NADPH-Diaphorase Histochemistry

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14.1 Introduction

The introduction of rectal suction biopsy, while making the procedure less traumatic for the patient, has made the diagnosis of Hirschsprung's disease (HD) more difficult for the pathologist. Many histopathologists are reluctant to make a positive diagnosis of HD on the basis of suction rectal biopsy results, using conventional H&E stains. This reluctance stems from doubt as to the amount of submucosa that must be scanned before the absence of ganglion cells can be confirmed, as well as the relative difficulty in accurately identifying smaller and sparse submucosal ganglion cells by comparison with the more compact and familiar ganglion cells of the intermuscular plexus.

The development of histochemical techniques for the detection of acetylcholinesterase (AChE) was a considerable advance in the investigation of HD [1]. However, because occasional false-negative results occur [2, 3], alternative diagnostic neuronal markers have been sought [4–7]. During the last few years, we have used many newer neuronal markers in our laboratory to investigate enteric plexus disorders. We have found nicotine adenine dinucleotide phosphate (NADPH) diaphorase histochemistry a particularly important technique for diagnosis of HD and its allied disorders.

14.2 Nitric Oxide and NADPH-Diaphorase

Nitric oxide (NO) is an important neurotransmitter that mediates relaxation of the smooth muscle within the gastrointestinal tract [8]. Its is synthesized from l-arginine in a reaction catalyzed by NO synthase (NOS). In 1990, Bult et al. [9] provided evidence that NO is released on stimulation of enteric nonadrenergic, noncholinergic (NANC) nerves. Since then, substantial evidence has emerged indicating that NO is the primary mediator of NANC neurotransmission in the intestinal tract in various species [10–12]. Numerous studies have shown the effectiveness of NO in evoking relaxation of the smooth muscle in different parts of the gastrointestinal tract [13, 14]. NO is released from bowel wall and stomach during nerve stimulation [15]. Exogenous NO mimics NANC nerve-evoked relaxation and hyperpolarization in the gastrointestinal muscle in the animal model and human jejunum and colon [16–18]. Inhibition of NO synthesis attenuates the effects of NANC nerve stimulation in animal models and in human sigmoid colon and internal anal sphincter [13, 19, 20]. Furthermore, NO is involved in neurogenic relaxation of the rectum, and NOS immunohistochemistry identified a subpopulation of neurons in the myenteric ganglia and immunoreactive fibers within both layers of the muscularis propria of the human rectum [21]. The mechanism by which NO mediates NANC inhibition of gastrointestinal muscle is understood only partly. NO acting as a neurotransmitter from a final inhibitory neuron binds to cytosolic guanylate cyclase and increases the production of 5'-cyclic guanosine monophosphate (cGMP) with subsequent relaxation of smooth muscle [22].

The above findings suggest that nerves innervating smooth muscle are able to release NO that will penetrate the cells to induce relaxation. Additional sources of NO other than neurons involved in NANC inhibitory transmission have also been proposed, e.g. interstitial cells of Cajal and smooth muscle cells [23].

Deficiency of the nitrergic innervation has been shown in different tissues from patients with infantile hypertrophic pyloric stenosis, HD, and internal anal sphincter achalasia, suggesting that a lack of NO release may be involved in the pathophysiology of these disorders [24–30].

In both brain and peripheral neuronal tissue, NOS has been shown to colocalize with reduced NADPH-diaphorase. Histochemical staining with NADPH-diaphorase, described in brain tissue by Scherer-Singler et al. has facilitated the identification of neuronal NOS [31]. Gabella was the first to describe NADPH-diaphorase staining in gastrointestinal ganglion cells in 1967 [32]. Neuronal NOS catalyses the oxidation of l-arginine to form l-citrulline and NO, a reaction that depends on Ca^2+/calmodulin and NADPH. NOS reduces nitroblue tetrazolium to water-insoluble, intensely blue formazan using NADPH as substrate. It has been shown that the activities of NOS and NADPH-diaphorase are identical [33, 34].

14.3 Tissue Preparation for NADPH-Diaphorase Histochemistry

Suction rectal biopsy or full-thickness bowel biopsy tissue is fixed in Zamboni's solution (0.21% picric acid, 2% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.3, for 15 min at 4°C). After fixation, the specimens are rinsed in 0.1 *M* phosphate buffer with 10% sucrose for 15 min, subsequently snap-frozen and embedded in Tissue-Tek OCT compound (Miles, Elkhart, Ind.). Frozen sections (10 μm thick) are cut serially and processed for NADPH-diaphorase histochemistry.

14.4 Whole-Mount Preparation Technique

Gut innervation has a complex, three-dimensional structure, which is difficult to appreciate on thin sections. The whole-mount preparation technique produces a three-dimensional picture to better show the structure of neuronal networks and the relationship of branching and interconnecting nerve fibers to each other and to the neighboring tissues [35]. This technique, therefore, is especially useful for the investigation of pathological changes in the submucosal and myenteric plexuses such as hyperplasia of the plexuses or giant ganglia. The great advantages for histological evaluation become obvious when whole-mount preparations are compared with regular sections. Histological sections only partially show the morphology of the nerve and glial cells, being dependent on orientation and localization. However, whole-mount preparations show the morphology of the plexuses in full, making possible changes easy to see. Whole-mount preparations of the longitudinal muscle layer and the myenteric plexus are made by separating the muscular layers from the submucosal layer, then removing the circular muscle layer from the longitudinal muscle layer with the adherent myenteric plexus. Subsequently, the mucosa is removed from the submucosal layer.

The submucosal and myenteric plexuses in healthy and diseased bowel can be visualized clearly in whole-mount preparations combined with NADPH-diaphorase histochemistry.

14.5 NADPH-Diaphorase Histochemistry

To stain for NADPH-diaphorase activity, sections or whole-mount preparations are incubated in 10 ml Tris buffer (pH 8.0) containing 0.3% Triton (Sigma), 10 mg β-NADPH (Sigma), and 1 mg nitroblue tetrazolium (Sigma) at 37°C for 60 min. Subsequently the specimens are rinsed and coverslipped with DAKO (Denmark) Glycergel mounting medium.

Recently, several investigators have studied the pattern of NADPH-diaphorase staining in the normal colon and colon from HD patients and have reported lack or deficiency of NOS-containing nerves in the smooth muscle of aganglionic colon [26–29, 36]. There was a strong NADPH-diaphorase staining of submucosal and myenteric plexus in the normal colon and the ganglionic colon of HD patients whereas in aganglionic bowel, weak staining of hypertrophic nerve trunks was found (Figs. 14.1 and 14.2). The lack of NO-producing nerve fibers in the aganglionic bowel contributes to the inability of the smooth muscle to relax, thereby causing the lack of peristalsis in HD. We have used NADPH-diaphorase histochemistry to stain suction rectal biopsies and found it valuable in the diagnosis (Figs. 14.3 and 14.4). There is a considerable lack of NADPH-diaphorase-positive fibers within the muscularis mucosae whereas hypertrophic submucosal fibers stain weakly but are clearly visible.

We have recently employed NADPH-diaphorase histochemistry for the intraoperative evaluation of the extent of the aganglionic segment during pull-through operations for HD [37]. For the intraoperative diagnosis of HD, the sections are incubated in the staining solution for 20 minutes instead of the conventional 60 minutes. NADPH-diaphorase histochemical staining provided 100% diagnostic accuracy regarding the extent of the aganglionosis in HD patients, including newborns. With this technique, it is easier to distinguish the normally innervated bowel segment from the hypoganglionic transitional zone.

Three-dimensional morphology of nitrergic innervation in HD has been investigated using the whole-mount preparation technique [35]. The whole-mount preparation of the ganglionic segment from rectosigmoid HD showed the typical three-dimensional NADPH-diaphorase mesh-like myenteric plexus consisting of nerve bundles with ganglia containing clustered ganglion cells (Fig. 14.5A). In contrast, the aganglionic segment showed absence of the typical architecture of the myenteric plexus

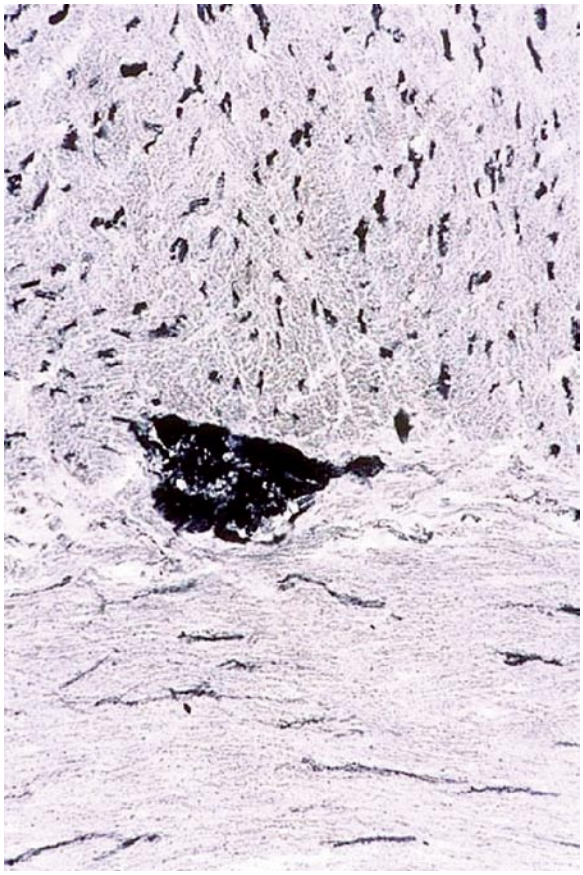


Fig. 14.1 NADPH-diaphorase staining of normal myenteric plexus and intermuscular nerve fibers

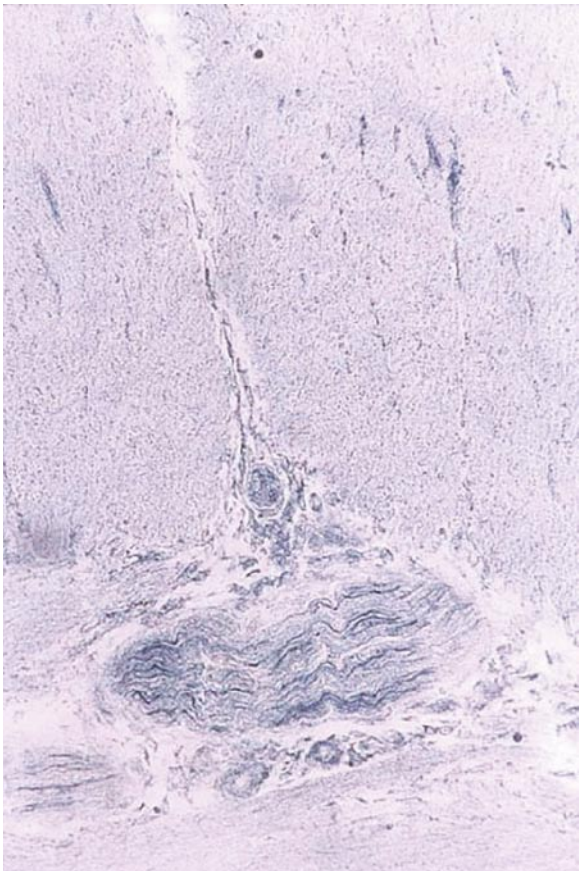


Fig. 14.2 NADPH-diaphorase staining of hypertrophic nerve fibers in aganglionic bowel of HD

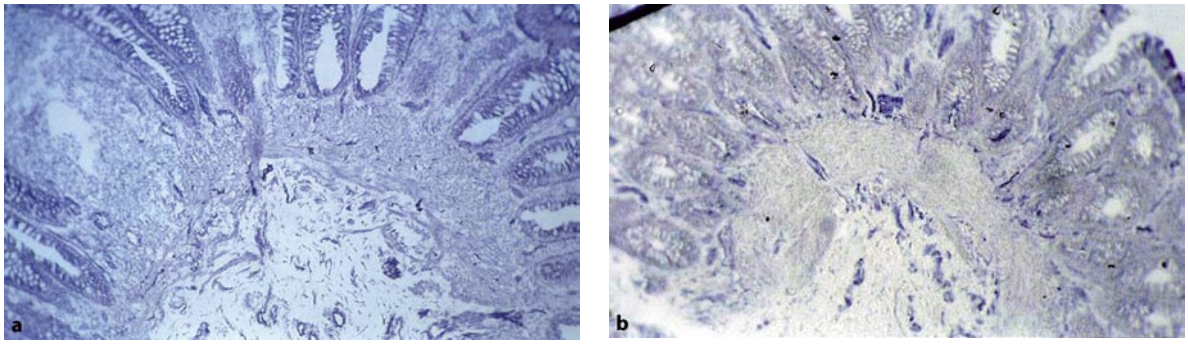


Fig. 14.3 Rectal suction biopsy (RSB). **a** Normal RSB with regular NADPH-diaphorase-positive small submucosal ganglia and normally expressed nerve fibers within the muscularis mucosae. **b** RSB in HD with clearly reduced NADPH-diaphorase-positive fibers within the muscularis mucosae and weakly stained hypertrophic submucosal nerve trunks

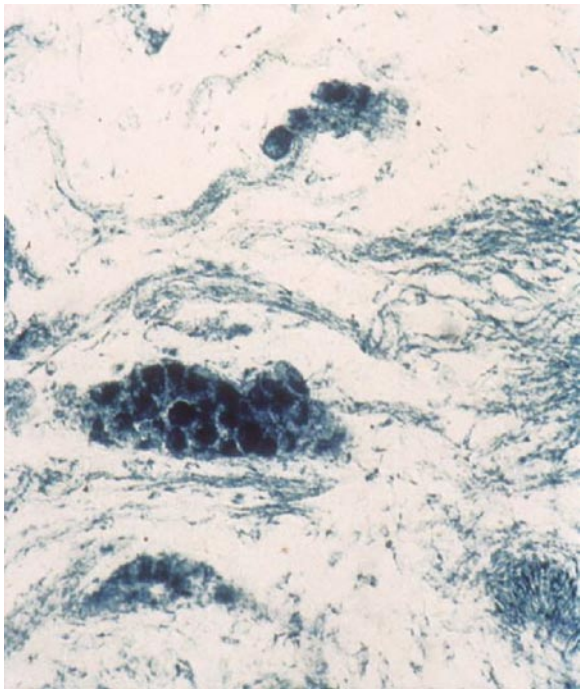
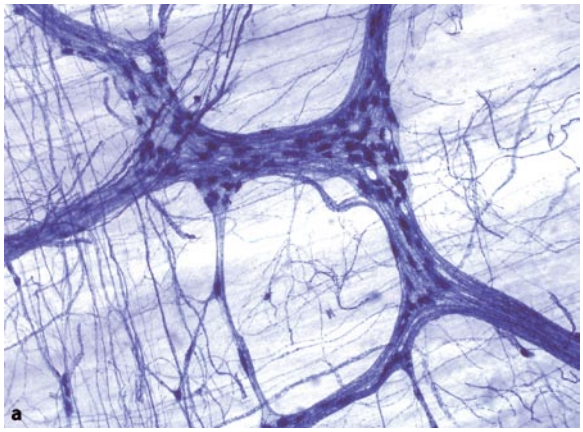
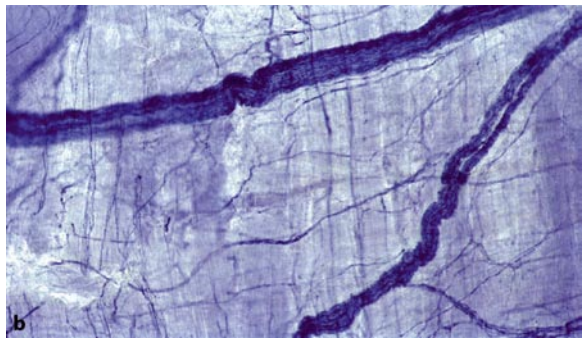


Fig. 14.4 NADPH-diaphorase-positive submucosal giant ganglion





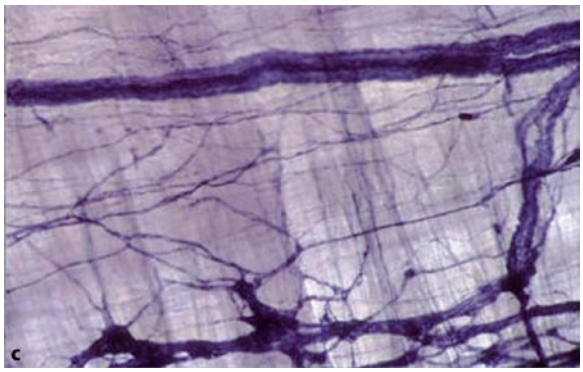


Fig. 14.5 NADPH-diaphorase, whole-mount preparation: **a** normal myenteric plexus; **b** hypertrophic fibers and absent ganglia in the aganglionic zone of HD; **c** hypertrophic fibers and defective small myenteric ganglia in the transitional zone of HD

and the presence of tortuous hypertrophied nerve trunks (Fig. 14.5B). Furthermore, NADPH-diaphorase combined with whole-mount preparation of the specimen is extremely useful to better display the morphological characteristics of the transitional zone in HD, in which there are defective ganglia and still present hypertrophic nerve fibers (Fig. 14.5C).

We have further used NADPH-diaphorase histochemistry in combination with whole-mount preparations to investigate full-thickness bowel biopsies from selected patients with chronic constipation. The specimens showed the characteristic findings of isolated hypoganglionosis [38]. NADPH-diaphorase histochemistry revealed sparse and small myenteric ganglia and a reduced number of nerve fibers in the circular muscle (Fig. 14.6). No hypertrophic nerve trunks were identified in the myenteric or submucous plexuses. Whole-mount preparations of normal bowel stained with NADPH-diaphorase demonstrated a dense mesh-work of nerve bundles in the myenteric plexus containing clusters of ganglion cells.

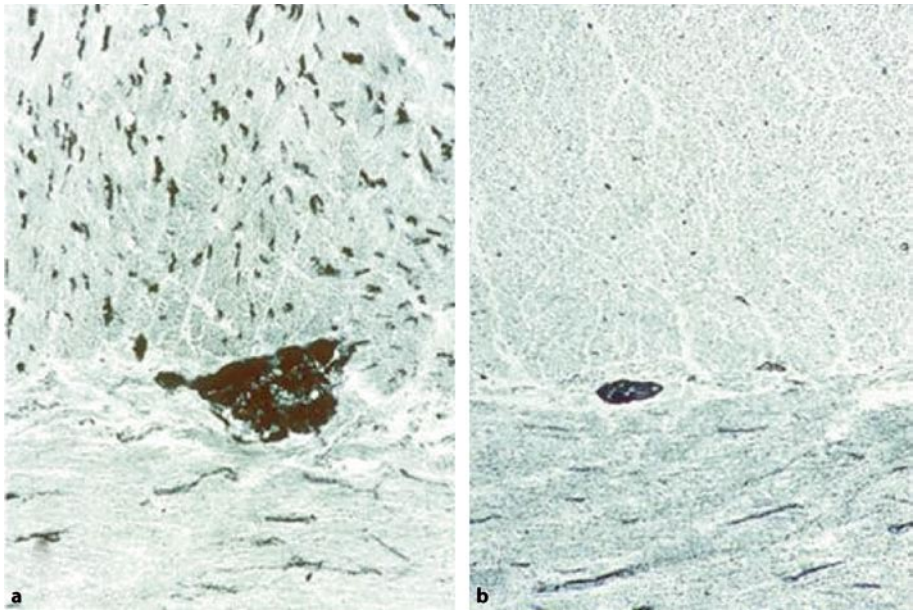


Fig. 14.6 Normal (**a**) and hypoganglionic (**b**) myenteric plexus (section, NADPH-diaphorase histochemistry)

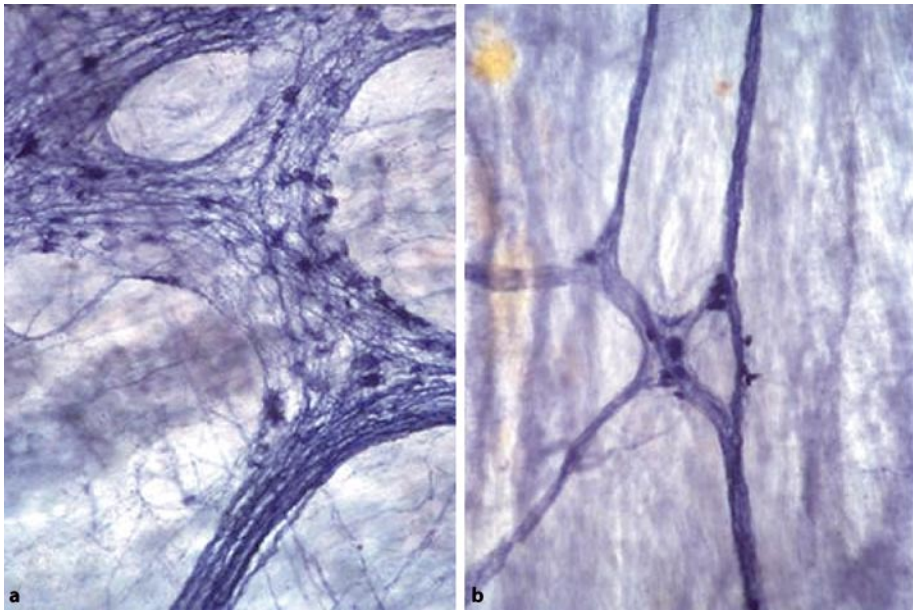


Fig. 14.7 Normal (**a**) and hypoganglionic (**b**) myenteric plexus (whole-mount preparation, NADPH-diaphorase histochemistry)

In hypoganglionosis the number of ganglion cells and thickness of nerve bundles in the myenteric plexus were markedly reduced compared to controls (Fig. 14.7).

Combination of whole-mount preparation and NADPH-diaphorase staining has been shown to be very valuable in patients with chronic constipation and histological signs of intestinal neuronal dysplasia (IND). We have used this technique to assess bowel specimens during and after surgery for persistent symptomatic constipation. Whole-mount preparations combined with NADPH-staining elegantly show the three-dimensional morphology of the normal submucous plexus (Fig. 14.8) and myenteric plexuses (Fig. 14.10) compared to the submucous and myenteric plexus in IND which demonstrate markedly a increased number of ganglion cells compared to controls (Figs. 14.9,11) [39]. This technique accurately identifies the hyperganglionosis of the myenteric and submucous plexuses which is characteristic of IND.

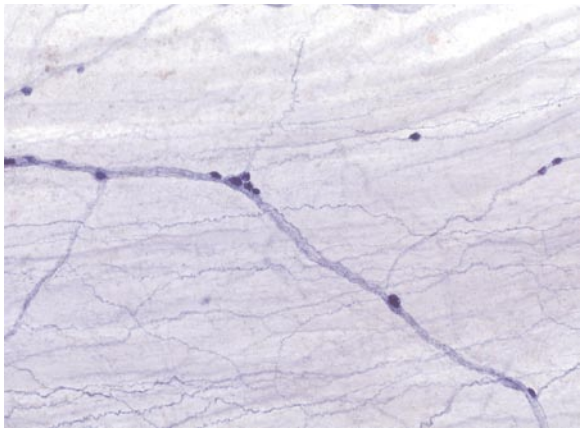


Fig. 14.8 Normal submucous plexus (whole-mount preparation, NADPH-diaphorase histochemistry)

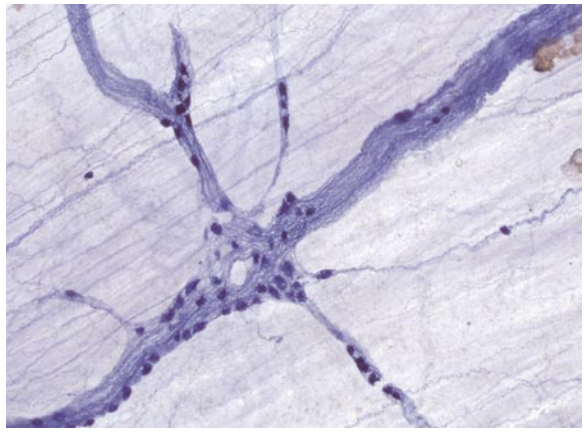


Fig. 14.9 Hyperganglionic submucous plexus (whole-mount preparation, NADPH-diaphorase histochemistry)

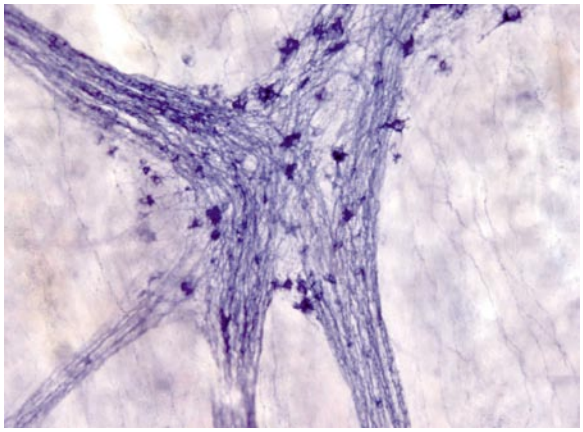


Fig. 14.10 Normal myenteric plexus (whole-mount preparation, NADPH-diaphorase histochemistry)

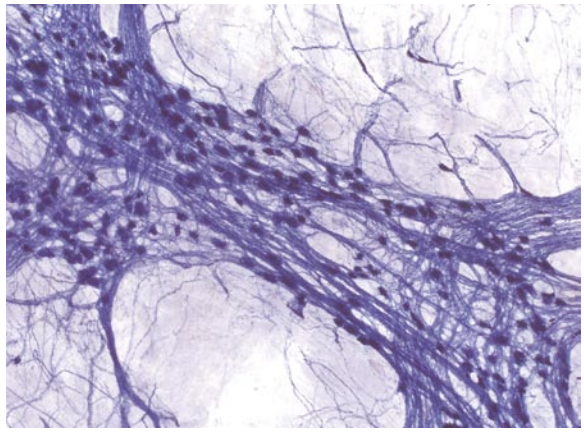


Fig. 14.11 Hyperganglionic myenteric plexus (whole-mount preparation, NADPH-diaphorase histochemistry)

References

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