies we found that B-50 immunoreactivity at the denervated motor endplates was strongly increased in comparison to innervated motor endplates in which B-50 immunoreactivity was hardly detectable. However, when a high concentration of B-50 antibodies was applied the normal innervated motor endplates were also B-50 immunoreactive. Muscle fibres did not display B-50 immunoreactivity. Northern blot analysis revealed elevated B-50 mRNA in denervated muscle and in degenerating nerve with respect to the controls. The B-50 mRNA levels in these non-neuronal tissues were very low compared to the intact and injured facial nucleus containing the neuronal cell bodies. Electron microscopy demonstrated that the B-50 protein was localized in the processes of Schwann cells covering axon terminals of intact and vacant motor endplates and in axon varicosities of sympathetic nerves. This study has confirmed that prior to reinnervation B-50 immunoreactivity is increased at denervated motor endplates and shows that B-50 is co-localized with S100 in Schwann cells. Therefore, upregulation of B-50 expression in Schwann cells may explain the early occurrence of B-50 immunoreactivity at the motor endplate.

Introduction

Injury to peripheral nerves results in a number of morphological and structural changes (reviewed in [28,46]). The succession of events in the distal nerve portion of an injured nerve, briefly referred to as Wallerian degeneration, starts with disintegration of the damaged axons and their myelin sheaths. Schwann cells multiply and form a cellular string within the endoneural tubes, the so-called bands of Büngner. In the course of Wallerian degeneration, the cellular debris is digested by proliferating non-myelinating Schwann cells [25,26,44] and by infiltrating macrophages [54]. In addition to their role as scavenger cells, Schwann cells express neurotrophic factors [25,26,43,60] and neural cell adhesion molecules that stimulate the outgrowth of regenerating nerve fibres into the distal nerve stump [59]. These newly formed nerve fibres eventually reinnervate their target cells in

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muscle and skin. The Schwann cells regain their post-mitotic status and remyelinate the newly formed axons. At the reinnervated neuromuscular junctions Schwann cells ensheath the newly formed presynaptic axon terminals.

In studies focusing on the biochemical changes in experimentally damaged peripheral nerves, synthesis and transport of the neuronal phosphoprotein B-50 was correlated with regenerative sprouting and target cell reinnervation [8,22,48,50,51]. These observations led to the concept that enhanced expression of B-50 is a prerequisite for successful axonal regeneration [50,51]. In this concept B-50 was designated a growth-associated protein, GAP-43 (also termed GAP-48, neuromodulin, P57, F1, pp46) [8,22,48].

B-50 is thought to play a crucial role in neuritogenesis, axonal regeneration, synaptic plasticity and neurotransmission [15,22,48,55,56]. This is based on studies of the biochemical interactions of B-50 in growth cones and in nerve terminals in vitro and in vivo. Dephosphorylated B-50 binds calmodulin at low intracellular Ca^{2+} levels [2,3,11]. However, increase of intracellular Ca^{2+} levels leads to a dissociation of the B-50/calmodulin complex, resulting in a stimulation of second messengers and vesicle release [19,31]. The phosphorylation of B-50 by protein kinase C (PKC) [4,11,14,52] and casein kinase II [41] is inhibited by calmodulin [2,5]. Palmitoylation of B-50 enhances its association with the membrane GTP binding protein, Go [49,58]. Depalmitoylation of B-50 results in dissociation and stimulation of second messengers mediated by the activated Go protein [56,57]. Phosphorylation of B-50 by PKC can inhibit phosphatidyl-inositol phosphate kinase activity in vitro [66]. PKC mediated phosphorylation of B-50 seems to be independent whether B-50 is associated with the membrane or not [12]. Though an impressive wealth of data on intracellular interactions of B-50 has become available, the physiological function of B-50 in neurons is still in debate.

In an immunocytochemical study on the distribution of B-50 during denervation and reinnervation of the adult rat soleus muscle we demonstrated that high B-50 immunostaining was present in newly formed nerve fibres invading the soleus muscle and at the newly formed neuromuscular junctions [67]. This elevated B-50 expression during axon growth supports the idea that B-50 might be a key component in the reconstruction of axons. However, an unexpected and unexplained increase in B-50 immunoreactivity was observed at denervated motor endplates during the first 3 days following denervation. B-50 immunostaining decreased up to day 6 and increased up to day 18 in a large fraction of the endplates following nerve crush. Subsequently the number of B-50 positive neuromuscular junctions gradually diminished and reached control values after reinnervation of the muscle. Studies by Benowitz and Lewis [7], Skene and Willard [50,51], and van der Zee et al. [65] have shown that when regenerating axons reinnervate their target the B-50 level drops to the normal low level in adult nerve.

Until recently B-50/GAP-43 expression has been considered to be restricted to neurons [29,48]. However, lately, it has been reported that B-50 immunoreactivity can also be observed, in vitro, in glial cells in culture [16–18,20] and, in vivo, in association with the bands of Büngner in a degenerating peripheral nerve [62] and in non-myelin forming Schwann cells [17]. This suggests that B-50 can be synthesized in non-neuronal cells. These considerations and observations inspired us to restudy the unexplained increase in B-50 immunoreactivity at denervated motor endplates observed 4 days after crush by applying double immunofluorescence of B-50 and S100, a marker protein for Schwan cell and immuno electron microscopy. Our study concerns the motor endplates of the rat facial muscle (i.e. the levator labii superioris muscle) during the initial stage of Wallerian degeneration. In addition, we monitored the consequences of nerve injury on the regenerating motoneurons in the facial nucleus after rat facial nerve crush. Some of the results have been published previously in abstract form [64].

Materials and Methods

Antibodies

Polyclonal anti-rat B-50 immunoglobulins (from rabbit 8920) were affinity-purified and characterized as previously described [34,37,38]. NM4, a monoclonal mouse anti-rat B-50 antibody has been characterized by Mercken et al. [34]. The other antibodies were purchased: monoclonal mouse anti-rat GAP-43 antibodies (Sanbio); polyclonal rabbit anti-bovine S100 antibodies (Sigma); monoclonal mouse anti-bovine synaptophysin antibodies (Dakopatts); biotin-conjugated horse anti-mouse IgG(H+L) (Vector); peroxidase-conjugated swine anti-rabbit IgG (Dakopatts); fluorescein (FITC)-conjugated streptavidin (Dakopatts); rhodamine (TRITC)-conjugated swine anti-rabbit immunoglobulins (Dakopatts).

Animal surgery

Female Wistar rats (120–140 g in weight) were obtained from Harlann Sprague–Dawley (Zeist, The Netherlands). The rats were housed five in a cage at 21–23°C and 60–70% relative humidity. They were fed Hopefarms pellet food and water ad libitum. Rats (n = 5) were deeply anaesthetized with Hypnorm, contain-

ing 10 mg fluanison and 0.315 mg fentanyl citrate per ml, obtained from Janssen Pharmaceutica B.V. (Tilburg, The Netherlands). The right facial nerve was crushed 3 mm distal from the stylomastoid foramen for 30 s with a haemostatic forceps. The facial nerve model is described by Kreutzberg [28]. The intact contralateral side served as a control. Four days after operation rats were heparinized under deep Nembutal anaesthesia, and transcardially perfused with 250 ml fixative warmed to 37°C and consisting of 2% paraformaldehyde, 0.1 M lysine and 0.2% periodate in 50 mM phosphate buffer at pH 7.4 [33,67].

The levator labii superioris muscles and the facial nuclei were dissected and cryoprotected by immersion in graded sucrose solutions (7.5%, 15% and 25%, pH 7.3; 3 h each) in phosphate buffered saline (PBS). After cryoprotection, the tissue was quickly frozen in liquid N_2-cooled iso-pentane and stored at −80°C.

Light microscopy

Four μm thick cryostat sections of the muscles were fixed on 0.5% formol-gelatin coated coverslips. Prior to the immunostaining, the sections were airdried and stained for acetylcholine esterase (AChE), using 5-bromoindoxyl acetate until microscopy showed the motor endplates to be clearly defined blue areas (10–15 min, 37°C, pH 7.2). Subsequently, the sections were washed at room temperature with phosphate-buffered saline (PBS, 2 × 5 min); 100% acetone (3 × 5 min); 0.2% Triton X-100 in PBS (2 × 5 min) and PBS (3 × 5 min).

Optimum conditions for immunocytochemical staining of the antibodies were determined in preliminary experiments. For the B-50 immunostaining increasing dilutions of polyclonal and monoclonal anti-B-50 antibodies were tested. The anti-B-50 antibodies together with the second antibody-conjugates used in these experiments appeared to detect more readily B-50 than those used by Verhaagen et al. [67]. All antibodies were diluted in 0.2% bovine serum albumin (BSA) in PBS. Double immunofluorescence staining was performed by incubating the sections with the monoclonal mouse anti-rat B-50 antibodies (1 : 3,000) together with the polyclonal rabbit anti-bovine S100 antibodies (1 : 800) overnight in a humid box at room temperature. In some experiments mono- or polyclonal anti-B-50 antibodies were diluted to 1 : 300 to detect B-50 in innervated muscle. After washing with PBS (3 × 5 min), the sections were incubated for 1 h with biotinylated horse anti-mouse immunoglobulins (1 : 220) with the addition of 2% normal rat serum to prevent non-specific binding. After washing with PBS, the sections were incubated for 1 h with fluorescein (FITC)-conjugated streptavidin (1 : 100) together with rhodamine (TRITC)-conjugated swine anti-rabbit immunoglobulins (1 : 30). After washing with PBS (3 × 5 min), the sections were embedded in 90% glycerol containing 10% veronal buffered saline at pH 9 and 10% 1,4-diazobicyclo-(2,2,2)-octane. The sections were viewed with a Leitz Orthoplan fluorescence microscope. Immunohistochemical controls were carried out by omitting the primary antibodies and continuing the staining sequence further. Preabsorption of the B-50 antibodies with excess of purified B-50 removed largely the specific immunostaining.

Electron microscopy

For pre-embedding immunocytochemistry 20 μm cryostat sections of muscles were fixed on 0.5% formol-gelatin coated coverslips. After each section a 6 μm serial section was made to locate the endplate regions by histochemical staining for AChE. When the 20 μm thick sections were almost dry they were incubated in 0.00002% Triton X-100 in PBS (2 × 5 min) and washed in PBS (2 × 5 min). Antibodies were diluted in PBS/BSA/0.00002% Triton X-100. The sections were incubated for 6 h in a humid box at room temperature with (i) the primary monoclonal to B-50 (1 : 3,000); (ii) polyclonal antibodies to B-50 (usually 1 : 3,000; sometimes 1 : 300); (iii) polyclonal antibodies to S100 (1 : 10,000) or (iv) monoclonal antibodies to synaptophysin (1 : 40). A few experiments were carried out with 1 : 300 diluted monoclonal anti-B-50 antibodies. After washing twice with PBS (2 × 5 min), the sections were incubated overnight with, respectively, the peroxidase-conjugated rabbit anti-mouse immunoglobulins (1 : 100) with the addition of 2% normal rat serum and the peroxidase-conjugated swine anti-rabbit immunoglobulins (1 : 100). After washing (3 × 5 min), the peroxidase reaction was performed for 6 min according to Adams [1]. After washing twice with PBS (2 × 5 min), the sections were fixed in 2% osmium tetroxide for 1 h [39], dehydrated in a graded acetone sequence, embedded in Epon 812 and polymerized overnight at 60°C. The endplate regions in the 20 μm sections were located by comparing them with the 6 μm serial sections. Ultrathin sections were cut on an LKB ultratome and counterstained for 1 min with lead citrate before observation with a Philips 301 electron microscope.

Northern blot analysis

Both facial nerves of the rat (n = 10) were crushed. Four days following the surgery, the facial nuclei, the facial nerves from 1 to 3 cm distal to the crush side and the levator labii superioris muscles were dissected, immediately frozen and stored at −80°C. Tissues from unoperated age-matched rats (n = 10) were used as controls. In order to obtain sufficient tissue for RNA

isolation, the facial nuclei of 3 rats and the facial nerves of 5 rats were pooled.

Total RNA was extracted using RNAzol® (Cinna/Biotecx, Friendswood,USA) according to manufacturers instructions. About 100 mg weighing tissue samples were homogenized in RNAzol (2 ml/100 mg tissue) in a glass tube with a tightly fitting teflon pestle by 10–20 strokes up and down at 0°C. Chloroform (0.2 ml) was added to 2 ml of homogenate. The samples were shaken vigorously (15 s) and put on ice (15 min). After centrifugation (12, 000 × *g*, 15 min, 4°C), the upper aqueous phase containing the RNA was transferred to a fresh tube and an equal volume of isopropanol was added. The RNA was allowed to precipitate for at least 45 min at −20°C. RNA was pelleted by centrifugation (12, 000 × *g*, 20 min, 4°C) and washed twice with 70% ethanol. The RNA pellet was briefly dried under vacuum and dissolved in sterilized water. The amount of RNA was quantified with a multiwave spectrophotometer by UV absorption at 260–280 Å; and the quality was examined on a 2% agarose gel stained with ethidium bromide. When the two ribosomal RNA bands of 18S and 28S were visible, RNA was judged to be of sufficient quality to be used for Northern blotting.

For Northern blot analysis, samples of approximately 20 μg total RNA were denatured with glyoxal and DMSO in 10 mM sodium phosphate buffer at 50°C for 60 min. Subsequently, samples were cooled on ice and run on 1% agarose gel in 10 mM sodium phosphate, pH 7.0, following by capillary blotting to nylon transfer membrane (Hybond N, Amersham, UK) in 20 × SSC (SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0). The membranes were exposed to UV light (2 min) and baked at 80°C for 2 h. Membranes were prehybridized for 3 h at 42°C in a hybridization solution containing (50 mM Tris-HCl, pH 7.5, 50% formamide, 1 M NaCl, 10% dextran sulphate, 0.1 mg/ml denatured salmon sperm DNA, 0.2% BSA, 0.2% polyvinylpyrrolidon, 0.2% Ficoll, 0.1% sodium pyrophosphate, 1% SDS) and hybridized overnight at 42°C in the same solution containing 1×10^6 cpm/ml of a [α-^{32}P]dCTP B-50 cDNA probe. The specific B-50 probe was an 1,120 bp B-50 cDNA isolated from clone pGBO [36]. The probe was labelled by using a random primed DNA labelling kit (Boehringer, Mannheim) and was purified on a Sephadex column. The membranes were washed with successively 2 ×, 1 ×, 0.2 × and 0.1 × SSC with the addition of 0.1% SDS in a shaking water bath (42°C, 20 min each). Kodak X-OMAT films were exposed to the membranes using intensifying screens (Amersham UK) and were developed after 1 to 8 days. The developed films were quantified using a computer connected to an LKB enhanced laser densitometer. To correct for variations in sample loading, membranes were stripped with 0.01 × SSC (100°C; 20 min) and reprobed with a cDNA probe encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [40]. Quantification of B-50 mRNA of the diverse samples was normalized by dividing the results by the GAPDH mRNA content of the respective sample.

Results

Detection of motor endplates and Schwann cells

Motor endplates in the muscle sections were visualized by staining for AChE and observed as clearly marked blue areas in the light microscope (Fig. 1A,D; Fig. 2A). Schwann cells were identified by immunostaining with anti-S100 antibodies [21,32]. The Schwann cells were found to cover the AChE-stained motor endplates (Fig. 1B,E; Fig. 2B).

B-50 immunoreactivity at control and denervated motor endplates

Light microscopy

Double immunofluorescence of muscle sections revealed that B-50 and S100 immunoreactivity were observed close to AChE stained endplates (Fig. 1). Immunostaining of sections of control muscle with monoclonal anti-B-50 antibodies gave only background and no immunofluorescence in muscle fibres (Fig. 1C). Sometimes little B-50 immunofluorescence was noticeable at AChE positive endplates. With less diluted (i.e. 1 : 300) polyclonal or monoclonal antibodies to B-50, some B-50 immunoreactivity could be detected in mature intramuscular nerves (Fig. 2C; double arrowheads) and in Schwann cells at most of the intact AChE positive endplates (Fig. 2A,B,C). The muscle fibres were negative. Results were similar for polyclonal and monoclonal B-50 antibodies. Since the used polyclonal and monoclonal anti-B-50 antibodies [34] do not cross react with Bovine B-50-Immunoreactive C-Kinase Substrate (also named neurogranin; [6,13]), the immunoreactivity detected is likely to represent B-50 and perhaps its breakdown products. In the control muscle sections anti-S100 antibodies stained areas (Fig. 1B; Fig. 2B) adjoining the hardly detectable B-50 immunoreactivity (Fig. 1C) or bright B-50 immunoreactivity (Fig. 2C). The S100 immunostaining was always associated with the AChE-positive endplate regions and around intramuscular axons (Fig. 1B; Fig. 2B).

Four days post crush, all AChE positive endplates of the denervated muscle displayed strong B-50 immunoreactivity (e.g. Fig. 1F), whereas the muscle fibres were

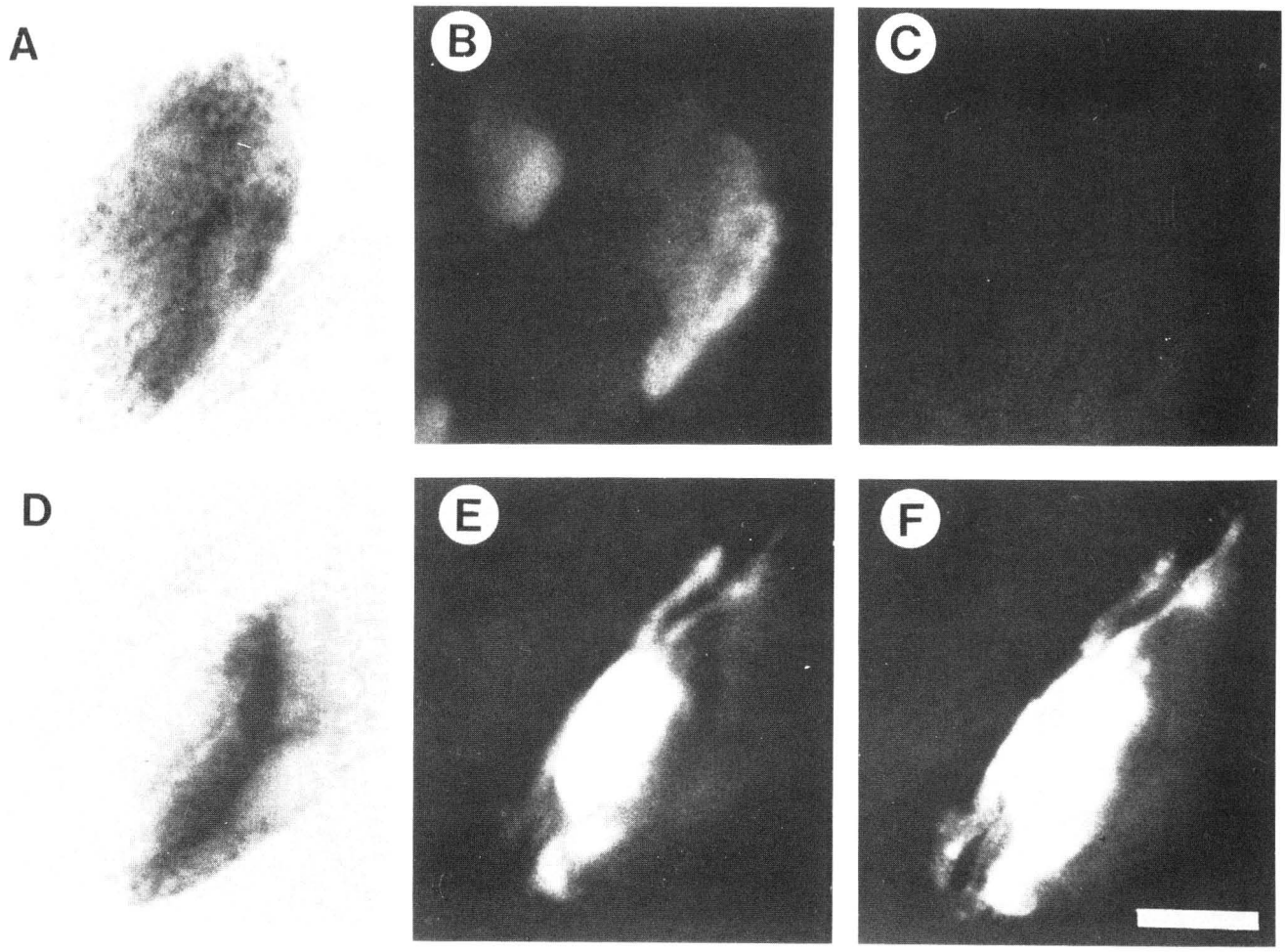

Fig. 1. Double immunofluorescence of B-50 and S100 at motor endplates of control (B,C) and denervated (E,F) facial muscle. Samples shown are derived from the ipsilateral and contralateral muscle of a rat 4 days after unilateral crush of the right facial nerve. Bright field micrograph of muscle fibres with AChE stained motor endplates (A,D) and the corresponding immunostaining of the same section with 1:800 diluted S100 antibodies (B,E) and 1:3,000 diluted B-50 antibodies (C,F). The AChE stained neuromuscular junctions are covered by S100-positive Schwann cells. In the intact muscle hardly any B-50 immunoreactivity (C) is present at the same sites as the Schwann cell at the neuromuscular junction (B). In contrast, in the denervated muscle (F) B-50 immunoreactivity is elevated at the endplates and clearly co-localized with S100 immunoreactivity of the Schwann cell (E). Bar = 10 μm.

devoid of B-50 immunoreactivity. This remarkable increase of B-50 immunoreactivity was noted at the denervated endplates from day 3 onwards after crush. Double immunofluorescence revealed that the contours of the cells covering the AChE positive endplates showing B-50 immunoreactivity and S100 immunoreactivity were similar (Fig. 1E,F). The double immunofluorescence studies were performed with 2 different monoclonal anti-B-50 antibodies, revealing an identical co-localization of B-50 immunoreactivity with S100 immunoreactivity. This co-localization demonstrates that increased B-50 immunoreactivity is present in the S100-positive Schwann cells. In addition, immunostaining for S100 in the cytosol and in the nucleus of Schwann cells (Fig. 1E) at the endplates of the denervated muscle appears to be elevated over that at endplates of controls (Fig. 1B). Immunocytochemical controls were always negative when the primary antibodies were omitted and when the B-50 antibodies were preabsorbed with excess of purified B-50.

Electron microscopy

In order to demonstrate that the increased B-50 immunoreactivity at the denervated motor endplates is localized within Schwann cells, Schwann cells immunostained with anti-S100 antiserum or anti-B-50 antibodies (diluted to 1:3,000) were analyzed by electron microscopy for co-localization. In control muscle Schwann cells and its processes (arrowheads) displayed some reaction product after staining with anti-S100 (Fig. 3A), and no staining with anti-B-50 (Fig. 3D). The cytoplasm and processes of Schwann cells were S100-positive. Schwann cells and their processes covered the nerve terminals (t) at the neuromuscular junctions. Nerve terminals were heavily stained by monoclonal antibodies to synapto-

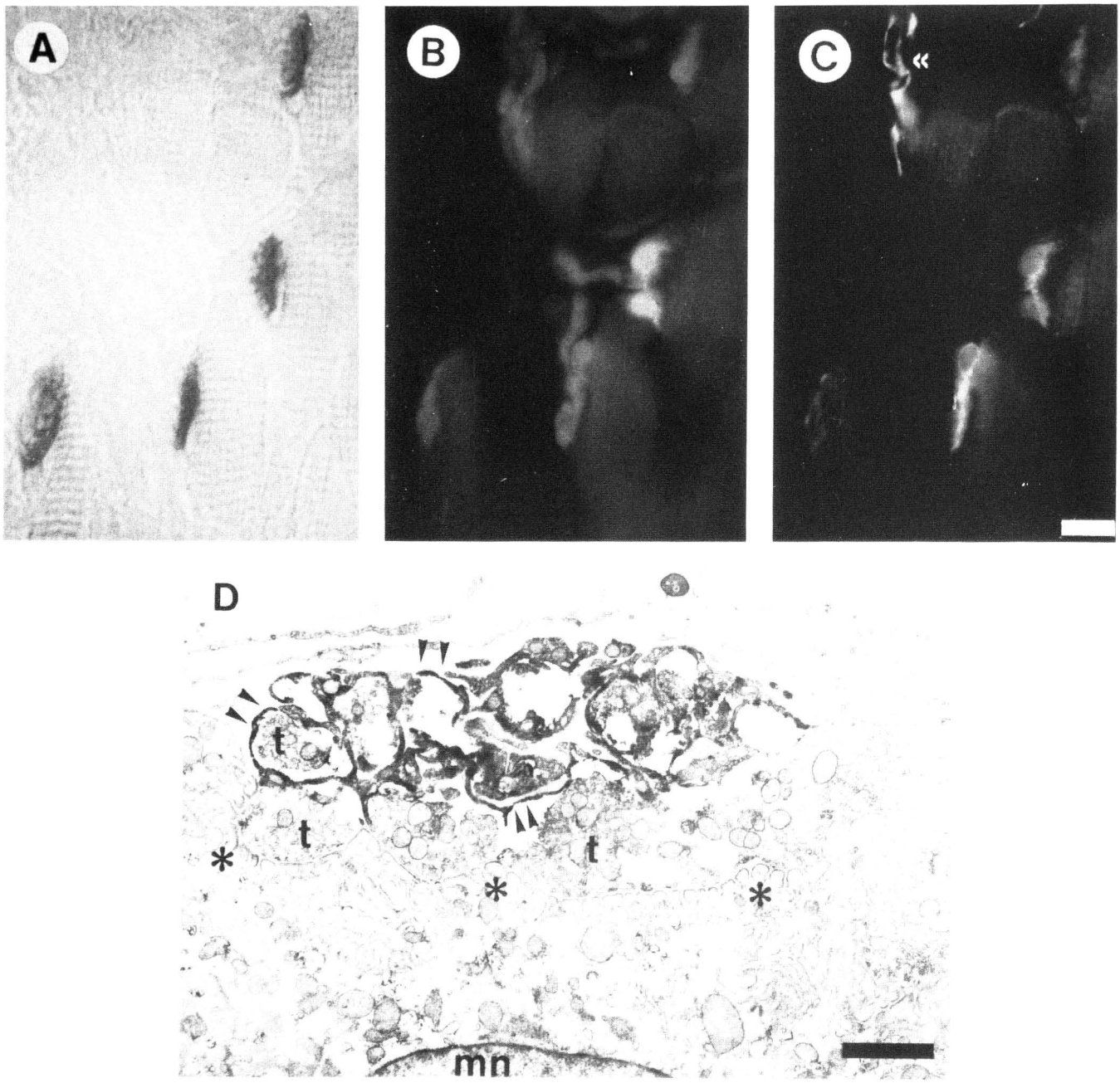

Fig. 2. Double immunofluorescence of B-50 and S100 at motor endplates of control facial muscle. Bright field micrograph of muscle fibres with AChE stained motor endplates (A) and the corresponding immunostaining of the same section with 1 : 800 diluted S100 antibodies (B) and 1 : 300 diluted B-50 antibodies (C). The AChE stained neuromuscular junctions are covered by S100-positive Schwann cells. In the intact muscle B-50 immunoreactivity (C) is present in intramuscular nerves (double arrowheads) and in particularly at the sites of the Schwann cells covering the neuromuscular junctions (B). In 2C, bar = 50 μm. Electron micrograph of the motor endplate of control muscle (D). Schwann cell processes (D; arrowheads) immunoreactive for B-50 cover the presynaptic axon terminals (t) opposed to the folded postsynaptic membrane (asterisks) in the muscle. Presynaptic terminals contain hardly any B-50 immunoreactivity. In 2D, bar = 2 μm. (mn: muscle nucleus.)

physin (Fig. 3C), but in these terminals hardly any B-50 immunoreactivity was detected (Fig. 3D) when the anti-B-50 antibodies were diluted to the usual applied concentration (1 : 3,000). However, in addition, with less (1 : 300) diluted anti-B-50 antibodies B-50 immunoreactivity could be readily detected in the processes of the Schwann cells at the neuromuscular junctions in controls (Fig. 2D; arrowheads) and in intramuscular nerves (data not shown). Under these conditions hardly any B-50 immunoreactivity could be detected in the nerve terminals (t) (Fig. 2D).

Four days after facial nerve crush motor endplates had lost all nerve terminals (Fig. 3B,E; see arrows indicating vacant postsynaptic junctions). In correspondence

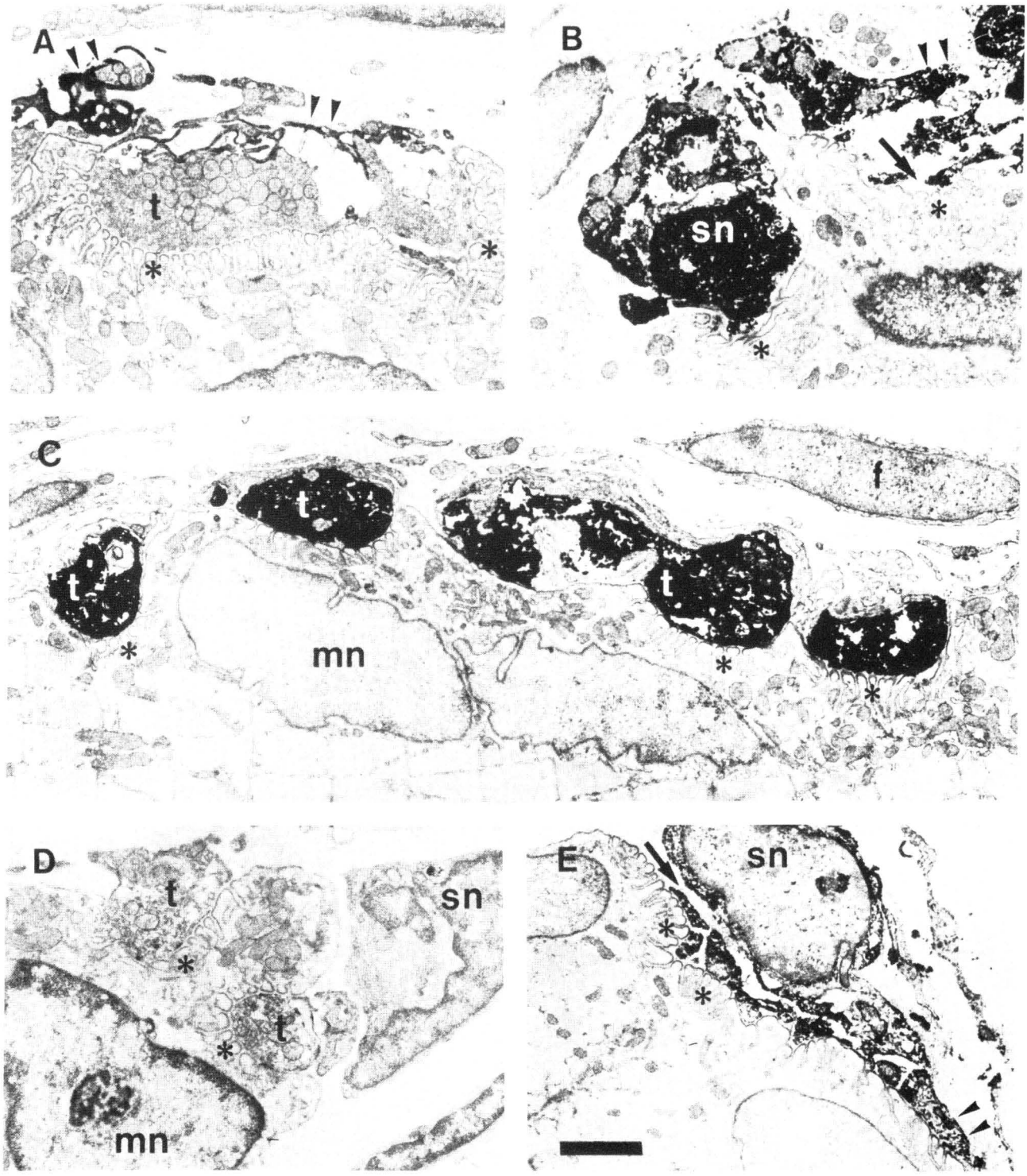

Fig. 3. Electron micrographs of the motor endplates of control (A,C,D) and denervated (B,E) muscle 4 days after crush. The Schwann cell and its processes (arrowheads), characterized by S100 immunoreactivity (A,B), cover the presynaptic axon terminal (t) opposed to the folded postsynaptic membrane (asterisks) in the muscle. Presynaptic axon terminals were stained by monoclonal antibodies to synaptophysin (C) resulting in reaction deposits around the synaptic vesicles inside the terminals. Hardly any B-50 immunoreactivity (D) was detected at the control neuromuscular junction. Four days post crush (B,E) motor endplates are devoid of axon terminals (see arrows indicating these vacant sites). These sites were covered by S100 (B) and B-50 (E) positive Schwann cells extending processes along the postsynaptic folds. Bar = 2 μm. (mn: muscle nucleus, sn: Schwann cell nucleus, f: fibroblast.)

with this absence antibodies to synaptophysin yielded no immunostaining at the neuromuscular junctions (data not shown). Opposed directly against the folded postsynaptic membrane (asterisks) of the muscle fibres S100-positive Schwann cells were located extending processes along the region vacated by nerve terminals and the endplate areas. The cytosol, nucleus and processes of the Schwann cells contained the reaction product of the immunoperoxidase staining by anti-S100 antibodies (Fig. 3B). B-50 immunoreactivity (Fig. 3E) was detected as patchy deposits in processes and the cytosol of the Schwann cells. Similar results were obtained with polyclonal and monoclonal anti-B-50 antibodies. In addition, B-50 immunoreactivity was detected in Schwann cells in the intramuscular nerve fascicles (results not shown).

B-50 immunoreactivity in non-denervated tissue

In undamaged facial nerve tissue little B-50 immunoreactivity was detected by 1 : 3,000 diluted anti-B-50 antibodies. With this commonly used dilution of anti-B-50 antibodies sympathetic nerves innervating blood vessel walls were strongly positive (Fig. 4A). Immunostaining with anti-synaptophysin antibodies of blood vessels revealed a similar staining pattern as that of the anti-B-50 antibodies (data not shown). Ultrastructural studies showed that nerve varicosities/endings of the sympathetic nerves in blood vessel walls were rich in B-50 immunoreactivity (Fig. 4B).

B-50 immunoreactivity in the motoneurons of the facial nucleus

Immunofluorescence studies of the motoneuron cell bodies of crushed facial nerve demonstrated slightly increased B-50 fluorescence (Fig. 5B) in comparison to that detected in the cell bodies at the contralateral side (Fig. 5A). Visual inspection of micrographs of the injured and intact facial nucleus revealed no clear-cut differences in B-50 immunoreactivity in the neuropil and in the perineuronal glial cells of the neuropil.

Northern blot analysis

To examine whether B-50 was synthesized by Schwann cells and other non-neuronal cells mRNA was extracted from samples of control and denervated tissue on day 4 after crush and analyzed by Northern blotting for the presence of B-50 mRNA (Fig. 6). In the denervated levator labii superioris muscles (Fig. 6, lane b) a four times higher level of B-50 mRNA was determined by Northern blotting compared to the control muscles (Fig. 6, lane a). Samples taken from the distal region of the degenerated facial nerve (Fig. 6, lane d) contained twice as much B-50 mRNA in comparison to samples from control rats (Fig. 6, lane c). The highest levels of B-50 mRNA were found in the facial nucleus (Fig. 6, lane e,f). The facial nucleus containing the cell bodies of the intact and injured nerves (Fig. 6, lane e, f) contained much more B-50 mRNA than the samples of nerve and

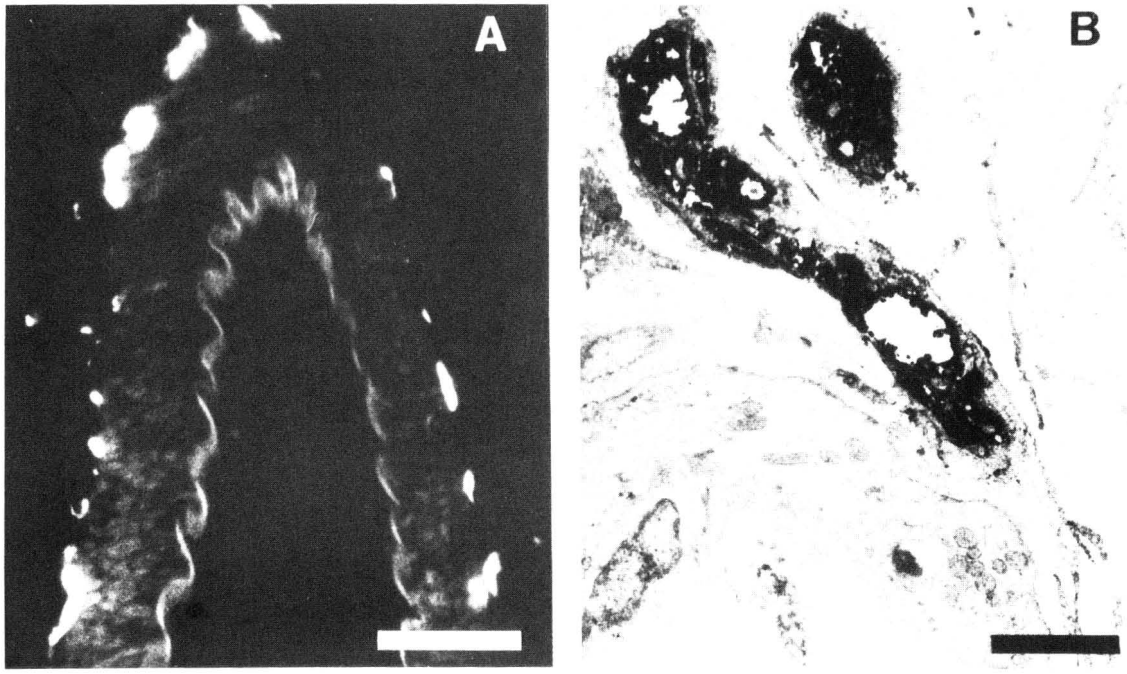

Fig. 4. Light (A) and electron (B) micrograph of B-50 immunoreactivity in sympathetic nerves innervating blood vessel walls. The B-50 immunostaining is concentrated in the nerves (A) and in the nerve varicosities/endings (B). In 3A, bar = 50 μm. In 3B, bar = 2 μm.

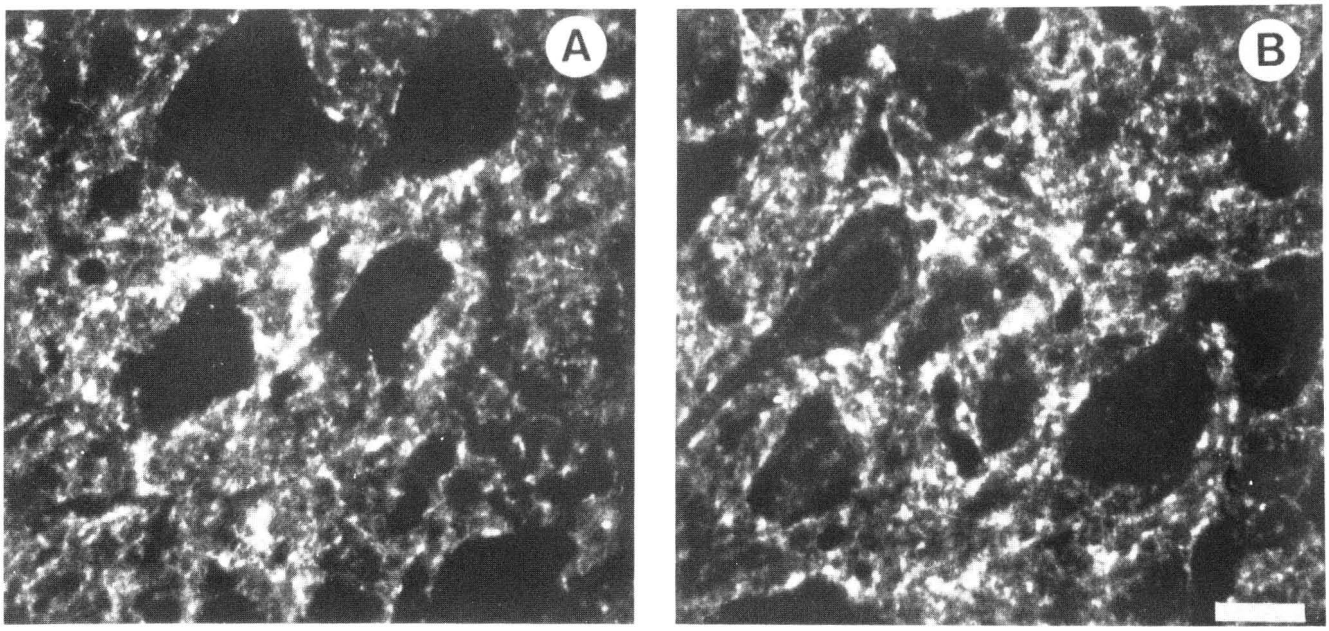

Fig. 5. B-50 expression in motoneurons of the intact (A) and crushed (B) facial nucleus 4 days post nerve injury. Note the slightly increased B-50 immunofluorescence (B) in the cytoplasm of the ipsilateral motoneurons in comparison to the intact contralateral side (A). B-50 immunofluorescence in the neuropil was not different for both conditions. Bar = 20 μm.

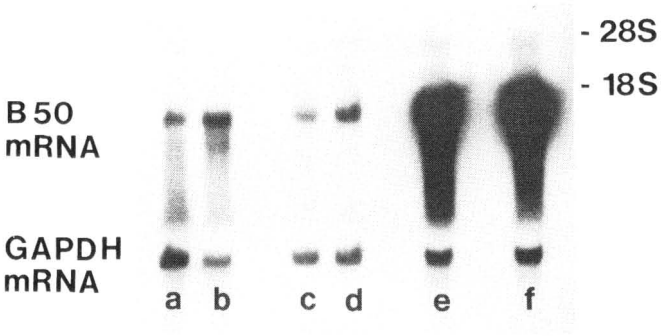

Fig. 6. Northern blot. *Upper row:* the denervated levator labii superioris muscles (lane b) contained a four times higher level of B-50 mRNA than control muscles (lane a). In the distal region of the degenerated facial nerves (lane d) a two times higher level of B-50 mRNA was determined compared to control nerves (lane c). Compared to the facial nuclei containing the motoneuron cell bodies of intact (lane e) and injured nerves (lane f) the non-neuronal cells (lane a,b,c,d) contained very low levels of B-50 mRNA. For quantification of the facial nucleus (lane e and f) a much shorter exposure time of the film to the blot was used. The level of B-50 mRNA in the facial nucleus at the crushed side (lane f) was twice as high compared to the control side (lane e). *Lower row:* To control for variation in sample loading the same mRNA blot was reprobed with cDNA encoding GAPDH. The levels of B-50 mRNA (approximately 1,400 bp, upper row) were standardized against the corresponding levels of GAPDH mRNA (approximately 1,300 bp, lower row). The bands of B-50 mRNA and GAPDH mRNA were located just below the 18 S ribosomal bands. The experiment was repeated twice.

muscle, both intact or injured. The B-50 mRNA of the facial nucleus was quantified by exposing films on the same Northern blot for much shorter times than that shown in Fig. 6. The B-50 mRNA of the facial nucleus at the crushed side was increased twice with respect to that of the control side. This analysis showed that, comparatively, the injured tissues had an increased B-50 mRNA expression above that of the uninjured tissues. In addition, it was demonstrated that the tissues containing neuronal cell bodies express much more B-50 mRNA than those comprising solely non-neuronal cells.

Discussion

Demonstration of B-50 protein in Schwann cells at denervated endplates

The aim of the present study was to clarify the origin of extraneuronal B-50 immunoreactivity following nerve injury [67]. The traumatized facial nerve of rat was used as experimental model. In particular the following question was studied: in which cellular compartment at the neuromuscular junction can B-50 immunoreactivity be demonstrated.

Four days after peripheral facial nerve crush, increased B-50 immunoreactivity was observed in S100-positive Schwann cells covering the denervated postsynaptic membranes of the neuromuscular junctions. At the ultrastructural level the localization of B-50 was confirmed to be present in the Schwann cells. This implies that the previously observed B-50 immunoreactivity at motor endplates in the denervated muscle by Verhaagen et al. [67] can be explained by the presence of B-50 in Schwann cells covering denervated endplates.

As a control of undamaged nerve in the same tissue we demonstrate that the injury was restricted to the facial nerve and not to unrelated nerves. We examined at both light and electron microscopic level B-50 immunoreactivity of the non-denervated sympathetic axons and nerve terminals innervating blood vessels and in skeletal muscle fibres. Indeed, we found no changes in B-50 immunoreactivity of the B-50-positive nerve terminals innervating capillaries and of B-50 negative muscle fibres. The presence of B-50 immunoreactivity in sympathetic nerves is consistent with the findings of Sharkey et al. [47] and, recently, Stewart et al. [53]. They reported that by light microscopy GAP-43/B-50 immunoreactivity is detected in various nerves of the autonomic nervous system.

With a dilution of 1:3,000 only a few weak B-50 positive Schwann cells were observed in the control tissue. Four days after nerve injury B-50 immunoreactivity was readily detectable in Schwann cells at the denervated endplates. This suggests that B-50 is upregulated in activated Schwann cells. In contrast, when we used concentrated anti-B-50 antibodies (dilution 1:300) we detected in control tissue specific B-50 immunostaining not only in intramuscular nerves, but also in most Schwann cells at intact motor endplates. Therefore, we suggest that a very low basal B-50 expression occurs in Schwann cells which appears to be upregulated under conditions of nerve injury resulting in loss of neuronal contact. The suggestion of upregulation of B-50 expression in Schwann cells is supported, though not proven (but see below, Woolf et al. [69]), by our findings of increased B-50 mRNA. However, since in the muscle only Schwann cells and intact sympathetic nerves were sources of B-50 immunoreactivity, we are confident that increased mRNA detected on a Northern blot indicates an increased expression of B-50 mRNA in Schwann cells associated with the endplates.

Neuronal expression of B-50

In addition, we have studied the effect of facial nerve crush, at four days post surgery, on the motoneuron cell bodies. After facial nerve crush, the motoneuron cell bodies lose contact with the target and with afferent synapses which are pushed aside by perineuronal microglial cells [9]. The affected motoneuron cell bodies showed an increase of B-50 expression. Cell bodies of injured peripheral nerves re-express B-50 as a part of their axonal regeneration program [61]. At the crushed side motoneuron cell bodies display elevated B-50 immunoreactivity in comparison to those in the contralateral intact facial nucleus. Northern blotting of the facial nucleus, containing motoneuron cell bodies and the neuropil, shows a two-fold increase in B-50 mRNA after nerve crush. The tissues containing the motoneuron cell bodies express much more B-50 mRNA than the nerve and muscle tissues comprising solely non-neuronal cells.

Non-neuronal expression of B-50

Recent papers have cast doubt on the neuron-specificity of GAP-43/B-50. Verhaagen et al. [67] observed an increase of B-50 immunoreactivity at denervated neuromuscular junctions two/three days following nerve crush or transection. As explanation for this finding the authors hypothesized an accumulation of B-50 protein at denervated motor endplates or phagocytosis of axonal debris by non-neuronal cells. Tetzlaff et al. [62] reported B-50 immunoreactivity distal to a peripheral nerve lesion in Schwann cells. Campbell et al. [10] performed a study of peripheral nerve grafts in the central nervous system and detected B-50 immunoreactivity on the Schwann cell plasma membrane. Both authors suggested that the B-50 protein was axonally derived. Recently, it has been shown that Schwann cells express GAP-43/B-50 when they lose axonal contact [17,70]. Mature myelin-forming Schwann cells in adult rats do not express B-50 [17]. Nonmyelin-forming Schwann cells grown in tissue culture from fetal and neonatal rat nervous tissue and in vivo, e.g. in normal adult rat sciatic nerve, display B-50 immunoreactivity and synthesize GAP-43/B-50 [17]. B-50 immunoreactivity in oligodendrocytes decreases during differentiation in vitro [20] and during development in rat brain tissue in vivo [16]. Normal and reactive astrocytes did not express B-50 immunoreactivity [16]. While we were completing our manuscript, a paper by Woolf et al. [69] appeared. This showed by immuno light microscopy that after sciatic nerve lesion nonmyelin-forming terminal Schwann cells at denervated motor endplates of the soleus muscle demonstrate B-50 immunoreactivity in their processes. Moreover, GAP-43 mRNA detected by in situ hybridisation was located in a number of the Schwann cells at the denervated soleus muscle [69] and in the Schwann cells of a degenerating sciatic nerve [42]. Our findings on B-50 protein and B-50 mRNA after facial nerve crush during the initial stage of Wallerian nerve degeneration are consistent with these observations and confirm at both light and electron microscopic level that B-50 is localized in Schwann cells at denervated motor endplates of the levator labii superioris muscle. Hall et al. [23] demonstrated very recently that at the ultrastructural level GAP-43 immunoreactivity is localized in denervated Schwann cells of the distal stump of transected rat sciatic nerve. Other studies indicate that non-neuronal B-50 expression (mRNA and protein) in the denervated distal nerve stump increases with time following peripheral nerve transection [42,70]. When reinnervation

occurs, e.g. after nerve crush, expression of B-50 mRNA returns to control levels of the intact nerve [42,70].

Neuron–glia interaction

After peripheral or central nerve injury B-50 was not observed in proliferating astroglia in brain tissue [16]. However, non-neuronal cells in culture are capable to express B-50. B-50 was shown to be present in cultured O-2A progenitor cells and in their differentiated offspring, type 2 astrocytes [18] and oligodendrocytes [16,20] in which B-50 is downregulated as they mature. Thus, glial cells without neuronal contact apparently do express B-50. During development of human intramuscular nerves B-50 immunoreactivity was not observed in Schwann cells having axonal contact [24]. We have detected only little B-50 in Schwann cells when in contact with intramuscular axons or nerve terminals and observed that de-differentiated Schwann cells at denervated endplates increase their B-50 expression.

De-differentiation of Schwann cells by loss of neuronal contact

Schwann cells respond to nerve injury by dramatic changes in gene expression, resulting in activation, proliferation and if myelin-forming then in return to the state of nonmyelination [27,44,54]. After loss of axonal contact, for example, synthesis of NGF, low-affinity NGF receptors [25,26,60] and cell surface molecules like N-CAM and L1 are upregulated [27,44,59]. The expression of proteins involved in myelin production, e.g. P30, Po and myelin associated glycoprotein is downregulated [59,63]. The altered gene expression in proliferating Schwann cells resembles in many ways the behaviour of these cells grown in culture [25,26,30,35]. In this study, an increase of S100 in the cytosol and nucleus of activated nonmyelin-forming Schwann cells was observed together with the elevated immunoreactivity of B-50. The here observed elevation of B-50 mRNA in denervated muscle and of B-50 protein in Schwann cells which have lost contact with nerve endings is in line with reported altered gene expression of Schwann cells after nerve injury.

Reynolds and Woolf [45] observed that the nonmyelin forming terminal Schwann cells at the muscle endplate elaborate extensive processes following denervation of the muscle. There appears to be, during extensive denervation (nerve transection), a relation between the further elongation of Schwann cell processes noticed by Reynolds and Woolf [45] and increase in GAP-43 observed by Woolf et al. [69] and Curtis et al. [17]. When reinnervation occurs B-50 levels drop and Schwann cell processes retract.

In non-neuronal cells B-50 may interact with actin filaments and promote transiently the formation of filopodia or process-like extensions [31,57,68,71]. B-50 appears to influence the cell surface behavior (shape and morphology) and its phosphorylation modulates its activity [57,68]. This would suggest that B-50 could be functionally involved in outgrowth of Schwann cell processes comparable to its role in axonal outgrowth.

Conclusion

We demonstrated both at light and electron microscopic level that low basal B-50 immunoreactivity was present in Schwann cells at the intact motor endplate. B-50 immunoreactivity in Schwann cells was elevated after denervation. Therefore, it appears that B-50 expression is upregulated in glia cells, when glia have lost neuronal contact in vivo and when glia develop without neuronal contact in vitro [17].

Further studies are needed to elucidate the stimuli regulating the B-50 expression during neurodegeneration and regeneration. Our results indicate that the dynamic state of the neural tissue in the animal regulates B-50 expression.

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Abbreviations

AChE	acetylcholine esterase
FITC	fluorescein isothiocyanate
GAP	growth associated protein
NGF	nerve growth factor
PBS	phosphate buffered saline
PKC	protein kinase C
SSC	standard saline citrate
TRITC	trimethylrhodamine isothiocyanate

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