THE JOURNAL OF COMPARATIVE NEUROLOGY 462:315–327 (2003)

Regeneration of Myenteric Plexus in the Mouse Colon after Experimental Denervation with Benzalkonium Chloride

MENACHEM HANANI,[1]* OREN LEDDER,[1] VLADIMIR YUTKIN,[1] RIBHI ABU-DALU,[1] TIAN-YING HUANG,[1] WOLFGANG HÄRTIG,[2] MARIA-GIULIANA VANNUCCHI,[3] AND MARIA-SIMONETTA FAUSSONE-PELLEGRINI[3]

[1]Laboratory of Experimental Surgery, Hadassah University Hospital, Mount Scopus, Jerusalem 91240, Israel

[2]Department of Neurochemistry, P. Flechsig Institute for Brain Research, University of Leipzig, Leipzig, D-04109 Germany

[3]Department of Anatomy and Forensic Medicine, University of Florence, I-50139 Florence, Italy

ABSTRACT

Recent reports suggest a far greater plasticity in nerve tissue than previously believed. As the digestive tract is exposed to a variety of insults, this question is relevant to enteric nerves, but little is known about their ability to recover from damage. To address this problem, we ablated the myenteric plexus of the mouse colon with the detergent benzalkonium chloride (BAC) and followed the ensuing morphologic changes for up to 60 days by using light- and electron microscopy. We found that, 2 days after BAC application, the treated area was essentially devoid of intact nerve elements. From day 7, new nerve fibers were observed within the denervated region. This growth progressed until, at days 30–60, newly grown nerve fibers were present in most of this region, and the pattern of muscle innervation was similar to the normal one. At least part of these fibers originated at neurons within intact ganglia surrounding the denervated region. The cross-sectional area of neurons near the denervated region at day 14 was 52% greater than controls. Glial cells were closely associated with the regenerating nerve fibers. From day 14 onward, we observed undifferentiated cells and differentiating neurons in ganglia surrounding the denervated region, and by day 30, new neurons were present in the myenteric region, along with regenerating nerve fibers. We conclude that the myenteric plexus is endowed with a considerable ability of regeneration and plasticity. The results provide evidence for the presence of stem cells and for an adult neurogenesis in this plexus. J. Comp. Neurol. 462:315–327, 2003.

Indexing terms: enteric nervous system; neuro-degeneration; neuro-regeneration; neuronal plasticity; ultrastructure; neuronal stem cells

Research in recent years revealed that the central nervous system has a considerable ability to regenerate. There is evidence that neural stem cells are present in the adult brain and that they are able to differentiate and generate new neurons (Reynolds and Weiss, 1992; Temple, 1999; van Praag, 2002). In the adult myenteric plexus, the persistence of undifferentiated or poorly differentiated cells capable of turning into neurons has been postulated (Benninghoff, 1951; Filogamo and Vigliani, 1954; Giacobini-Robecchi et al., 1985). This hypothesis was based on a marked increase in number of myenteric neurons in animals in which a stenosis of the small intestine was induced. Thus, it is possible that cells, probably residing in myenteric ganglia and lacking some typical aspects of nerve cells, might differentiate into neurons under

Grant sponsor: US-Israel Binational Science Foundation; Grant number: BSF, 98-00185.

*Correspondence to: Menachem Hanani, Laboratory of Experimental Surgery, Hadassah, University Hospital, Mount Scopus, Jerusalem 91240, Israel. E-mail: hananim@cc.huji.ac.il

Received 11 September 2002; Revised 22 January 2003; Accepted 10 March 2003

DOI 10.1002/cne.10721

Published online the week of June 9, 2003 in Wiley InterScience (www.interscience.wiley.com).

certain conditions. Definite evidence for the presence of stem cells in the postnatal gut was recently obtained by Kruger et al. (2002), who used flow cytometry to purify the cells.

As the enteric nervous system (ENS) is exposed to a variety of chemical and mechanical insults, as well as to foreign organisms, the question of plasticity and regeneration of enteric nerves is highly relevant. Alterations of the ENS due to disease, age, or other conditions lead to impairment of gastrointestinal function (De Giorgio et al., 2000). An effective way to ablate the ENS is to apply the detergent benzalkonium chloride (BAC) to the serosal surface of the large (Sato et al., 1978) or small intestine (Fox and Bass, 1984; Holle and Forth, 1990; Ramalho et al., 1993; Luck et al., 1993; Parr and Sharkey, 1997). This ablation is accompanied with increased muscle thickness (See et al., 1990; Holle, 1991, 1998; Zucoloto et al., 1991; Hadzijahic et al., 1993) and mucosal hypertrophy and hyperplasia (See et al., 1988; Holle, 1991; Hadzijahic et al., 1993). Functional changes in the smooth muscle were also noted (Herman and Bass, 1987; Fox and Bass, 1993; Osinski and Bass, 1995). Despite this important effort, the question of neuronal regeneration after BAC treatment received little attention. It has been reported that, after BAC treatment, nerve fibers regenerated within the circular muscle and that their source may be the submucosal plexus (Luck et al., 1993). Ramalho et al. (1993) described an initial reduction in the number of neurons in the treated region, followed by an increase.

In this study, we address questions on regeneration and plasticity in myenteric plexus after its destruction. We applied BAC on the serosal surface of a restricted area of mouse colon to destroy the myenteric plexus and performed staining methods appropriate for following neuronal and glial re-growth and to determine the site of origin of the new nerve fibers. By using electron microscopy, we inspected the presence of stem cells and differentiating neurons.

MATERIALS AND METHODS

Denervation procedure

The experimental protocols were approved by the Animal Care and Ethics Committee of the Hebrew University-Hadassah Medical School and follow NIH guidelines. One hundred forty-five young adult Balb/C mice (17–22 g) of either sex were used for the denervation experiments, and an additional 50 mice served as controls. For each staining method and time point after treatment, the animals were divided into groups of four to eight mice. Animals were anesthetized with 45 mg/kg pentobarbitone (Abbott Laboratories, N. Chicago, IL), and the descending colon was exteriorized through a 1-cm midline incision. Cotton wool soaked in 0.05% BAC in Krebs solution was wrapped around the exteriorized colon through a small hole in the mesentery and left for 20–30 minutes, after which the treated area and the peritoneum were thoroughly flushed with Krebs solution. The wound was then sutured in two layers. The Krebs solution had the following composition (in mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, NaH_2PO_4 1.5, $NaHCO_3$ 14.5, glucose 11.5, $CaCl_2$ 2.5.

The animals were killed by cervical dislocation at 2, 7, 14, 30, and 60 days after BAC treatment. The distal colon including the treated area was resected from the animal, opened along the mesenteric border, stretched, and pinned flat in a dish with a Sylgard (Dow Corning) bottom in Krebs solution; the mucosa and most of the submucosa were dissected away. The resulting preparation, consisting mainly of the external muscle and the myenteric plexus, was used for all the staining procedures.

Staining methods

Staining for reduced nicotinamide adenine dinucleotide phosphate–diaphorase (NADPH-d) was done as described previously (Hanani et al., 1995). Briefly, the tissues were fixed for 30 minutes in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4), rinsed, and then incubated at 37°C in 0.1 M Tris containing 0.3% Triton-X 100, pH 7.4, for 20 minutes. The specimens were then incubated in the same solution containing 0.2 mg/ml nitroblue tetrazolium and 0.5 mg/ml NADPH (both from Sigma, St. Louis, MO) for 30 minutes. The tissues were photographed with a digital camera (Pixera 120es, Pixera Corp., Los Gatos, CA) and mounted on an Axioskop microscope (Zeiss, Jena, Germany). The regenerated nerve fibers were traced from the NADPH-d–stained specimens by using camera lucida.

To label the neuronal components, we used a monoclonal mouse antibody directed against protein gene product 9.5 (PGP9.5, Ultraclone, Isle of Wight, UK) diluted 1:200. A polyclonal rabbit antiserum directed against S100β (East Acres, Southbridge, MA) diluted 1:3,000 was used to label glial cells. The staining method was similar for all the antibodies used. The colon was dissected as described above. The specimen was fixed in 4% paraformaldehyde in PBS for 4 hours. The tissue was rinsed three times with 0.1 M Tris-buffered saline, pH 7.4 (TBS), and a blocking solution (5% normal goat serum in TBS containing 0.3% Triton X-100) was added. A mixture of both primary antibodies (diluted in the blocking solution) was applied to the tissues at room temperature for 24 hours. After rinsing with TBS, the tissue was processed with carbocyanine (Cy)3-tagged goat anti-rabbit immunoglobulin (Ig) G and Cy2-conjugated goat anti-mouse IgG (both from Dianova, Hamburg, Germany, 20 μg/ml) for 1 hour. Finally, the tissue was rinsed with TBS, briefly washed with distilled water, mounted onto fluorescence-free slides, air-dried, and cover-slipped with Entellan (Merck, Darmstadt, Germany).

Electron microscopy

For electron microscopy, three animals were used for each time point and three for control. The tissue was fixed for 4 hours in buffered glutaraldehyde (1.6% in 0.05 M cacodylate buffer, pH 7.4). After four rinses in the cacodylate-buffered solution containing 0.22 M sucrose, full-thickness strips, 10 × 3 mm, containing the denervated area and its normal surrounding region were post-fixed with 1% phosphate-buffered OsO_4 (pH 7.4), dehydrated with acetone, and embedded in Epon using flat molds. Semithin sections, obtained with a LKB NOVA ultramicrotome, were stained with a solution of toluidine blue in 0.1 M borate buffer and then observed under a light microscope. Ultrathin sections of the selected areas were obtained with the LKB NOVA ultramicrotome by using a diamond knife and stained with an alcoholic solution of uranyl acetate, followed by a solution of concentrated bismuth subnitrate. These sections were examined under a JEOL 1010 electron microscope.

Morphometry and statistical analysis

The cross-sectional area of NADPH-d–stained neurons was measured in whole-mount preparations by using

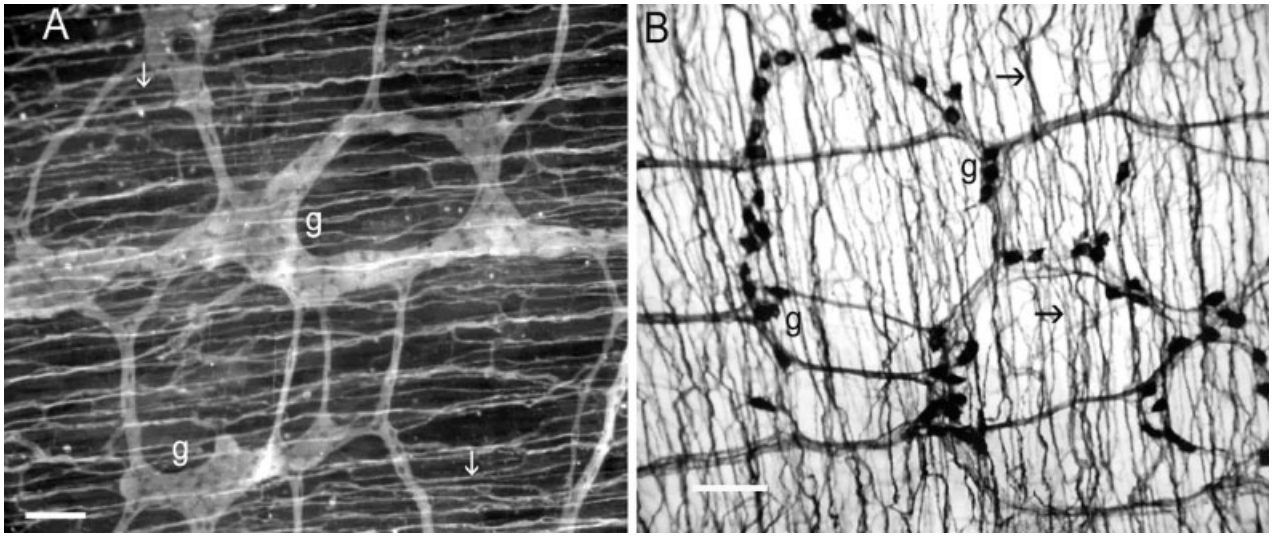

Fig. 1. The normal morphology of the myenteric plexus in the mouse distal colon. **A:** Immunostaining for the neuronal marker PGP9.5. **B:** Histochemical staining for nicotinamide adenine dinucleotide phosphate–diaphorase. The arrows indicate nerve bundles, and g, individual ganglia. Scale bars = 100 μm in A,B.

Image-Pro Plus software (Media Cybernetics, Rockville, MD). Tissues from two mice, 14 days after BAC treatment, were used. The areas of 227 neurons from within 100 μm from the treated area were measured. Two types of control were used, ganglia from two treated animals, approximately 1 cm from the lesioned regions (239 neurons measured), and ganglia from two Krebs-treated animals (228 neurons). Analysis of variance or *t* tests was used to compare means; $P < 0.05$ was considered statistically significant.

RESULTS

Macroscopic observations

Two days after BAC treatment, the descending colon was narrower than the rest of the colon, and also in comparison with the corresponding region in control animals. It was always empty, whereas the proximal part of the colon was dilated and packed with fecal material. This dilation was less prominent at longer periods after the BAC treatments. In general, in all the series, the animals did not appear ill during the first few days after BAC application, consistent with the observation that the BAC treatment did not lead to complete colonic obstruction.

Microscopic observations

Phase 1: neurodegeneration. The basic observations on myenteric plexus changes were made by staining for PGP9.5, and further information was obtained by staining for NADPH-d. In control animals, observed 2–30 days after the operation, the myenteric ganglia and the innervation of the muscle appeared identical to those observed in untreated animals. The ganglia were packed with PGP9.5-immunopositive neurons, and the internodal strands and intramuscular nerve fibers were clearly stained (Fig. 1A). A smaller number of neurons was observed when staining for NADPH-d, as only nitric oxide–producing ones are stained by this method, but individual neurons and nerve fibers could be seen in greater detail (Fig. 1B).

The denervated area was usually elliptical, and its size varied from 2.7 × 2.2 mm to 5.5 × 11.0 mm. Two days after BAC application, this area appeared by using staining for PGP9.5, to contain damaged ganglia that were filled with cell debris (Fig. 2A). At the edges of the treated area, there were abnormally looking nerve fibers and somata (Fig. 2B). With NADPH-d staining, the damaged area appeared virtually empty of any nerve, and even the cellular debris seen with PGP9.5 staining were not observed, probably because of loss of the enzymatic activity (Fig. 2C,D). With both staining methods, normally appearing neurons were absent within the treated area. Between the denervated and the normal regions, there were one to two rows of ganglia, which we termed the "transition zone." This zone contained cell debris, damaged neurons, and nerve fibers, as well as a variable number of normally appearing neurons. Beyond the transition zone ganglia appeared normal (Fig. 2C,D). The lack of S100β-immunoreactivity in the lesioned area indicated the absence of glial cells.

Electron-microscopic examination verified that, 2 days after BAC application, there was only neuronal and glial cell debris in the treated area (Fig. 3A). In the transition zone, in addition to neuronal debris and normal neurons, there were neurons with swollen mitochondria, dilated cisternae of the rough endoplasmic reticulum (RER), and electron-dense cytoplasm (Fig. 3B). In deeper regions of the tissue, such as the submucosa and the adjacent circular muscle layer, where BAC did not fully penetrate, and also in the transition zone, some nerves were still present but most of the nerve endings were altered: some of them were empty, some others contained a floccular material, and others contained swollen mitochondria and/or bodies containing sequestered, degenerating synaptic vesicles (Fig. 3C). Glial cells were rich in rough cisternae and

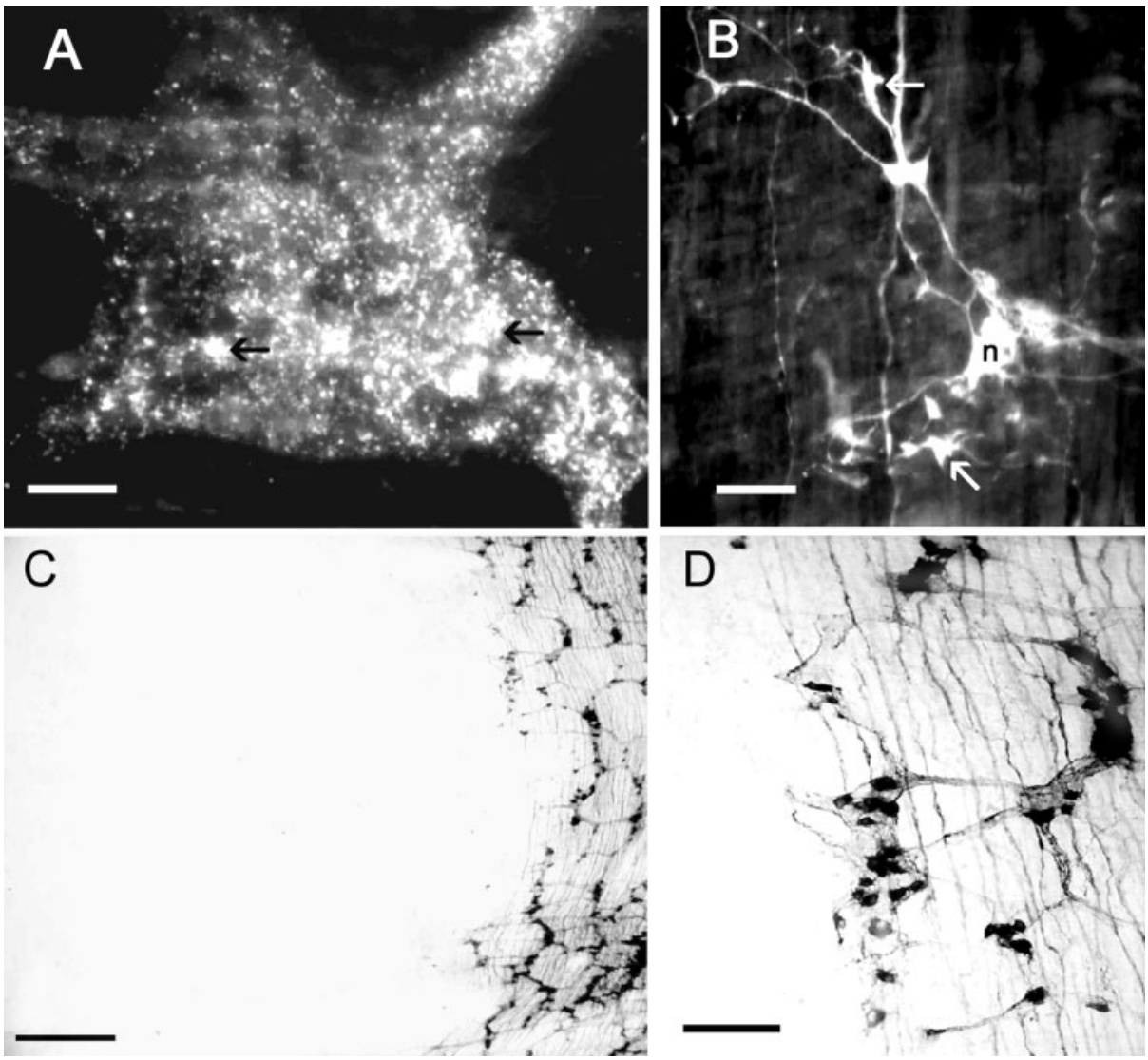

Fig. 2. The appearance of the colonic myenteric plexus 2 days after treatment with benzalkonium chloride. **A:** Myenteric ganglion within the denervated area seen with PGP9.5 staining. The ganglion is filled with cell debris, and the arrows indicate the remains of neurons. **B:** Neurons and nerve fibers in the transition zone, stained for PGP9.5; n indicates a neuron, and the arrows point to swollen nerve fibers. **C:** Low-magnification photomicrograph showing a large denervated area, and to its right the transition zone and normal ganglia. Nicotinamide adenine dinucleotide phosphate–diaphorase (NADPH-d) staining. **D:** A higher magnification photomicrograph showing the transition zone and the denervated area. NADPH-d staining. Scale bars = 25 μm in A,B, 400 μm in C, 100 μm in D.

lysosomes (Fig. 3A,C). In areas where nerve fibers were missing, glial cells were also absent.

Two days after BAC application, inflammatory cells (macrophages, granulocytes) accumulated at the serosal surface of the lesioned area and were also close to the degenerating/degenerated nerve structures (Fig. 3A,C). From day 7, these cells were markedly reduced in number and were practically absent at later times, either at the myenteric plexus level or intramuscularly. The regenerating nerve structures never showed close relationships with such cells.

Phase 2: neuroregeneration. At 7 days after BAC treatment, light microscopy revealed a clear growth of nerve fibers from the undamaged area into the denervated one (Fig. 4). Nerve fibers grew in the directions of either the circular or longitudinal muscle cells, but the former predominated. Many of the growing fibers had club-like structures in their tips (Fig. 4), similar to growth cones described in various developing neural systems (Jacobson, 1991). Most of the fibers grew in a straight line, but there were cases where they changed courses, mainly turning in 90 degrees (Fig. 5A). Staining the same tissues for nerve and glial cells by using antibodies for PGP9.5 and S100β, respectively, demonstrated that regions where nerve growth took place contained elongated glial cells that were usually associated with growing nerve fibers. Somata of glial cells were commonly present at points where nerve fibers branched or changed the direction of growth (not shown).

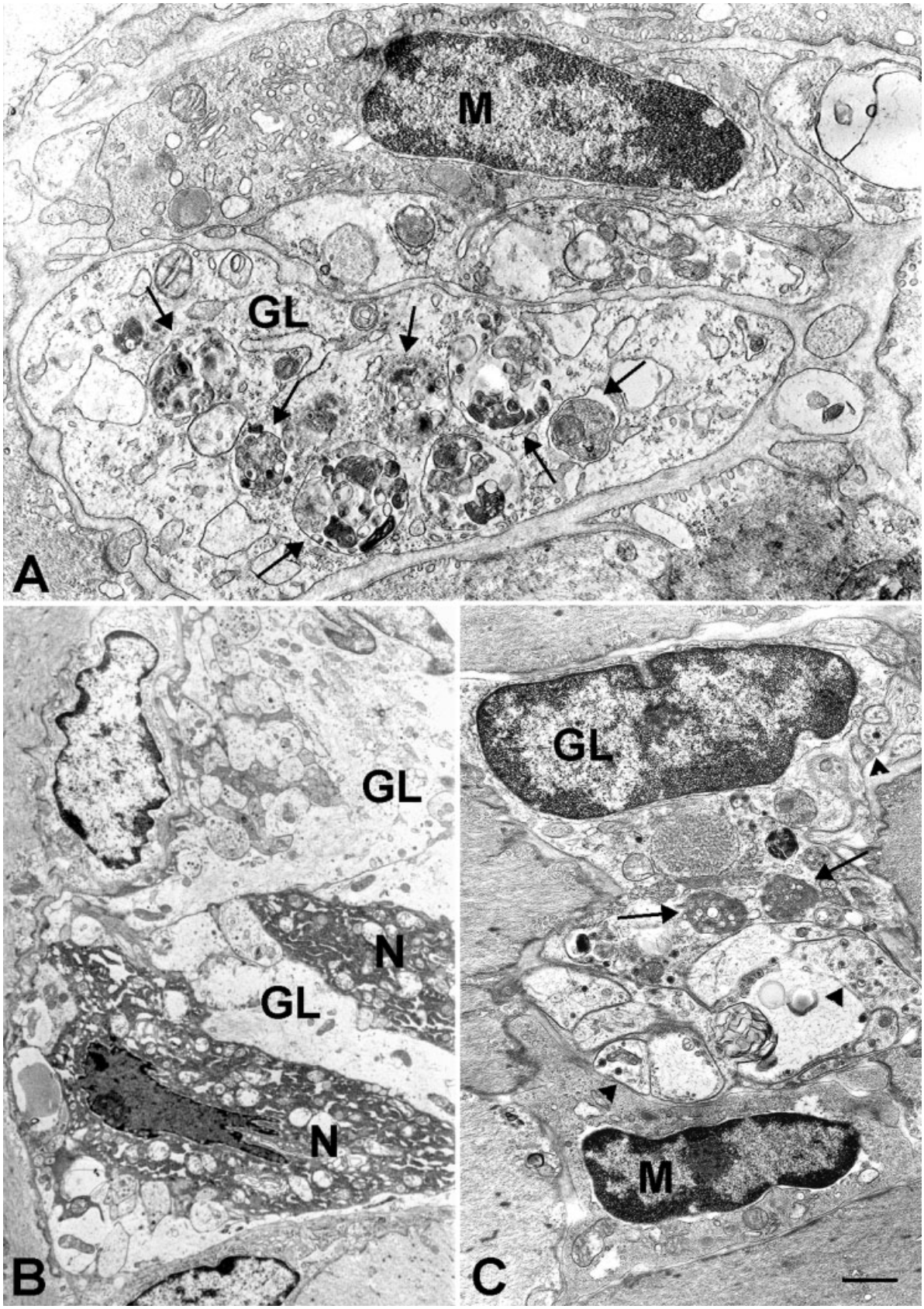

Fig. 3. Two days after benzalkonium chloride (BAC) treatment. Transmission electron microscopy. **A:** Remnants of a degenerating nerve fiber at the myenteric plexus level in the treated area. All of the axons (arrows) are degenerated, and the enveloping glial cell (GL) has vacuoles, lysosomes, and swollen mitochondria. A macrophage (M) is located close to the degenerating nerve fiber. **B:** A myenteric ganglion in the transition zone. Neurons (N) have a dense cytoplasm, dilated rough endoplasmic reticulum cisternae and swollen mitochondria. Glial cells (GL) have a clear, empty cytoplasm. **C:** An intramuscular nerve fiber in the transition zone. Most of the axons (arrows) are degenerated and the enveloping glial cell (GL) has several lysosomes. Arrowheads indicate axons with apparently normal features. A macrophage (M) is located close to the degenerating nerve fiber. Scale bar in C = 0.5 μm in A, 1.5 μm in B, 0.6 μm in C.

Because of the high density of nerve fibers, it was impossible to trace all individual fibers for long distances; however, in many cases, we could follow fibers from the denervated area to myenteric neurons in the transition zone. Figure 5A shows such an example in a tissue 7 days after BAC. This point was investigated further by drawing the nerves with a camera lucida, which again demonstrated that these fibers originated in neuronal cell bodies in the transition zone (Fig. 5B). At day 14, the growing fibers were considerably longer and they extended into much of the denervated area (Fig. 6A,B). The degree of re-innervation depended on the size of the denervated area. When this area was small (less than 3×3 mm), the whole denervated region was filled with nerve fibers, but when it was larger than approximately 5×5 mm, its central portion did not contain nerve fibers, as seen in Figure 6B. The extent of nerve fiber growth was assessed quantitatively by measuring the density of the NADPH-d–positive nerve fibers in the edge (Fig. 7A) and center (Fig. 7B) of the denervated area.

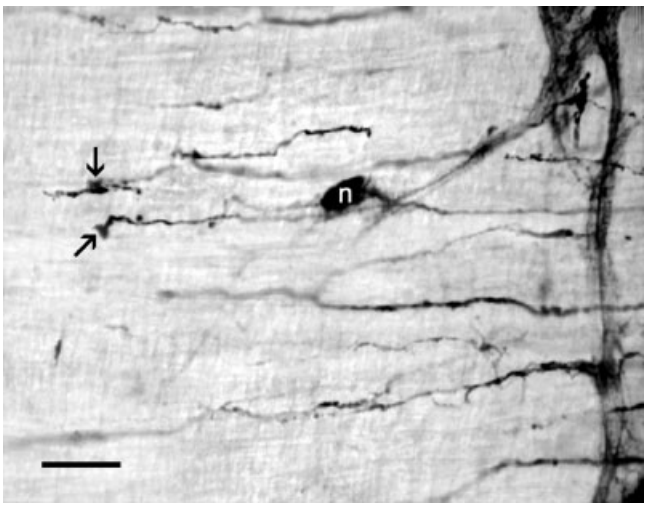

Fig. 4. Changes in the pattern of nerves in the denervated region 7 days after benzalkonium chloride treatment. Nicotinamide adenine dinucleotide phosphate–diaphorase staining. An outgrowth of nerve fibers is seen to arise from the transition zone (on the right side). Some of the growing fibers have club-like thickenings on their tips (arrows). n indicates a neuron. Scale bar = 50 μm.

Electron microscopic examination showed that, beginning at 7 days after BAC treatment, the remnants of nerve tissue in the denervated myenteric plexus area disappeared progressively. In the transition zone, neurons showing altered features, such as electron-dense cytoplasm, dark nucleus, swollen mitochondria, and dilated cisternae of the RER, were rarely seen. By day 14, most of the neurons in the transition zone appeared normal, and some showed hypertrophic features, as evidenced by the abundance in either RER cisternae or polyribosomes and also by the presence of numerous Golgi complexes (Fig. 8). Glial cells were very large by day 7, possessed two to three large nucleoli and clear cytoplasm. Morphometry of NADPH-d–stained neurons confirmed that surviving neurons adjacent to the denervated region underwent hypertrophy. In control tissues, the cross-sectional areas of the neurons were 233.0 ± 72.0 and 230.7 ± 67.2 μm^2 (see Materials and Methods section), whereas 14 days after BAC treatment, the value was 352.9 ± 139.3 μm^2, which is a 52% increase ($P < 0.0001$, n = 227–239 neurons in each group). This change may be related to the enhanced metabolic activity of the regenerating neurons.

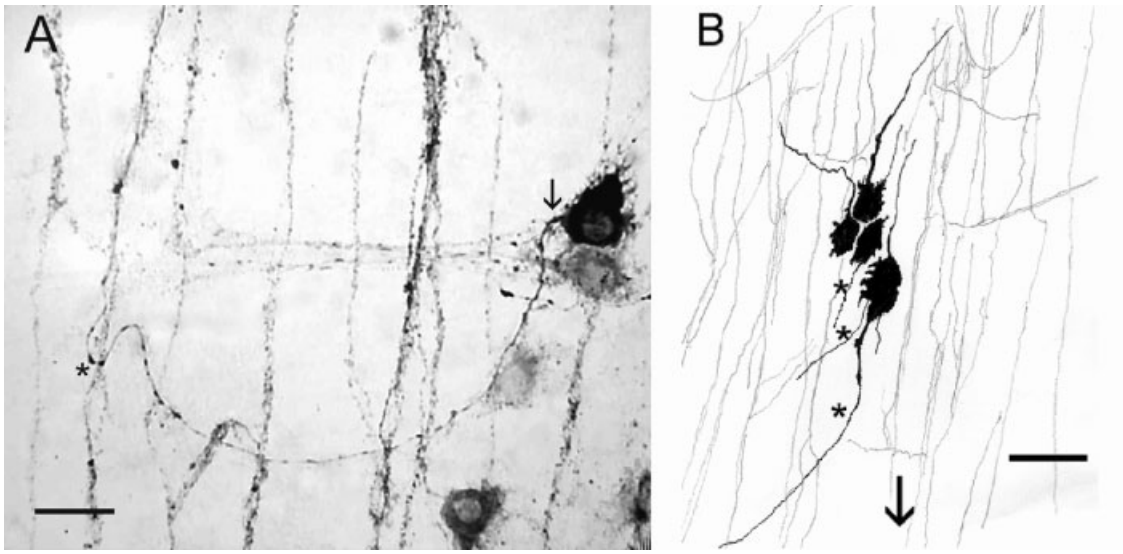

Fig. 5. Tracing the origin of the regenerating nerve fibers. **A:** Photomicrograph of nicotinamide adenine dinucleotide phosphate–diaphorase (NADPH-d) -stained tissue 7 days after benzalkonium chloride (BAC) treatment. A darkly stained neuron sends an axon (arrow) into the denervated region. This axon turns approximately 90 degrees (asterisk) and joins other axons innervating the circular muscle. **B:** Camera lucida drawing from a tissue 14 days after BAC treatment and NADPH-d staining. Four neurons were seen in this region (dark shading), and two of them send processes (asterisks) into the denervated area, the direction of which is indicated with an arrow. Scale bars = 25 μm in A, 50 μm in B.

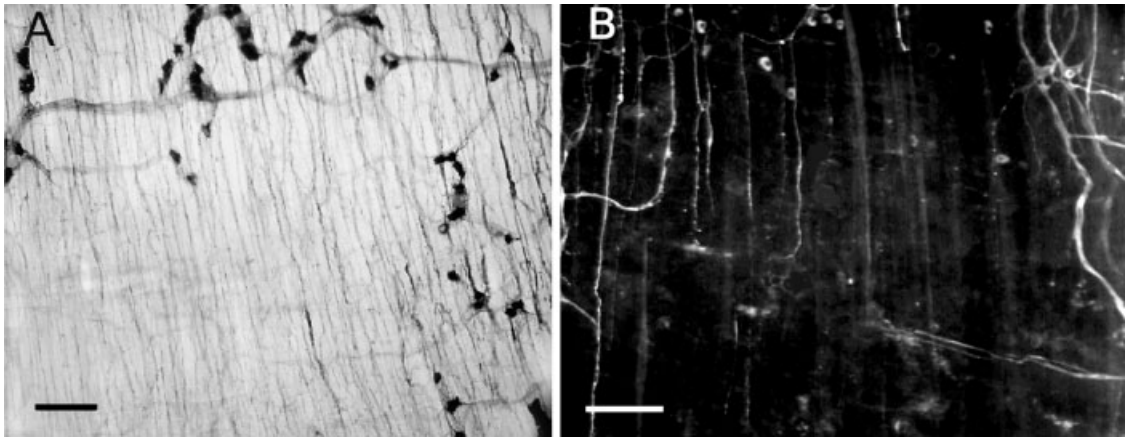

Fig. 6. Changes in the pattern of nerves in the denervated region 14 days after benzalkonium chloride treatment. **A:** Nicotinamide adenine dinucleotide phosphate–diaphorase staining showing a massive growth of nerve fibers from the transition zone into the aganglionic region. **B:** Growth of nerve fibers near the center of the denervated region. Some fibers can be seen along the direction of the circular muscle, but the central region contains no nerves. PGP9.5 staining. Scale bars = 100 μm in A, 25 μm in B.

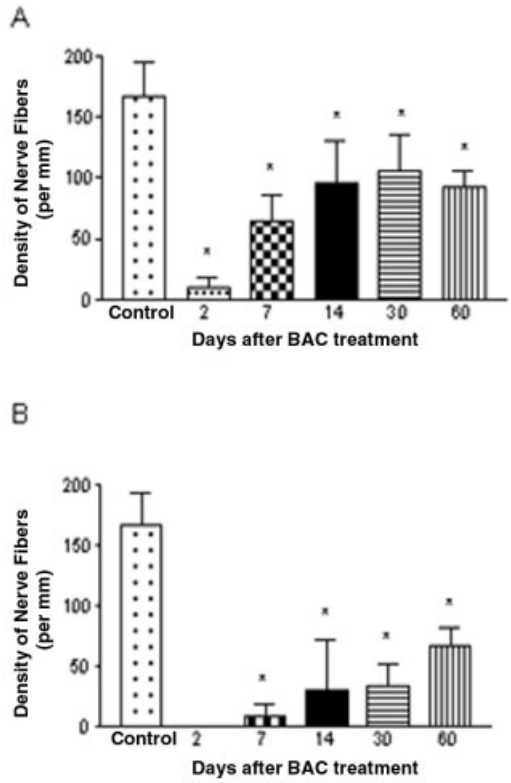

Fig. 7. Quantitative assessment of nerve growth 2–60 days after benzalkonium chloride (BAC) treatment. The density of nerve fibers per millimeter was measured in four to nine preparations at each time point in the periphery (**A**) and in the center (**B**) of the denervated area. Note the total absence of nerves in the center at 2 days. In the periphery, most of the growth takes place between 2 and 14 days. The bars indicate standard deviation, and the asterisks indicate statistically significant differences from controls ($P < 0.05$).

The ganglia in the transition zone also contained small cells with poorly differentiated cytoplasm and a very large nucleolus (Fig. 9A). Some of them had a cilium and a large Golgi apparatus. At days 14 and 30, cells resembling differentiating neurons were also seen. These cells were small in size, but had a large Golgi apparatus, numerous ribosomes, and some RER cisternae (Fig. 9B). The most differentiated ones contained synaptic vesicles and the nucleus was round and had the typical clear neuronal chromatin (Fig. 9C).

From day 14, nerve fibers ensheathed by glial cells were seen to invade the circular muscle layer of the aganglionic region (Fig. 10A,B). Some axons were rich in microtubules and had no typical varicosities, but small, dilated segments, in which occasional synaptic vesicles were seen (Fig. 10A). Synaptic vesicles were also scattered in the intervaricose portions. Some other axons had nerve varicosities apparently normal in features but contained few synaptic vesicles (Fig. 10B). At day 30, varicosities with morphology identical to controls, as well as ramified nerve endings, were filled with synaptic vesicles (Fig. 10C). By 30 up to 60 days, there was a further growth of nerve fibers into the denervated region, and they occupied 50% or more of the total area (Fig. 11A). However, as shown in Figure 5, even at day 60, there was no full recovery of the fiber density to control levels, and there was no significant difference between densities at 30 and 60 days. In the growth areas, we frequently observed structures resembling myenteric ganglia (Fig. 11B). These structures were formed by nerve fibers in the myenteric plexus area and were connected to similar structures by nerve fiber bundles but always lacked neurons. However, isolated neurons, stained for either PGP9.5 or NADPH-d, were occasionally observed along the nerve bundles in the aganglionic area (Fig. 11C). In virtually all tissues at days 30 and 60, such neurons were present, but because their numbers were small (1–3 in each tissue) no quantitative analysis of their density was undertaken.

DISCUSSION

Plasticity in the enteric nervous system

The results of this work provide new insights into the process of re-innervation that follows neuronal destruction by applying BAC onto the serosal surface of the

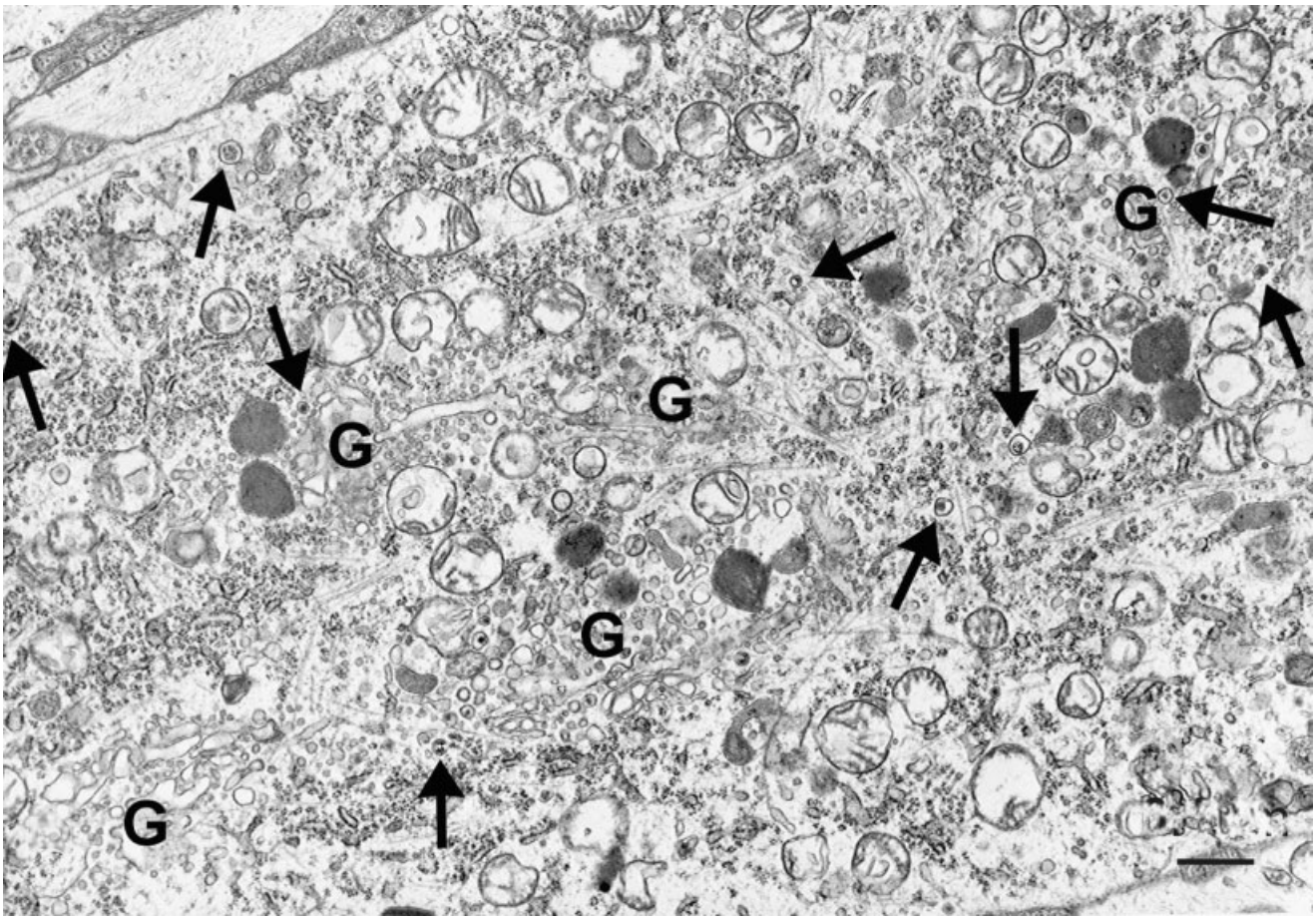

Fig. 8. Transition zone at 14 days after benzalkonium chloride (BAC) treatment. Transmission electron microscopy. A myenteric neuron with hypertrophic features, including large size, rough endoplasmic reticulum cisternae, polyribosomes, and microtubules well developed and scattered throughout. G, large Golgi complex. Arrows point to synaptic vesicles. Scale bar = 0.6 μm.

mouse large intestine. As reported by other authors, we also found that BAC is very effective in destroying neurons and nerve fibers of the myenteric plexus (Sato et al., 1978; Fox and Bass, 1984; Luck et al., 1993; Ramalho et al., 1993; Parr and Sharkey, 1997). These authors, however, gave little information on later events. Only one study reported the regeneration of nerve fibers within the circular muscle (Luck et al., 1993), and Ramalho et al. (1993) observed an increase in the number of neurons in the treated area. Some information on neurodegeneration followed by neuroregeneration is available for other experimental models, such as intestinal obstruction and ileal resection followed by end-to-end anastomosis (Jew et al., 1989; Tokui et al., 1994; Brookes et al., 1996; Karaosmanoglu et al., 1996).

Our results indicate that, from day 7 up to day 60 after ablation, nerve fibers regenerated and reinnervated the muscle. We traced nerve fibers from the reinnervated area to the transition zone and found that at least some of the growing fibers originated from myenteric neurons around the denervated region. These results do not rule out the possibility that some of the regenerating fibers originated in the submucosal plexus, as suggested by Luck et al. (1993).

It is likely that the surviving myenteric neurons underwent trophic changes to enable them to restore, at least partly, the innervation of the damaged region. In agreement with this idea, we found in the transition zone a 50% increase in the cross-sectional area of the neurons. Also, electron microscopy revealed large-sized neurons particularly rich in RER cisternae and having numerous Golgi complexes. Neuronal hypertrophy after nerve damage has been described in other autonomic systems (see Purves, 1988, for review). Gabella et al. (1992) showed that, after destruction of one pelvic ganglion, the contralateral ganglion sent processes into the denervated region, which was followed by hypertrophy of the neurons in this ganglion.

At days 30 and 60 after BAC application, most of the denervated region contained nerve fibers. However, the density of the fibers in the denervated area did not return to control levels even at 60 days. The pattern of the muscle innervation was quite similar to that of the normal tissue, as the new fibers were arranged mostly in parallel to the circular or longitudinal muscle cells and also formed ganglion-like structures reminiscent of normal ganglia, although they did not contain neurons. Electron microscopic examination showed that, at first, the regrowing nerve fibers had small dilated portions instead of typical

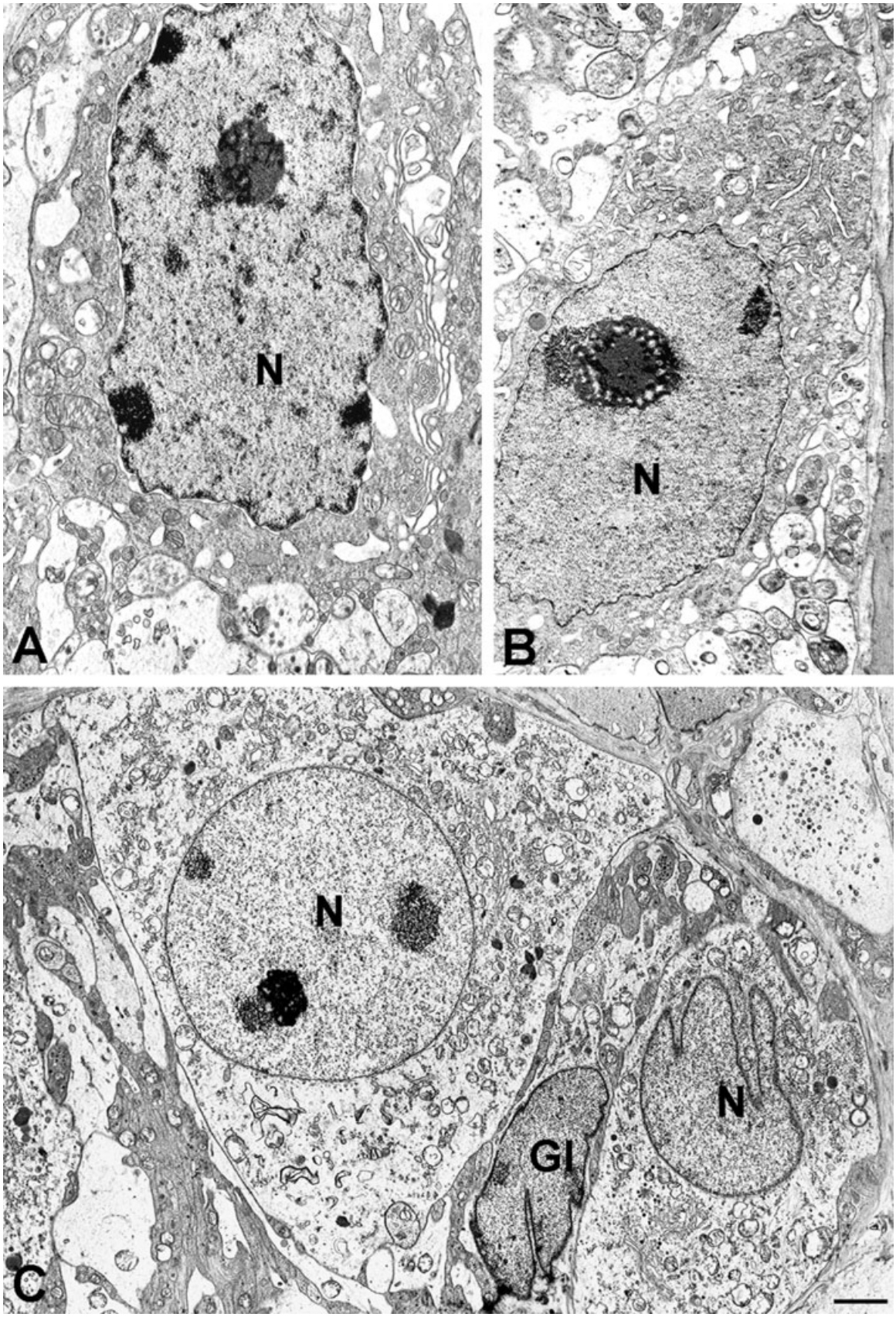

Fig. 9. Neuroregeneration in the transition zone. Transmission electron microscopy. A,B: Two presumptive neuroblasts (N) at the myenteric plexus area, 14 days after benzalkonium chloride (BAC) treatment. **A:** A small and poorly differentiated cell, with a large, dark, and nucleolated nucleus and a well-developed Golgi apparatus (right side). **B:** A cell that looks like a differentiating neuron, with numerous rough endoplasmic reticulum cisternae and a large, clear, and nucleolated nucleus. **C:** Thirty days after BAC treatment. A myenteric ganglion with two young neurons (N), which have features similar to those of the differentiated ones but are smaller in size and have few organelles. GL, glial cell. Scale bar in C = 0.8 μm in A, 1 μm in B, 1.5 μm in C.

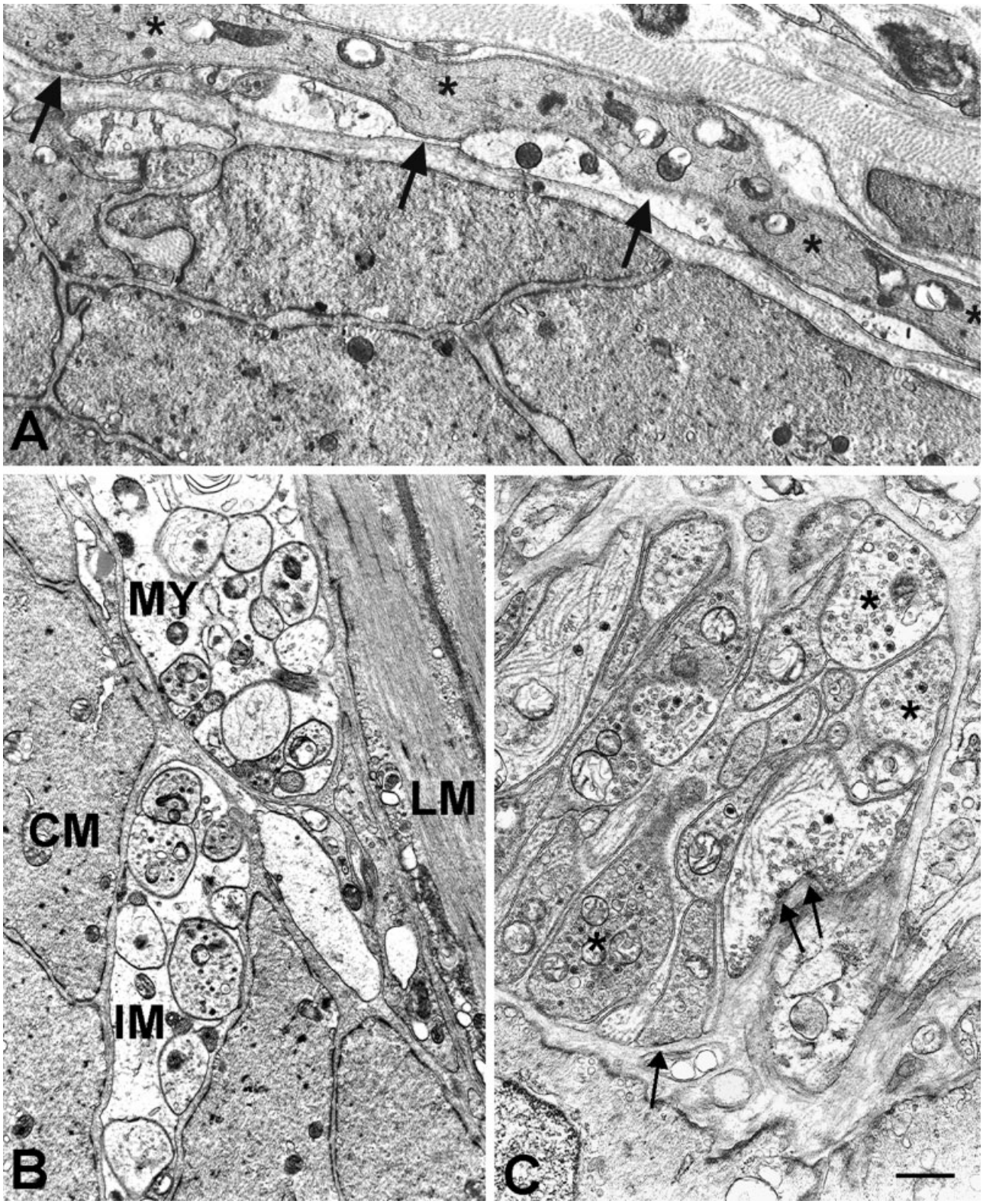

Fig. 10. Neuroregeneration in the reinnervated zone. Transmission electron microscope. **A:** A regenerating intramuscular nerve fiber, 14 days after benzalkonium chloride (BAC) treatment. The asterisks indicate dilated segments of the axon, occasionally containing sparse synaptic vesicles. Microtubules and neurofilaments fill either dilated or undilated axonal portions. The arrows indicate the ensheathing glial cell. **B:** Fourteen days after BAC treatment. An intramuscular (IM) nerve fiber with several axons, some of which contain synaptic vesicles, presumably coming from a myenteric (MY) nerve bundle. LM, longitudinal muscle layer; CM, circular muscle layer. **C:** Thirty days after BAC treatment. A typical nerve fiber containing several axons and nerve endings (asterisks), some of which are large and filled with synaptic vesicles. The synaptic vesicles are of both granular and agranular type. Arrows indicate accumulation of these vesicles at the releasing site of the nerve endings. Scale bar in C = 0.6 μm in A, 0.8 μm in B, 0.5 μm in C.

varicosities, and few synaptic vesicles scattered either in the dilated or nondilated axonal portions. At day 30, they became typical varicosities, where synaptic vesicles accumulated. These data indicate that the growing nerve fibers at first are poorly differentiated and gradually become capable to transport and release synaptic vesicles. Altogether, the present findings provide strong support for plasticity of myenteric nerves.

The mechanism that guides the reinnervation of the muscle is not known. Our experiments do not answer this question, but some useful ideas were provided by Nguyen et al. (2002), who studied the mechanism of the precise reinnervation of skeletal muscle after nerve injury. Two main models are possible: guidance by the target, and guidance by the path itself. Nguyen et al. (2002) obtained evidence for the latter, that is, axons retraced their preinjury paths according local cues, whose nature is not yet clear. We propose that, similarly, enteric nerves are guided by local cues, which may be remnants of the nerve or glial cells. The presence of ganglion-like structures in the denervated region is consistent with this idea, because it suggests that "signatures" of ganglia remain in the myenteric plexus region and guide the growing nerves. Glial cells may have a role in the regeneration process, because glial cell bodies are frequently seen at bifurcation point of nerve fibers. A similar observation was made in cocultured myenteric neurons and glial cells (Hanani et al., 1994). The alternative idea of guiding signals from the muscle cells cannot be discounted, and a combination of the two models is also possible. A role for inflammatory cells on this guidance is not supported by the present data, as inflammation resolved very early and close relationships were not seen between inflammatory cells and regenerating nerve structures.

Stem cells in the enteric nervous system

We obtained electron microscopic evidence that, 2 weeks and later after treatment with BAC, there were undifferentiated cells in the transition zone as well as differentiating ones. The latter were identified as cells belonging to the neuronal lineage by their ultrastructural features (Faussone-Pellegrini et al., 1996, 1999; Vannucchi and Faussone-Pellegrini, 2000). By day 30, under the light microscope, PG9.5- and NADPH-d–stained cells and, under the electron microscope, newly differentiated neurons were seen close to nerve fibers within the treated region. These neurons might be the result of differentiation either in the transition zone, which then migrate into the lesioned area, presumably following the new nerve fibers to the target area, or in the lesioned area from presumed progenitor cells. There are reports that the adult nervous system contains progenitor cells (Reynolds and Weiss, 1992; Gritti et al., 1996) that are able to differentiate into neurons and glia (Morrison et al., 1997; McKay, 1997). The presence of a reserve pool of neurons also in the myenteric plexus might be at the basis of the increase in the number of neurons in the treated area after an early

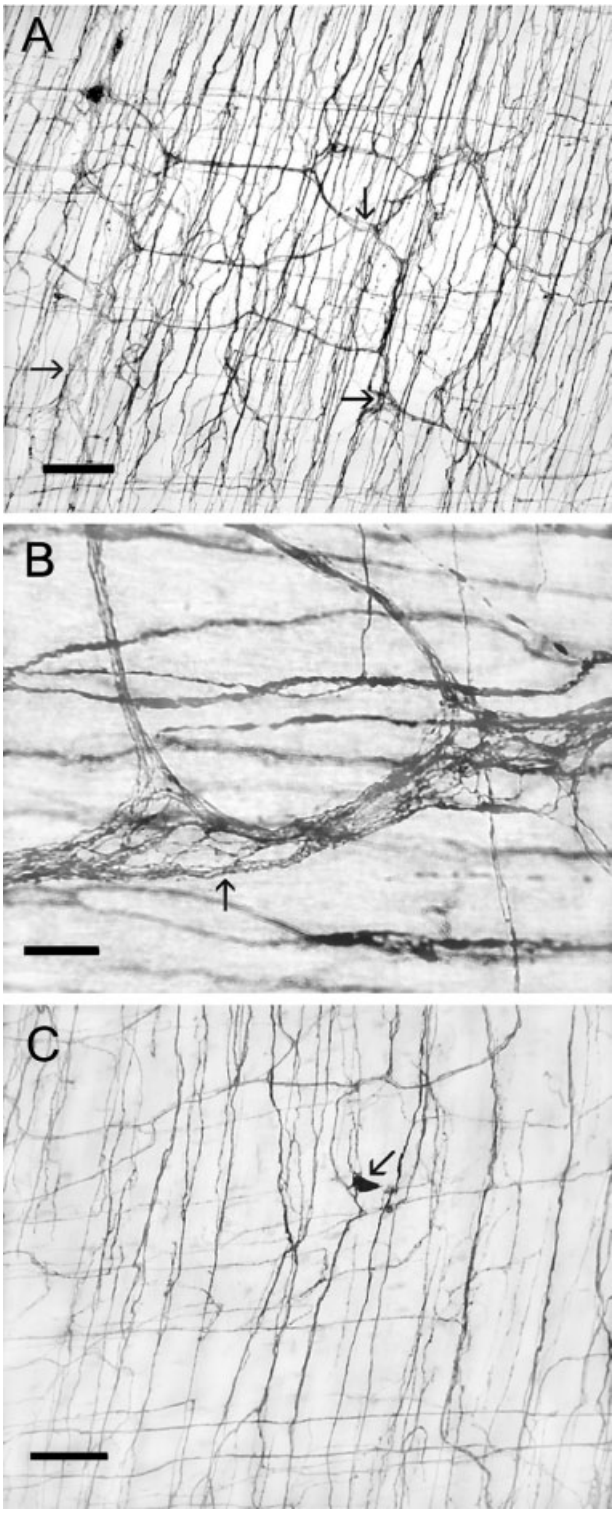

Fig. 11. The pattern of nerve fibers in the denervated area 30 days after benzalkonium chloride (BAC) treatment. **A:** Staining for nicotinamide adenine dinucleotide phosphate–diaphorase reveals a well-developed nerve fiber network. Most of the fibers are within the circular muscle layer, and they run parallel to the muscle cells. A nerve network is visible between the circular and longitudinal muscle layers. This network contains structures made up by nerve fibers that resemble myenteric ganglia (arrows) but contain no neurons. **B:** Photomicrograph showing a ganglion-like structure (arrow). **C:** The central part of a denervated region showing nerve fibers and a single neuron (arrow). Scale bars = 100 μm in A,C, 25 μm in B.

reduction after BAC treatment (Ramalho et al., 1993). The presence of undifferentiated cells and differentiating neurons is in agreement with this hypothesis. Increase in the number of myenteric neurons was also demonstrated after stenosis (Benninghoff, 1951; Filogamo and Vigliani, 1954; Giacobini-Robecchi et al., 1985), and evidence for the presence of a reserve pool was found in adult rats after partial intestinal obstruction (Filogamo and Cracco, 1995). These authors concluded that the adult intestine contains undifferentiated cells, which, under the influence of trophic factors, differentiate into neurons. These results, together with the present ones, indicate that the adult ENS contains stem cells. Recently, Kruger et al. (2002) succeeded in identifying in vitro stem cells from the ENS of adult rats, providing for the first time direct evidence for the presence of stem cells in the ENS. They ended their study by asking whether neurogenesis occurs in the adult gut, and whether it occurs in response to injury. Our results indicate a positive answer to both these questions, although much further work is required.

Is the BAC method a suitable model for disease?

Sato et al. (1978) proposed that ablation of enteric nerves with BAC can serve as a model for Hirschsprung's disease (HD), which is a congenital disease where enteric neurons are missing, usually in the distal colon (Lernau and Hanani, 1995). However, the BAC method does not produce a faithful model for HD because of the following: (1) In HD, the ganglia of both myenteric and submucosal plexuses are missing, whereas BAC eliminated only the myenteric plexus, apparently because the detergent did not penetrate into the deeper layers of the intestine. (2) In HD, the denervated area extends in a continuous manner from the anus, whereas in the BAC model, only an isolated island within the colon is denervated. (3) In HD, not only the enteric neurons are missing, but the surviving nerves are grossly abnormal, and the muscle is not properly innervated. In contrast, after ablation with BAC, nerves grow into the denervated region and the muscle innervation is qualitatively similar to the normal one. This finding is in accord with studies showing that, in a mouse model of HD, the microenvironment does not support normal development of nerves (Jacobs-Cohen et al., 1987). Although BAC treatment does not produce a faithful model for HD, it is very useful for simulating the absence of the myenteric plexus in a well-defined intestinal region. As myenteric nerves are essential for intestinal motility control, it is expected that BAC treatment will induce motility disturbances. Indeed, we observed that, 2 days after BAC application, the treated segment was constricted and the proximal one was dilated. At longer periods after BAC, these signs were less prominent, which can be correlated with the reinnervation of the muscle.

CONCLUSION

We showed that after ablation, the myenteric plexus has a considerable capacity to regenerate. The results suggest the presence of stem cells that can differentiate into neurons. The ability to regenerate contributes to recovery from injury and may have a potential for therapy of diseases associated with damage or degeneration of enteric nerves.

LITERATURE CITED

Benninghoff A. 1951. Vemehrung und Vergrösserung von Nervzellen bei Hypertrophie des Innervationgebietes. Z Naturforsch 6:38–44.

Brookes SJH, Lam TC, Lubowski DZ, Costa M, King DW. 1996. Regeneration of nerve fibers across a colonic anastomosis in the guinea pig. J Gastroenterol Hepatol 11:325–334.

De Giorgio R, Stanghellini V, Barbara G, Corinaldesi R, De Ponti F, Tonini M, Bassotti G, Sternini C. 2000. Primary enteric neuropathies underlying gastrointestinal motor dysfunction. Scand J Gastroenterol 35: 114–122.

Faussone-Pellegrini MS, Matini P, Stach W. 1996. Differentiation of enteric plexuses and interstitial cells of Cajal in the rat gut during pre- and postnatal life. Acta Anat 155:113–125.

Faussone-Pellegrini MS, Matini P, DeFelici M. 1999. The cytoskeleton of the myenteric neurons during murine embryonic life. Anat Embryol (Berl) 199:459–469.

Filogamo G, Cracco C. 1995. Models of neuronal plasticity and repair in the enteric nervous system: a review. Ital J Anat Embryol 100(Suppl 1): 185–195.

Filogamo G, Vigliani F. 1954. Ricerche sperimentali sulla correlazione tra estensione del territorio di innervazione e grandezza e numero delle cellule gangliari del plesso mienterico (di Auerbach) nel cane. Riv Patol Nerv Ment 75:1–32.

Fox DA, Bass P. 1984. Selective myenteric neuronal denervation of the rat jejunum. Gastroenterology 87:572–577.

Fox DA, Bass P. 1993. Pharmacological characterization of rat jejunal contractility after chronic ablation of the myenteric plexus. J Pharmacol Exp Ther 238:372–377.

Gabella G, Berggren T, Uvelius B. 1992. Hypertrophy and reversal of hypertrophy in rat pelvic ganglion neurons. J Neurocytol 21:649–662.

Giacobini-Robecchi MG, Cannas M, Filogamo G. 1985. Increase in the number and volume of myenteric neurons in the adult rat. Int J Dev Neurosci 3:673–675.

Gritti A, Paroti EA, Cova L, Frolichsthal P, Galli R, Wanke E, Farvelli L, Morassutti DJ, Roisen F, Nickel DD, Vescovi AL. 1996. Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. Neuroscience 16:1091–1100.

Hadzijahic N, Renehan WE, Ma CK, Zhang X, Fogel R. 1993. Myenteric plexus destruction alters morphology of rat intestine. Gastroenterology 105:1017–1028.

Hanani M, Xia Y, Wood JD. 1994. Myenteric ganglia from the adult guinea-pig small intestine in tissue culture. Neurogastroenterol Motil 6:103–118.

Hanani M, Louzon V, Udassin R, Freund HR, Karmeli F, Rachmilewitz D. 1995. Nitric oxide-containing nerves in bowel segments of patients with Hirschsprung's disease. J Pediatr Surg 30:818–822.

Herman JR, Bass P. 1987. Temporal changes in mechanical properties of rat jejunal smooth muscle after myenteric plexus ablation. Am J Physiol 253:G745–G750.

Holle GE. 1991. Changes in the structure and regeneration mode of the rat small intestinal mucosa following benzalkonium chloride treatment. Gastroenterology 101:1264–1273.

Holle GE. 1998. Changes in muscularis externa of rat small intestine after myenteric ablation with benzalkonium chloride. Dig Dis Sci 43:2666–2675.

Holle GE, Forth W. 1990. Myoelectrical activity of small intestine after chemical ablation of myenteric neurons. Am J Physiol 258:G519–G526.

Jacobs-Cohen RJ, Payette RF, Gershon MD, Rothman TP. 1987. Inability of neural crest cells to colonize the presumptive aganglionic bowel of ls/ls mutant mice: requirements for a permissive microenvironment. J Comp Neurol 255:425–438.

Jacobson M. Developmental neurobiology. 3rd ed. New York: Plenum. 776 p.

Jew JY, Williams TH, Gabella G, Zhang MQ. 1989. The intestine as a model for neuronal plasticity. Arch Histol Cytol 52(Suppl):167–180.

Karaosmanoglu T, Muftuoglu S, Dagdeviren A, Durgun B, Aygun B, Ors U. 1996. Morphological changes in the myenteric plexus of rat ileum after transection and end-to-end anastomosis. J Anat 188:323–331.

Kruger G, Mosher J, Bixby S, Joseph N, Iwashita T, Morrison S. 2002. Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness. Neuron 35:657–669.

Lernau OZ, Hanani M. 1995. Hirschsprung's disease: clinical and neurophysiological aspects. In: Korczyn AD, editor. Handbook of autonomic nervous system dysfunction. New York: Academic Press. p 117–128.

Luck MS, Dahl JL, Boyeson MG, Bass P. 1993. Neuroplasticity in the smooth muscle of the myenterically and extrinsically denervated rat jejunum. Cell Tissue Res 271:363–374.

McKay RDG. 1997. Stem cells in the central nervous system. Science 276:66–71.

Morrison SJ, Shah NM, Anderson DJ. 1997. Regulatory mechanisms in stem cell biology. Cell 88:287–298.

Nguyen QT, Sanes JR, Lichtman JW. 2002. Pre-existing pathways promote precise projection patterns. Nat Neurosci 5:861–867.

Osinski MA, Bass P. 1995. Myenteric denervation of rat jejunum alters calcium responsiveness of intestinal smooth muscle. Gastroenterology 108:1629–1636.

Parr EJ, Sharkey KA. 1997. Multiple mechanisms contribute to myenteric plexus ablation induced by benzalkonium chloride in the guinea-pig ileum. Cell Tissue Res 289:253–264.

Purves D. 1988. Body and brain. A trophic theory of neural connections. Cambridge, MA: Harvard University Press.

Ramalho FS, Santos GC, Ramalho LN, Kajiwara JK, Zucoloto S. 1993. Myenteric neuron number after acute and chronic denervation of the proximal jejunum induced by benzalkonium chloride. Neurosci Lett 163:74–76.

Reynolds BA, Weiss S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710.

Sato A, Yamamoto M, Imamura K, Kashiki Y, Kuneida T, Sakata K. 1978. Pathophysiology of aganglionic colon and anorectum: an experimental study on aganglionosis produced by a new method in the rat. J Pediatr Surg 13:399–405.

See NA, Epstein ML, Schultz E, Pienkowski TP, Bass P. 1988. Hyperplasia of jejunal smooth muscle in myenterically denervated rat. Cell Tissue Res 253:609–617.

See NA, Epstein ML, Dahl JL, Bass P. 1990. The myenteric plexus regulates cell growth in the rat jejunum. J Auton Nerv Syst 31:219–230.

Temple S. 1999. CNS development: the obscure origin of stem cells. Curr Biol 9:R397–R399.

Tokui K, Sakanaka M, Kimura S. 1994. Progressive reorganization of the myenteric plexus during one year following reanastomosis of the ileum of guinea pig. Cell Tissue Res 277:259–272.

Vannucchi MG, Faussone-Pellegrini MS. 2000. Synapse formation during neuron differentiation: an in situ study of the myenteric plexus during murine embryonic life. J Comp Neurol 425:369–381.

Van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. 2002. Functional neurogenesis in the adult hippocampus. Nature 415: 1030–1034.

Zucoloto S, Silva JC, Oliveira JSM, Muccillo G. 1991. The chronological relationship between the thickening of smooth muscle, epithelial cell proliferation and myenteric neural denervation in the rat jejenum. Cell Prolif 24:15–20.