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Morphology and Serotonergic Innervation of Physiologically Identified Cells of the Guinea Pig's Myenteric Plexus[1]

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Abstract

Ganglion cells of the myenteric plexus of the guinea pig were physiologically classified as to cell type using intracellular microelectrodes containing horseradish peroxidase (HRP). Interganglionic fiber tracts were then stimulated in an attempt to elicit slow excitatory postsynaptic potentials (EPSPs) in the impaled cells. The presence or absence of a slow EPSP was noted, following which the cells were injected with HRP through the recording micropipette and finally were incubated with tritiated 5-hydroxytryptamine ([^{3}H]-5-HT; 0.5 μM) and desmethylimipramine (10 nM). The preparations were examined by light and electron microscopy (EM) for which the physiologically identified, HRP-injected neurons were demonstrated histochemically and serotonergic nerve terminals were simultaneously demonstrated radioautographically. Forty-seven cells were physiologically identified, injected with HRP, and studied by light microscopy. Of these, 22 were also fully analyzed by electron microscopy. The sample included 13 type I/S cells (6 analyzed by electron microscopic radioautography), 16 type II/AH cells (10 analyzed by electron microscopic radioautography), and 18 nonspiking (NS) cells (6 analyzed by electron microscopic radioautography). Slow EPSPs were only observed in type II/AH cells. All five of the fully analyzed subset of type II/AH cells that manifested a slow EPSP were contacted by serotonergic terminals. These terminals were found on 7 of 10 type II/AH cells, 2 of 6 type I/S cells, and 0 of 6 NS cells. Serotonergic terminals, therefore, contacted type II/AH cells ($p < 0.05$) and especially those that showed a slow EPSP ($p < 0.005$) more frequently than other types of ganglion cells. Morphologically, cells with short, stubby dendrites were reproducibly found to be type I/S cells, and glia could be recognized by their astrocytic appearance; however, cell shape did not otherwise correlate with physiological cell type. These data are compatible with and provide support for the hypothesis that 5-HT is one of the mediators of slow EPSPs in the myenteric plexus.

Serotonin was first proposed as a transmitter in the enteric nervous system (ENS) by Gershon et al. (1965). Since then, a considerable body of evidence has been obtained that supports this suggestion. For example, 5-hydroxytryptamine (5-HT) is present in enteric neurons (Dreyfus et al., 1977; Costa et al., 1982, Furness and Costa, 1982a), is synthesized from the dietary amino acid, L-tryptophan, by these neurons (Dreyfus et al., 1977; Gershon and Dreyfus, 1980), and is released from them in a Ca^{2+}-dependent manner by electrical stimulation (Jonakait et al., 1979; Gershon and Tamir, 1981). In addition, enteric serotonergic neurons specifically and avidly take up 5-HT, providing an adequate inactivating mechanism for the amine as a transmitter (Gershon and Altman, 1971; Robinson and Gershon, 1971; Gershon et al., 1976; Gershon and Jonakait, 1979). On a gross level, 5-HT also mimics many of the authentic neurally mediated responses of the gut (Brownlee and Johnson, 1963; Bulbring and Gershon, 1967; Furness and Costa, 1973; Costa and Furness, 1979a; Julé, 1980); however, considerable controversy has arisen over whether these gross effects can be traced to physiological actions of 5-HT (see Costa and Furness, 1979b; Gershon, 1982) and over whether nerve-activated, slow excitatory postsynaptic potentials (EPSPs) in enteric neurons are mediated by 5-HT (Wood and Mayer, 1979b; Johnson et al., 1981).

Neurons of the myenteric plexus have been categorized as type I or S, type II or AH (Holman et al., 1972; Nishi and North, 1973; Hirst et al., 1974), and, more recently, types III (non-spiking) and IV (delayed spiking; Wood, 1983). The type I/S cells fire repetitively when depolarized by intracellular injection of current, whereas the type II/AH cells do not. The type II/AH cells show, instead, a pronounced hyperpolarization that follows an initial spike and makes these cells refractory and unable to discharge repetitively. The after hyperpolarization (AH) has been shown to be due to a calcium-activated potassium conductance (North, 1973; Wood and Mayer, 1979a; Grafe et al., 1980). This post-spike increase in conductance is abolished when a slow EPSP is elicited in type II/AH cells by stimulating either an interganglionic connective (Wood and Mayer, 1979a; Grafe et al., 1979a) or by intraganglionic focal stimulation (Johnson et al., 1980a, b). The slow EPSP can be mimicked either by 5-HT (Wood and Mayer, 1979b) or by peptides, such as substance P (SP; Katayama and North, 1978; Katayama et al., 1979). Since desensitization of type II/AH cells to 5-HT also blocks the slow EPSP, Wood and Mayer (1979b) originally concluded that 5-HT was a mediator of this response. This conclusion now has been called into doubt by the more recent work of Johnson et al. (1981) and Bornstein et al. (1984) whose observations are compatible with mediation of the slow EPSP by a peptide rather than by 5-HT. It is difficult to resolve the controversy because of the absence of a specific antagonist of the enteric neural actions of 5-HT (Costa and Furness, 1979b). It is possible that the slow EPSP is mediated in some cells by a peptide and in others by 5-HT. The different means of eliciting a slow EPSP used by different investigators might selectively activate one or another input to the neurons that display a slow EPSP. Wood and Mayer (1979b) stimulated interganglionic connectives, whereas Johnson et al. (1981) and Bornstein et al. (1984) used intraganglionic focal stimulation. Since 5-HT fibers are long and run between ganglia (Furness and Costa, 1982a), whereas

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SP fibers are short and extremely numerous within a single ganglion (Costa et al., 1980), it might be expected that interganglionic stimulation of the fiber tracts in interganglionic connectives would selectively activate the serotonergic innervation, while focal stimulation would have a better chance of activating SP-containing elements.

The current study was designed to test the hypothesis that serotonergic axons preferentially contact those myenteric neurons that display a slow EPSP. Such a distribution would be expected if 5-HT is one of the mediators of the slow EPSP. In fact, if serotonergic synapses could be shown to be absent from cells that manifest slow EPSPs, then 5-HT could be eliminated as a potential mediator of this response. Intracellular records were made in order to identify cells by physiological class: types I/S, II/AH, or non-spiking (NS). Interganglionic connectives were stimulated in order to activate serotonergic axons and to evoke a slow EPSP. The presence or absence of that response was noted and the cells were marked by intracellular injection of horseradish peroxidase (HRP). Preparations were subsequently exposed to [^{3}H]-5-HT under conditions in which the nonspecific uptake of that amine by noradrenergic axons was blocked. Finally, serotonergic axons and the HRP-labeled cells were located simultaneously by electron microscopic radioautography and cytochemistry, respectively. The hypothesis that 5-HT is a mediator of the slow EPSP would predict that cells that show the slow EPSP would actually receive serotonergic synapses. Conversely, if these cells were not to receive serotonergic synapses, the hypothesis could be rejected. Other myenteric ganglion cells were also studied in this way and served as controls.

Materials and Methods

Dissection of the myenteric plexus. Adult male guinea pigs (about 600 gm) were stunned and exsanguinated. A short segment of jejunum was removed, washed through the lumen with Krebs solution, and cut open along the mesenteric border. The resulting rectangular sheet of gut was pinned out flat, mucosal surface up, in a dish that was lined with a silicone elastomer (Sylgard 184; Dow Corning, Midland, MI) and had iced Krebs solution recirculating through it. The mucosal and submucosal layers of the bowel were gently removed with forceps under microscopic observation, leaving only the muscularis externa pinned to the dish. The circular layer of smooth muscle was then gradually teased away, a few fibers at a time, in order to expose the myenteric plexus which remained adherent to the underlying longitudinal muscle.

Physiological recording and cell marking. For intracellular recording, the longitudinal muscle with attached myenteric plexus was maintained in a low volume chamber (2.5 ml) which was perfused with Krebs solution at high speed (10 to 15 ml/min, 37°C). Individual ganglia were visualized using reflected light and differential interference contrast microscopy. Illumination was from above, employing fiberoptics to project light through the objective onto a mirrored surface below the specimen (Fig. 1). Because of the spontaneous contractions of the longitudinal muscle, each ganglion that was to be recorded from had to be immobilized. This was accomplished by pressing two L-shaped wires, called pressure feet, onto the smooth muscle next to and parallel to the ganglion (Fig. 1). Once the ganglion was immobilized, intracellular impalements were made with glass microelectrodes which were positioned under visual guidance with a micromanipulator. The microelectrodes were filled with a solution containing HRP (10%; type VI, Sigma Chemical Co., St. Louis, MO) and 0.2 M KCl in 50 mM Tris-HCl buffer (pH 7.0 at 37°C). After pulling, the back ends of the electrodes were first immersed in the electrolyte solution without HRP. This filled the long tips of the electrodes without bubbles. The HRP-containing solution was then injected into the back of the electrode and allowed to diffuse overnight in a humid chamber until the tips were completely filled with the viscous HRP. This two-step procedure was needed to prevent incomplete filling of the electrodes with HRP. The following day, the electrodes were beveled, using a jet stream beveler (Ogden, 1978), from approximately 200 megohms to a final resistance of 100 megohms. Membrane and action potentials were measured with the HRP-filled intracellular microelectrodes, using techniques similar to those of Wood and Mayer (1979a, b). Synaptic input and antidromic activation of neurons were elicited by individually stimulating the interganglionic connectives with a monopolar extracellular electrode (25-μm Teflon-coated platinum wire; referenced to the bath). Cells were filled with HRP by passing depolarizing constant current and/or pressure pulses through the recording microelectrode (0.5 to 2.0 nA and/or 5 to 40 psi for 500 to 750 msec at 0.5 to 1.0 Hz for 5 to 30 min).

Incubation with [^{3}H]-5-HT. The experimental preparations were removed from the recording chamber after intracellular injections had been completed and transferred to a vial containing 5 ml of oxygenated Krebs solution maintained at 37°C in a shaking water bath. The incubating solution contained the monoamine oxidase inhibitor, pargyline (0.1 mM), the norepinephrine uptake blocker, desmethylimipramine (10 nM), and [^{3}H]-5-HT (0.5 μM; 20 to 25 Ci/mmol; New England Nuclear Corp., Boston, MA). Incubation was continued for 30 min, during which the solution was constantly bubbled with a mixture of 95% O_2 and 5% CO_2, maintaining the pH at 7.4. This procedure specifically labels enteric serotonergic neuronal cell bodies and processes (Gershon and Sherman, 1982a, b). After incubation the tissue was rinsed for 5 min in warm Krebs solution (50 ml) containing desmethylimipramine and pargyline to remove extracellular [^{3}H]-5-HT (Gershon and Ross, 1966a, b). Iced fixative (2.5% glutaraldehyde and 3% sucrose in a 0.1 M sodium

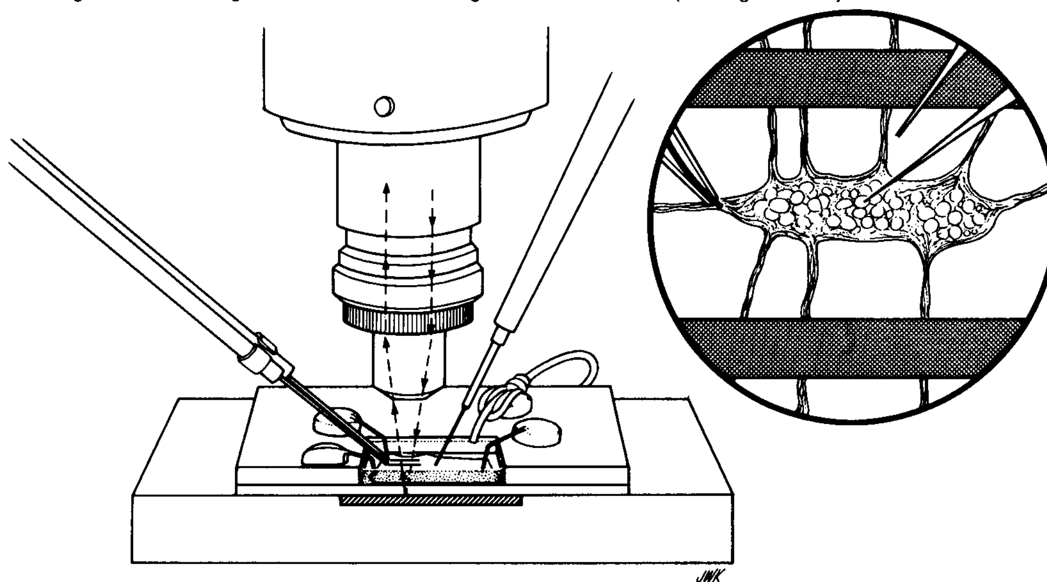

Figure 1. Apparatus used to record from neurons of the myenteric plexus. The longitudinal muscle-myenteric plexus is stretched flat and held with magnetized clips in a chamber with a transparent bottom through which oxygenated Krebs solution at 37°C is circulating. Light (pathway indicated by *dashed line*) is directed through the tissue from above and reflected back from a mirrored surface under the recording chamber. A ganglion of the myenteric plexus (*inset*) is immobilized by means of pressure feet (at *left* on the *diagram* and *stippled* in *inset*). Note that this method leaves intact at least some interganglionic fiber tracts. One or more of these were selected for stimulation with the platinum extracellular electrode (at *left* of *inset*).

phosphate buffer at pH 7.4) (Gershon and Ross, 1966a, b) was poured into the dish. The fixative was changed once and fixation was continued at 4°C for 2 to 12 hr. Little movement of intracellular [^{3}H]-5-HT occurs when hypertonic aldehyde fixation is used (Fishman and Gershon, 1964; Gershon and Ross, 1966a, b). This fixation prevents extraction of [^{3}H]-5-HT by any of the solutions needed for the subsequent steps of tissue processing.

Demonstration of HRP. After fixation, the preparations were rinsed very thoroughly with sucrose-containing phosphate buffer to remove residual glutaraldehyde. A solution containing 3,3′-diaminobenzidine (1 mg/ml; Sigma) and H_2O_2 (0.01%; Sigma) in Tris-HCl buffer (pH 7.4; Sigma) was then added to the dish. The tissue was agitated in the dark for 30 min at room temperature. Following examination, the tissue was detached from the dish and postfixed, in suspension, with 2% OsO_4 containing 3% sucrose in 0.1 M sodium phosphate buffer at pH 7.4 at 4°C for 1 hr before processing for electron microscopy.

Embedding. The fixed preparations were rinsed in buffer and dehydrated by serial transfer through an ascending series of alcohols at 0°C. After dehydration, the preparation was cleared in propylene oxide and embedded in epoxy resin (Epon 812) as a sandwich between two disks of Sylgard (35 mm in diameter and 10 mm in thickness). The Sylgard was then peeled away from the polymerized resin to leave a transparent flat wafer of plastic containing the tissue. Wafers were examined microscopically in order to locate the ganglion containing the cells that had been injected with HRP. These ganglia were studied and photographed in position as a whole mount in plastic. The photographed ganglia were then cut out and glued to a dummy block of Epon for thick and thin sectioning. By embedding the preparation flat in this way, it was possible to cut sections through the plane of the myenteric plexus and thus to maximize the number of ganglion cells included in each section.

Radioautography. For light microscopic radioautography, sections were cut at 0.5 μm through ganglia containing HRP-filled cells and mounted on slides that had been coated with chromium alum gelatin. The slides were dipped in photographic emulsion (Ilford L-4; diluted 1:1 with H_2O), allowed to dry, and then placed in light-tight slide boxes with a dessicant. After 1 or 2 weeks of exposure at 4°C the slides were developed in Kodak D-19 (5 min), fixed with Kodak Fixer (5 min), washed, and stained with toluidine blue.

For electron microscopic radioautography, ultrathin silver sections were cut with a diamond knife and placed on collodion-coated glass slides. The sections were stained on the slides with uranyl acetate and lead citrate. After staining, a thin layer of carbon was evaporated over the sections. The slides were then coated in the dark with a monolayer of Ilford L-4 emulsion and placed in a light-tight box under an atmosphere of dry CO_2. After 10 to 14 days of exposure, the slides were developed with Kodak Microdol-X for 3 min. Following fixation, the collodion film containing the sections and the overlying emulsion was scored and floated off. The sections were picked up on a slot grid and examined in a JEOLCO JEM 100C electron microscope.

Results

Twenty-two cells were fully analyzed. This analysis included physiological classification as to cell type, testing for the presence or absence of a slow EPSP upon stimulation of an interganglionic connective, intracellular filling with HRP, incubation with [^{3}H]-5-HT, and electron microscopic radioautography on near-serial sections of the entire cell. In addition to this group, 25 additional cells were classified physiologically and filled with HRP for light microscopic analysis alone.

Classification of cells

Type I/S cells were defined as those cells that spiked repetitively with a frequency that was proportional to stimulus strength when injected intracellularly with depolarizing current pulses. No AH was seen after a spike. Examples of records obtained from a type I/S cell are shown in Figure 2. Many of these cells exhibited frequent spontaneous fast EPSPs. Of the 22 cells fully examined, 6 were classified as type I/S. In addition, 7 cells classified as type I/S were filled with HRP and analyzed light microscopically, so that a total of 13 type I/S cells were studied.

Type II/AH cells were defined as those cells that failed to spike repetitively (in the absence of a slow EPSP) when injected intracellularly with depolarizing current pulses. Spike frequency was not proportional to the strength of the injected current. Spontaneous fast EPSPs were not seen. All of these cells displayed a prominent AH which was their most distinguishing characteristic. Input resistance (<50 megohms) was lower than that of type I/S cells; however, because of the extremely high resistance of the HRP-filled electrodes, accurate measurement of input resistance was difficult. Examples of records obtained from a type II/AH cell are shown in Figure 3. Ten of the total sample of 22 fully examined cells were classified as type II/AH. Six additional type II/AH cells were filled with HRP for light microscopic examination only, so that a total of 16 type II/AH cells were studied.

No attempt was made to classify non-spiking cells as types III, IV, or glia. Instead, one category of non-spiking cell, NS, was established. These cells had stable resting membrane potentials of about −70 mV. Some occasionally displayed a fast EPSP upon stimulation of an interganglionic connective. However, it was not possible to evoke this response often enough to make it possible on physiological grounds alone (see below) to distinguish non-spiking neurons from glia. Six of the 22 cells completely examined were NS cells, and 12 additional cells classified as NS were filled with HRP for light microscopic analysis alone, so that a total of 18 NS cells were studied.

The slow epsp

Slow EPSPs were seen in this study only in type II/AH cells (6 of 16). The slow EPSP was elicited by 1- to 5-sec trains of 200-μsec pulses applied to these connectives at 10 Hz. Examples of slow EPSPs are shown in Figure 4. Note the repetitive spiking of type II/AH cells during the duration of the slow EPSP. The physiological criteria for cell classification plus the slow EPSP were used to divide the 22 HRP-injected and [^{3}H]-5-HT-labeled specimens into four classes for subsequent morphological analysis. These classes were type II/AH cells with a slow EPSP (5 cells); type II/AH cells not displaying a slow EPSP (5 cells); type I/S cells with no slow EPSP (6 cells); and NS cells with no slow EPSP (6 cells).

Morphological analysis

Type II/AH cells. Injected type II/AH cells showed a limited array of morphologies. At the light microscopic level these cells were most often multipolar with smooth appearing somal outlines and extremely long, thin processes (Fig. 5). In accordance with the report of Hodgkiss and Lees (1983), there were an average of about five or six processes per neuron, of which the majority initially extended in the circumferential direction (along the axis of the ganglia); however, close examination of these processes revealed that many apparently circumferential processes passed out of their ganglion of origin and, having done so, turned to run with an oral-aboral orientation. In addition, such tracing of filled fibers revealed that some processes were damaged in making the *in vitro* preparations. These damaged processes sometimes terminated in swollen bulbous expansions that, when examined electron microscopically (Fig. 6), appeared to be formed by cut fibers that had resealed. The resealed processes were expanded and were filled with vesicles, membranous structures, and mitochondria that had evidently accumulated in them during the course of the experiments. These bulbous structures were called retraction bulbs. Two main causes of fiber damage were identified. Only one of these consistently resulted in the formation of retraction bulbs. One type of damage that rarely led to retraction bulb formation was due to the crushing of orally-aborally oriented interganglionic connectives by the pressure feet needed to immobilize the preparation to permit intracellular penetrations to be made. The second type of damage, that seemed invariably to lead to the formation of retraction bulbs, was the tearing of communicating branches running from myenteric ganglia, not to other ganglia of the myenteric plexus, but to other layers of the bowel dissected away in making the longitudinal muscle-myenteric plexus preparations. These deeper layers of the gut include the circular muscle, the deep muscle plexus, and the submucosal plexus. Retraction bulbs thus were found at the interface between intact and dissected tissue. Nevertheless, despite these limitations due to unavoidable specimen

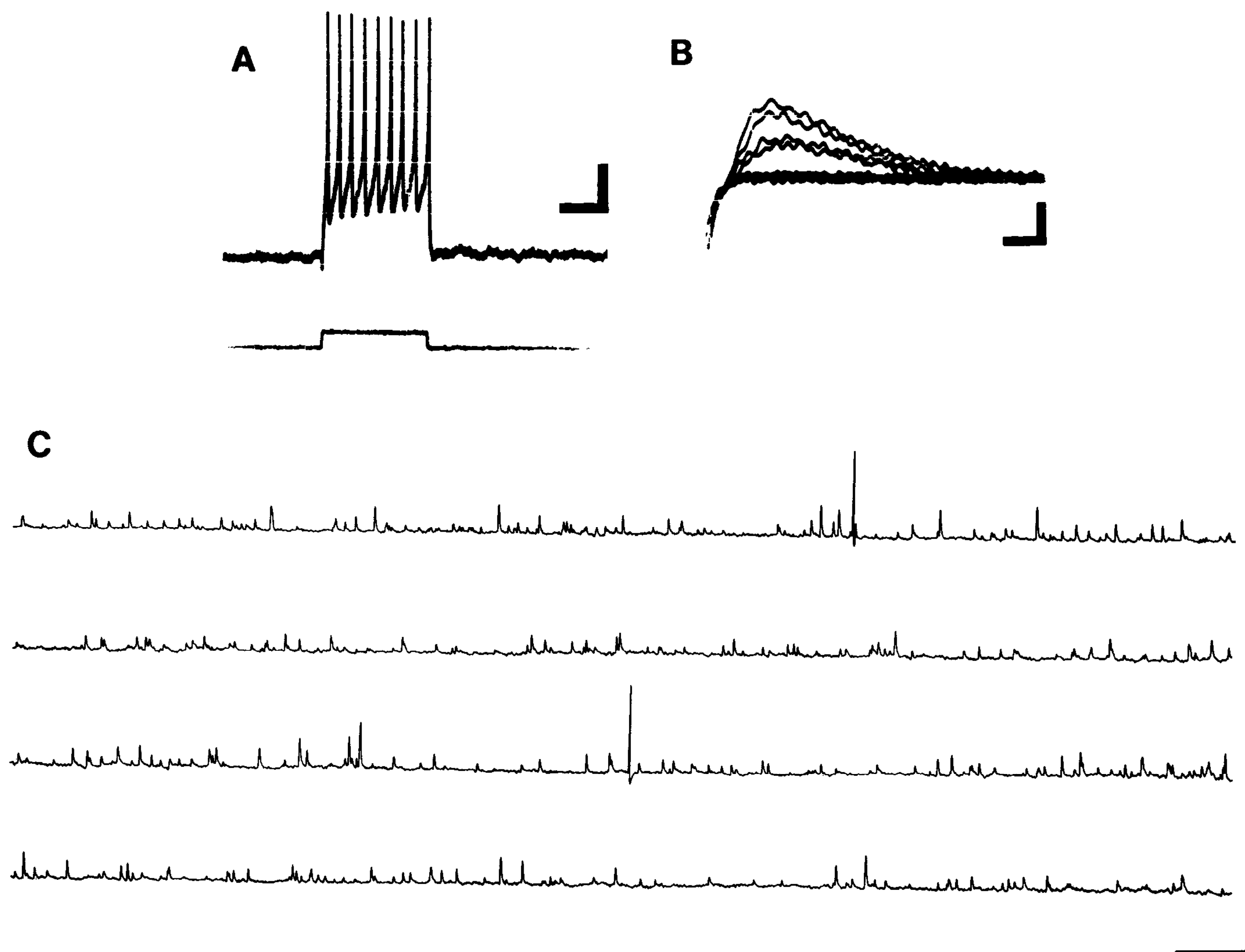

Figure 2. Typical recordings obtained from type I/S cell of the myenteric plexus. *A, Lower trace:* Current monitor; *upper trace:* intracellular membrane potential. Injection of depolarizing current through the recording micropipette causes repetitive spiking. *B,* Stimulation of an interganglionic connective with an extracellular electrode induces fast EPSPs. *C,* Spontaneous synaptic input (EPSPs) can be recorded from these cells. In this continuous record, two spontaneous action potentials also appear. *Calibrations: A,* 100 msec/10 mV/0.5 nA; *B,* 5 msec/10 mV; *C,* 1 sec/20 mV. The cell from which this record was obtained was injected with HRP and is shown in Figure 11, *A* and *B*.

damage, excellent visualization of the intraganglionic extent and branching of HRP-filled neurons was obtained, and nondamaged processes could often be traced in the oral-aboral direction through two to three rows of ganglia.

At the electron microscopic level, considerable distortion of the intracellular ultrastructure appeared to have occurred in filled cells, either by the recording electrode, the process of filling, or more likely, the cytochemical reaction necessary to demonstrate the HRP. Vacuoles were found and the interior of cells was nearly opacified by the dense HRP reaction product. Nevertheless, cellular outlines remained quite clear, revealing in the type II/AH cells numerous somal spines and other thin, short, irregular projections from the cell bodies. Moreover, no damage had apparently been caused by the filling process to neighboring cells or to the synaptic inputs received by filled cells. In addition, the preservation of the intraganglionic architecture and the ultrastructure of unfilled cells was relatively good despite the long maintenance of the surviving tissue *in vitro* during the experiments. This favorable preservation made it possible to cut through ganglia, retaining for examination as many of the near-serial sections as possible, and to trace filled cell outlines and processes through entire ganglia. It was not possible, however, to study the detailed ultrastructure of the interior of neurons injected with HRP.

Radioautography done with [^{3}H]-5-HT revealed that both soma and processes of type II/AH cells were contacted by varicosities that labeled with [^{3}H]-5-HT (Figs. 7 and 8). In no instance was an HRP-filled process doubly labeled by [^{3}H]-5-HT. Not all of the contacts between [^{3}H]-5-HT-labeled varicosities and HRP-filled cells and processes exhibited clear pre- and postsynaptic membrane specializations. The dense HRP reaction product tended to obscure these specializations, and they are rare and rudimentary, in any case, in the ENS (Baumgarten et al., 1970; Cook and Burnstock, 1976; Manber and Gershon, 1979); nevertheless, favorable sections did reveal evidence of synaptic specialization for many of these contacts. In evaluating the experiment, varicose contacts were recorded as being present if [^{3}H]-5-HT-labeled varicosities made direct contact with HRP-filled cells or processes and there was no separation between them by either a gap of greater than 25 nm or an intervening glial cell process. Of the five fully analyzed type II/AH cells that displayed a slow EPSP, all turned out to be contacted by [^{3}H]-5-HT-filled varicosities. All parts of the HRP-filled type II/AH cells contained membrane covered by these varicosities, and prominent examples were found of axoaxonic, axosomatic, and axodendritic [^{3}H]-5-HT-type II/AH cell contacts. In one cell, the initial segment of the axon was surrounded by a necklace of [^{3}H]-5-HT-labeled varicosities (Fig. 7C).

Of the five fully analyzed type II/AH cells in which a slow EPSP

Figure 3. Typical intracellular recordings obtained from a type II/AH cell of the myenteric plexus. *A*, Antidromic activation of the cell by stimulation of its axon in an interganglionic connective with an extracellular electrode. Action potentials are followed by a prolonged AH during which antidromic activation fails to elicit action pontentials. *B*, Injection of constant depolarizing current pulses (current record on *lower trace*) induces action potentials followed by an AH. Current pulses injected during the AH fail to elicit action potentials. *C*, Intracellular injection of a long current pulse (current record on *lower trace*). Two action potentials can be seen at the onset of current injection followed by a decrease in input resistance during which there is spike failure. *D*, A slow EPSP has been elicited in the same cell by fiber tract stimulation. During the slow EPSP the AH is diminished, input resistance increases, and the cell now spikes repetitively throughout the duration of injection of a depolarizing current pulse (current record on *lower trace*). *Calibrations: A*, 2 sec/10 mV; *B*, 2 sec/20 mV/1 nA; *C* and *D*, 200 msec/10 mV/1 nA. The cell from which this record was obtained was injected with HRP and is shown in Figure 7.

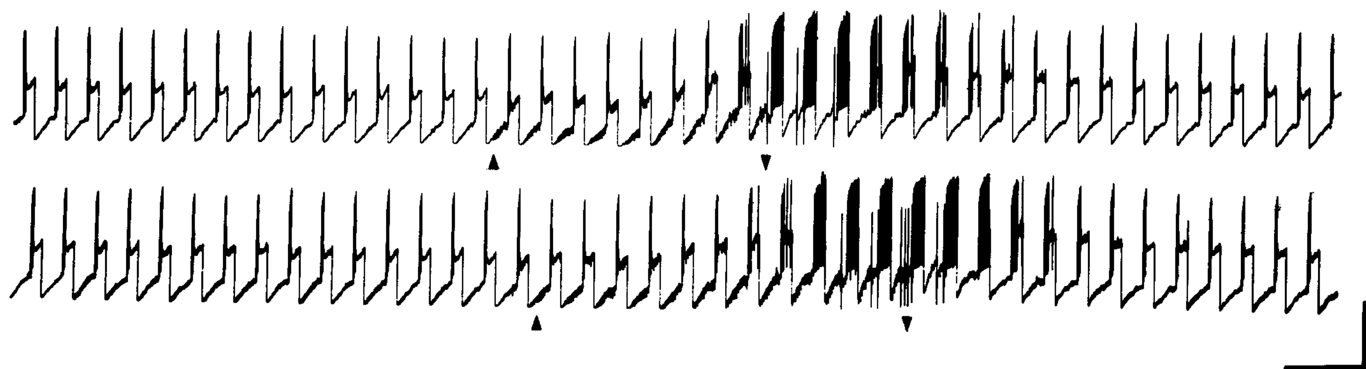

Figure 4. Slow EPSPs are induced by stimulation of an interganglionic connective (between *arrowheads*). A continuous record is shown. Constant depolarizing current pulses are injected through the recording micropipette. Spikes are elicited only at the onset of current injection except during the slow EPSPs (see also Fig. 3, *C* and *D*). During the slow EPSP there is an increased excitability of the type II/AH cell that outlasts fiber tract stimulation. *Calibration:* 5 sec/25 mV). The cell from which this record was obtained is illustrated in Figure 7.

could not be elicited, two received contacts by varicosities labeled by [^{3}H]-5-HT. No morphological features were observed that distinguished type II/AH cells without a slow EPSP from cells of this class that did show this response, except for the higher incidence of contacts by [^{3}H]-5-HT-labeled varicosities in the slow EPSP group.

Type I/S cells. Injected type I/S cells showed varying morphologies; however, the most frequently encountered cell was one that had many short, stubby dendrites extending from the cell soma and a single long, thin process (thought to be the axon) that projected with an oral-aboral orientation for a considerable distance out of its ganglion of origin (Fig. 9). All cells with short stubby dendrites of this configuration were found to be type I/S cells; however, in addition to this form of type I/S cell, other morphologies included cells that resembled type II/AH cells (above) and cells with several long, fat, knobby intraganglionic processes (Fig. 10). As with type II/AH cells, damage was sometimes done to the processes of type I/S cells by the pressure feet used in recording from them and by dissecting the longitudinal muscle-myenteric plexus preparation. Again, the latter type of damage gave rise to the formation of retraction bulbs.

At the electron microscopic level, type I/S cells, like type II/AH cells, displayed obvious cellular outlines (Fig. 11*A*) and received many synaptic contacts on both somata (Fig. 11, *B* to *D*) and dendrities (Fig. 11*E*). Many of the processes of the type I/S cells extended to the edges of the ganglion containing them and exposed surfaces not covered by glial processes to the periganglionic basal lamina. In sections, an extremely complex pattern of arborization of the dendrites of type I/S cells was revealed. In addition to the short stubby dendrites visible in the light microscope, a large number of finer secondary and tertiary processes could also be discerned. These processes were scattered about in the ganglionic neuropil

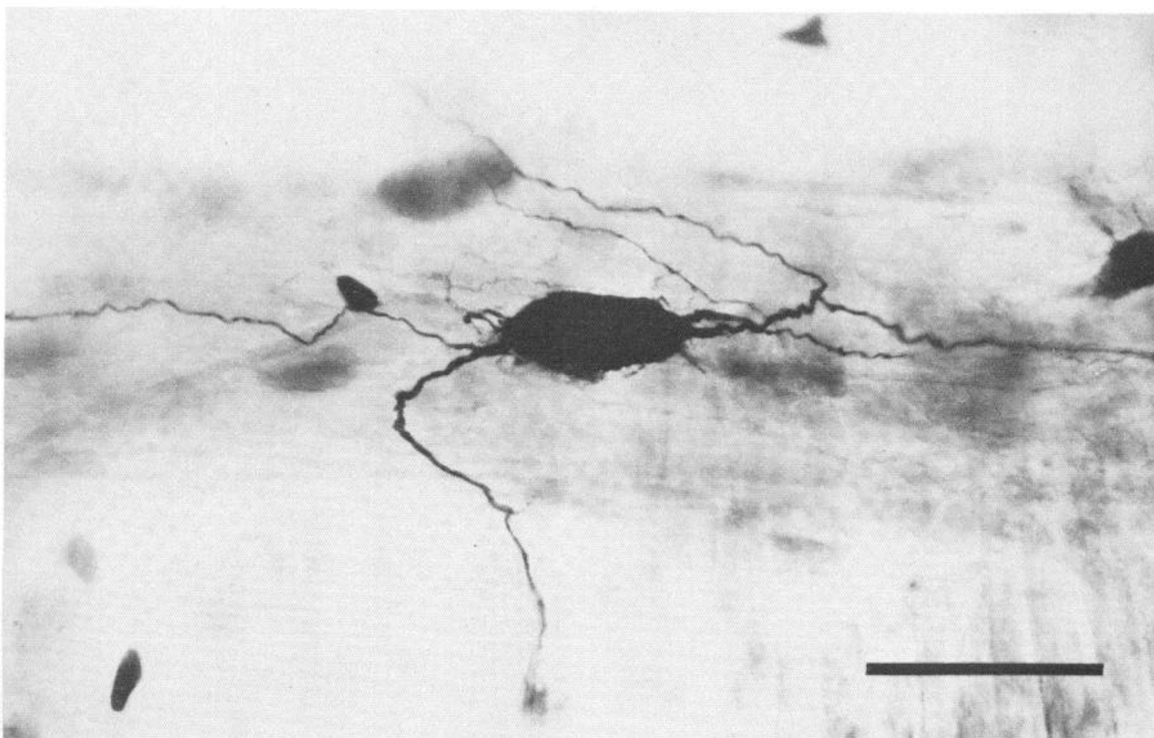

Figure 5. An identified type II/AH cell injected with HRP through the recording micropipette. The cell is multipolar and extends many fine neurites. Long, sometimes branching, processes extend circumferentially (*horizontal axis*) before leaving the ganglion; other processes extend in the oral-anal direction (*vertical axis*). *Bar* = 50 μm.

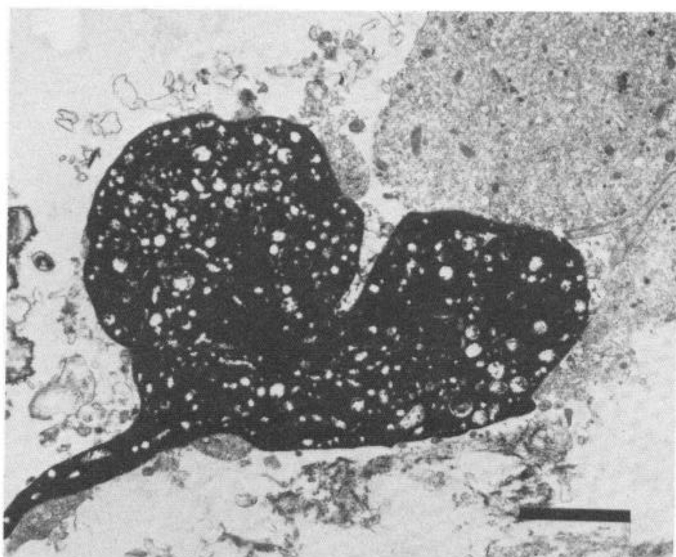

Figure 6. An electron micrograph of the cut end of an HRP-filled process of an identified type II/AH cell that extended out of the ganglion toward the circular muscle which was dissected away in making the preparation. Note the buildup of membrane-limited vesicles and the expanded bulbous end of the damaged neurite. These damaged processes appear button-like at the light microscopic level and are called "retraction bulbs." *Bar* = 2 μm.

and were present in much of the ganglion of origin. [^{3}H]-5-HT-labeled varicosities were found in contact with some type I/S cells. Of the population of six type I/S cells fully analyzed, two received [^{3}H]-5-HT-labeled contacts. Neither received as extensive a coverage with such varicosities as did type II/AH cells.

NS cells. Injected NS cells were easily divided on the basis of their appearance in light and electron micrographs into neuronal and glial categories. NS neurons (4 of 18) ranged in outline from cells with smooth somata (Fig. 12*B*) and small numbers of processes to cells with highly irregular somata that bristled with many short processes (Fig. 12*A*); however, all had one long process, apparently an axon, that extended, with occasional branches, for long distances through the ganglionic neuropil. Occasionally, these processes ended in varicose terminals at the boundary of the myenteric plexus with the periganglionic basal lamina. In contrast, glia (14 of 18) were characterized by a central soma giving rise to a complex astrocyte-like arbor composed of a multiplicity of processes (Fig. 13; see also Gabella, 1971). Some of these processes terminated in feathery expansions, or end-feet. At the electron microscopic level, the NS neurons were found to receive synapses (Fig. 14), whereas glia did not. The synapses on NS cells were both axosomatic and axodendritic in type. Glial processes, filled with HRP, were often found wrapped around neuronal processes of all sorts (Fig. 15*A*), including terminal varicosities (Fig. 1*B*). None of the HRP-injected cells, neither neurons nor glia, were found in contact with varicosities or processes labeled by [^{3}H]-5-HT (three neurons, three glia fully analyzed).

Correlation of [^{3}H]-5-HT input with physiological classification of cell type

Results correlating the radioautographic detection of [^{3}H]-5-HT-labeled contacts with HRP-marked, physiologically identified cells are summarized in Table I. The incidence of [^{3}H]-5-HT labeling of contacts on cells with a slow EPSP was found to be significantly greater than the incidence of [^{3}H]-5-HT-labeled contacts on cells without a slow EPSP ($p < 0.005$ is the two-tailed probability estimated by Fisher's exact test). If cells morphologically classified as glia were excluded retroactively as cells that could neither show a slow EPSP nor receive serotonergic synapses, the incidence of [^{3}H]-5-HT labeling of varicosities on neurons with a slow EPSP was still significantly greater than that on neurons that did not show the response ($p < 0.02$). The incidence of [^{3}H]-5-HT labeling of contacts on all type II/AH cells (without regard to the slow EPSP) is greater than that on other cell types ($p < 0.05$ being the two-tailed probability estimated by Fisher's exact test).

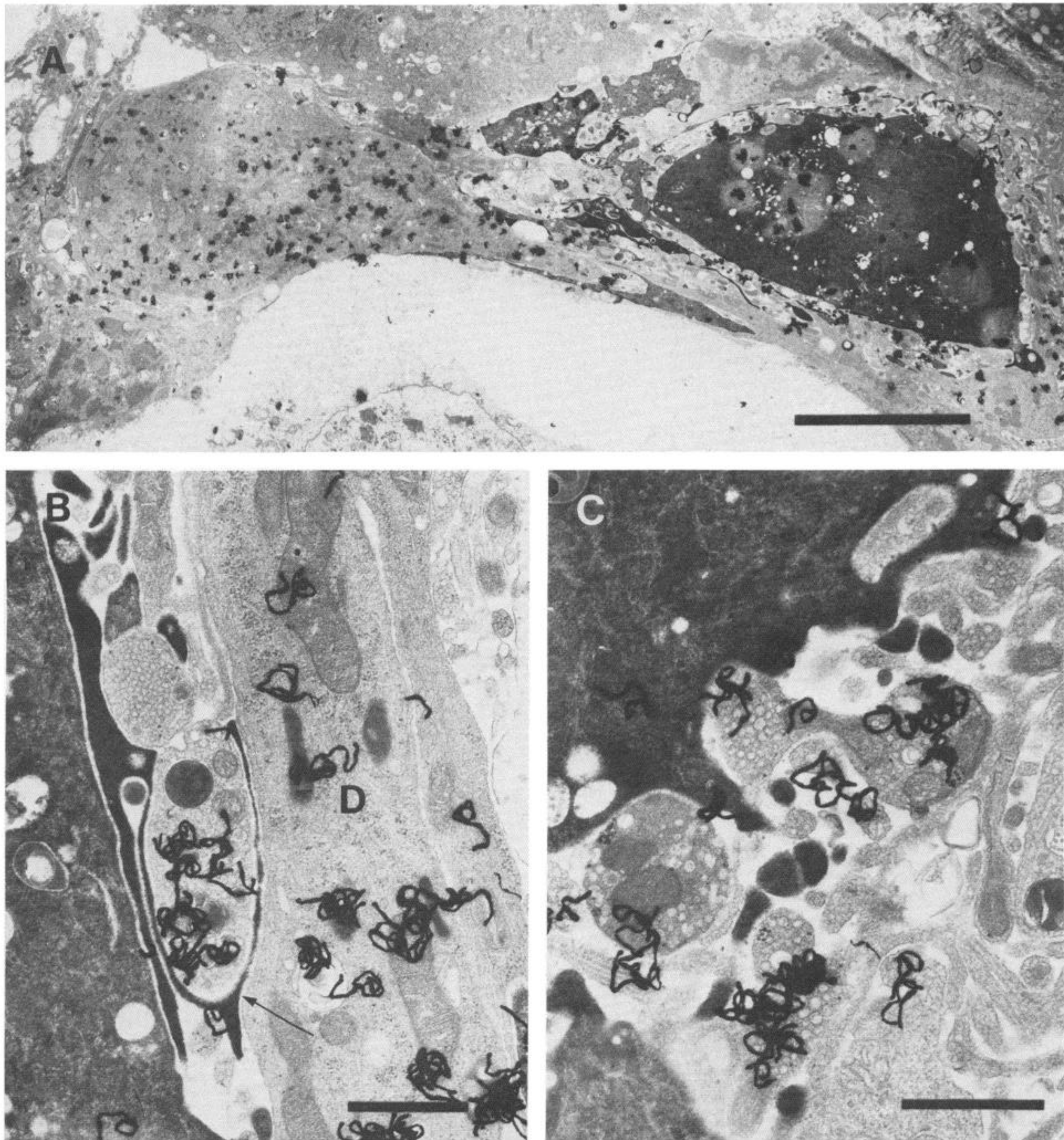

Figure 7. Electron micrographs of an identified type II/AH cell filled with HRP and serotonergic elements radioautographically labeled with [^{3}H]-5-HT. *A,* A serotonergic neuron (*left*) is located in close proximity to the dark, HRP-filled, type II/AH cell (*right*). *B,* The HRP-filled cell is at the extreme *left* and a [^{3}H]-5-HT-labeled dendrite (*D*) appears on the *right*. Between them there is a recurrent HRP-filled process that contacts the type II/AH cell's soma and also partially encircles a heavily [^{3}H]-5-HT-labeled varicosity (*arrow*). The [^{3}H]-5-HT-labeled varicosity contains large dense-cored vesicles and small clear vesicles as well as small mitochondria. Note that the vesicular content of the serotonergic varicosity differs from that of the unlabeled varicosity above it that also contacts the HRP-filled neurite of the type II/AH cell. *C,* A necklace of [^{3}H]-5-HT-labeled varicosities surrounds the initial segment of the axon (traced further in serial sections not shown) of the HRP-filled type II/AH cell. The serotonergic varicosities contact the type II/AH cell in this region. *Bars: A,* 10 μm; *B* and *C,* 1 μm. Physiological records from this cell are shown in Figures 3 and 4.

Discussion

At the outset of this study the hypothesis was framed that 5-HT is one of the transmitters responsible for the mediation of slow EPSPs in the myenteric plexus. To test this hypothesis, cells of the plexus were physiologically identified, marked by intracellular injection of HRP, subjected to specific labeling with [^{3}H]-5-HT, and analyzed electron microscopically. Near-serial sections were cut and the previously identified injected cells were simultaneously visualized along with serotonergic terminals by combining the cytochemical detection of HRP with the radioautographic demonstration of [^{3}H]-5-HT. It was reasoned that the hypothesis would be supported if cells that manifested the slow EPSP actually received serotonergic contacts, especially if they did so to a greater extent than cells of the plexus, which did not manifest a slow EPSP. These other cells were viewed as a control population for these purposes. On the other hand, if cells that exhibited a slow EPSP were not to receive serotonergic contacts, or if they were to do so to a significantly lesser extent than other cells of the myenteric plexus, the hypothesis that 5-HT is a mediator of the slow EPSP could be rejected. A

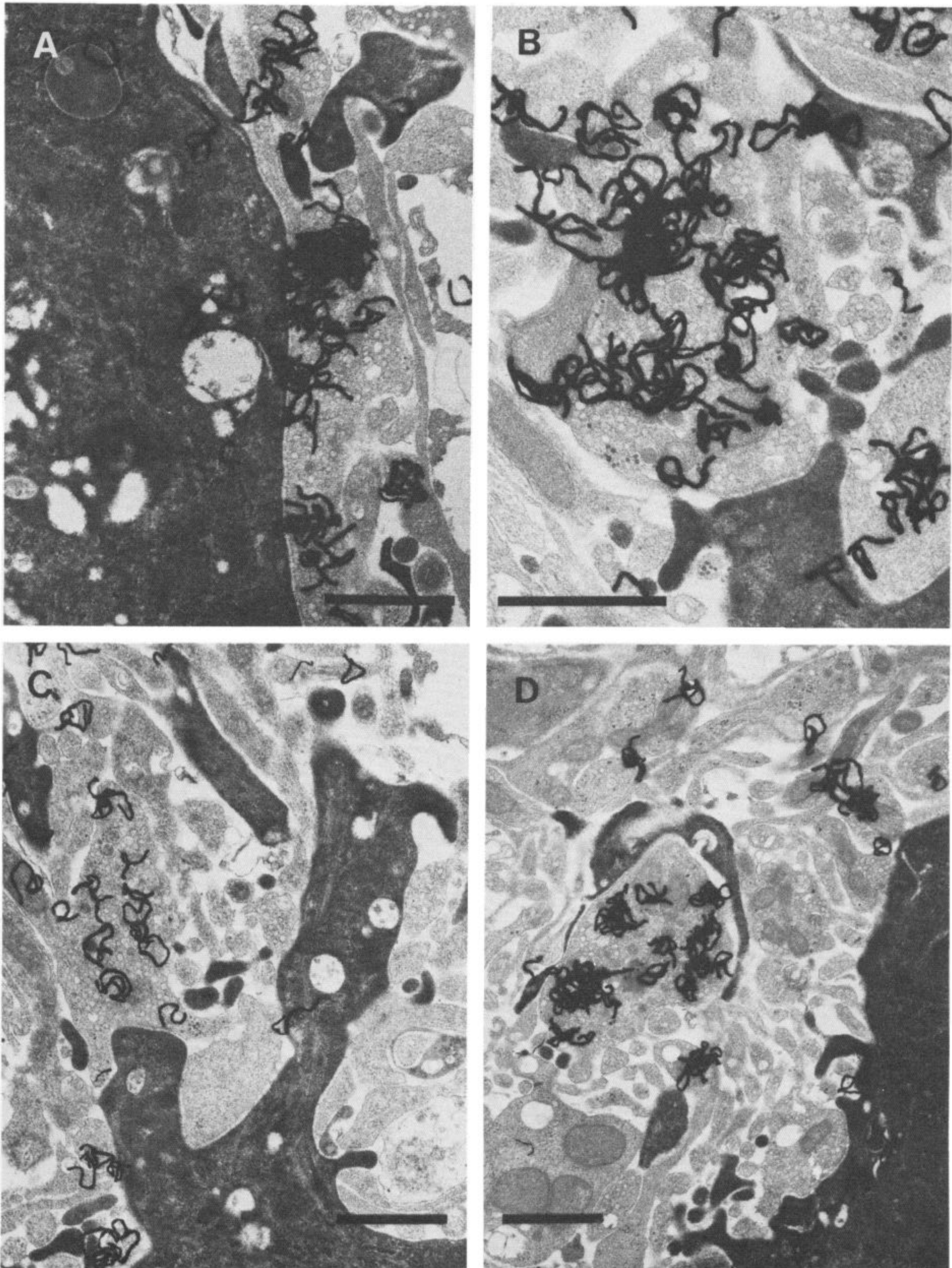

Figure 8. Illustrations of contacts made by [^{3}H]-5-HT-labeled serotonergic varicosities with an HRP-filled type II/AH cell. *A*, Axosomatic contact; *B*, axodendritic contact; *C*, contact with a somal spine; *D*, axodendritic contact. Note the similar vesicular content of all [^{3}H]-5-HT-labeled varicosities. *Bars* = 1.0 μm.

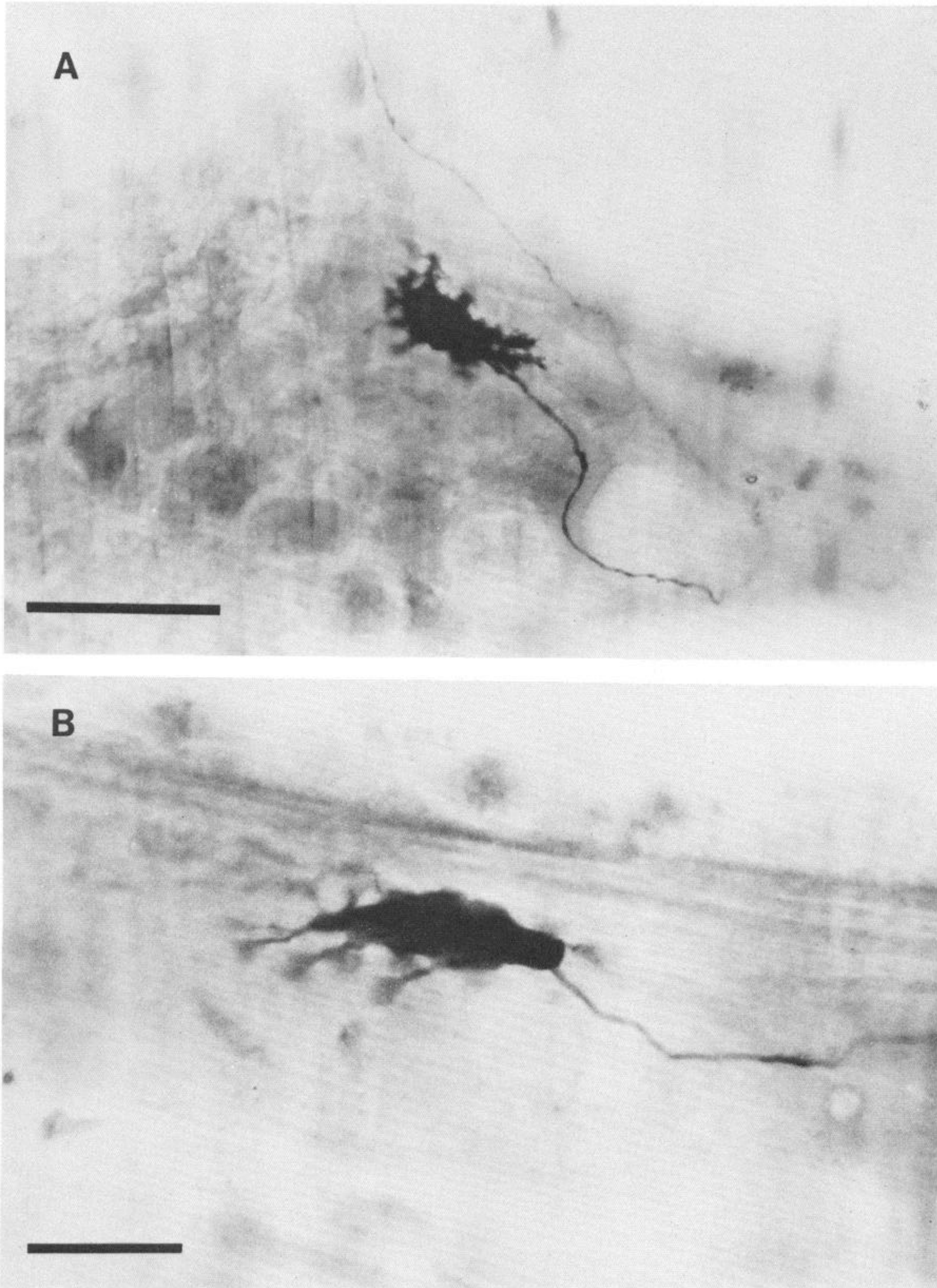

Figure 9. Two identified type I/S cells of the myenteric plexus injected with HRP through the recording micropipette. The cells have many short, stubby, paddle-shaped dendrites and a single long process, probably the axon, that runs circumferentially (longitudinal axis) before turning to project in the oral-anal direction. (vertical axis) out of the ganglion of origin. *Bars: A* = 50 μm; *B* = 25 μm.

secondary purpose of the study was to correlate neuronal morphology with physiological identification of enteric neurons and to examine the relationship of all classes of enteric neurons to the enteric sertonergic innervation.

All cells that exhibited a slow EPSP were found to be type II/AH cells and all received terminals labeled by [^{3}H]-5-HT. Because of the difficulty of the experiments and the long time necessary to complete the morphological analyses, relatively few cells could be fully analyzed. There were only five cells in the slow EPSP group; however, the probability of obtaining serotonergic synapses on 5 of 5 cells

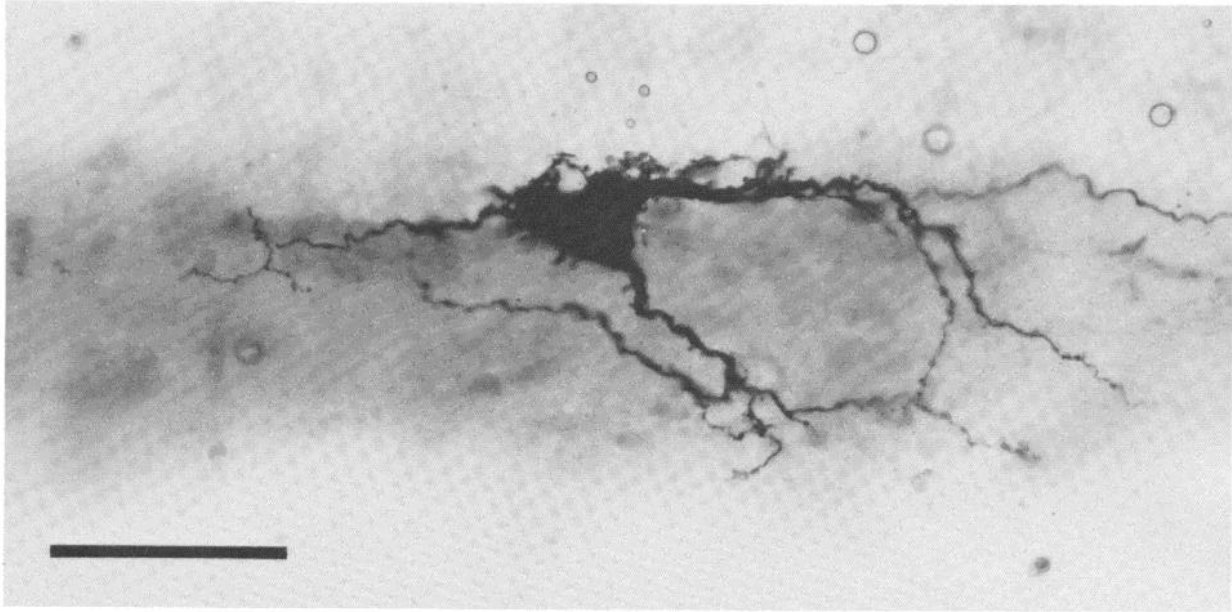

Figure 10. An identified type I/S cell injected with HRP through the recording micropipette. This cell has several long, knobby processes that branch and are mainly intraganglionic. Note the difference in morphology between this type I/S cell and the type I/S cells illustrated in Figure 9. *Bar* = 50 μm.

with a slow EPSP but on only 4 of 17 cells without this response is <0.005. It can be concluded, therefore, that cells that manifest a slow EPSP do receive a serotonergic input and do so significantly more frequently than cells that do not. In fact, the type II/AH cell category as a whole was significantly more likely to receive serotonergic contacts than the rest of the myenteric ganglion cell population ($p < 0.05$). It seems likely that the high proportion of type II/AH cells receiving a serotonergic input is related to the slow EPSPs of some of the cells of this set. This response was only exhibited by type II/AH cells, and some of the five type II/AH cells that were analyzed but did not manifest a slow EPSP probably failed to do so because of technical difficulties encountered in eliciting the response. For example, the relevant fiber tract carrying the input might not have been found and stimulated, or the tract might have been destroyed in setting up the preparation. Damage to fiber tracts did occur as a result of dissection or crushing by the pressure feet which were needed to immobilize the ganglia to obtain intracellular impalements. Morphological evidence of such damage was present in electron micrographs of the experimental tissue. In addition, connectives were deliberately stimulated to avoid activating SP-containing fibers. Since there are also connections between serotonergic cells and other cells of the same ganglion, it is likely that some of these intraganglionic serotonergic inputs to type II/AH cells would not have been stimulated in our experiments. In fact, one type II/AH cell that was immediately adjacent to a serotonergic neuron demonstrated frequent spontaneous slow EPSPs. It is thus possible that one or both of the five type II/AH cells in which a slow EPSP could not be elicited, but which did receive a serotonergic input, were actually capable of a slow EPSP and might have shown one if the appropriate input had been stimulated.

One aspect of the current study which should be noted is that slow EPSPs were not observed in type I/S cells. These potentials were seen neither in the small group of six type I/S cells examined electron microscopically, nor in the additional type I/S cells examined only by light microscopy. In fact, using the conditions described in the present study, slow EPSPs were not observed in type I/S cells, even in the 4-fold larger sample of cells that were not properly filled and thus were not morphologically examined. These data reproduce the observations of Wood and Mayer (1979a) (see also the review by Wood, 1981). In contrast to these results, Johnson et al. (1980a) have reported a higher proportion of type I/S cells than of type II/AH cells with a slow EPSP. These differing findings are probably attributable to differences in methods of stimulation. The current study utilized fiber tract stimulation, as did Wood and Mayer (1979a), and obtained similar results. Johnson et al. (1980a) utilized focal intraganglionic stimulation. As noted earlier, different ganglionic inputs probably are activated selectively by the two techniques. Focal ganglionic stimulation seems to result in a much higher percentage of cells that display a slow EPSP (see also Bornstein et al., 1984). An additional possibility is that there may be different types of slow EPSP with different mediators. North and Tokimasa (1982) have reported that a single pulse stimulus could elicit a slow EPSP in type I/S cells but not in type II/AH cells. The single-pulse, slow EPSPs in type I/S cells, but not the slow EPSPs evoked by repetitive stimulation in type II/AH cells, were abolished by hyoscine. The single-pulse, slow EPSPs in type I/S cells, therefore, are presumably cholinergic, whereas the slow EPSPs not blocked by hyoscine in type II/AH neurons are noncholinergic. The current experiments are only relevant to the noncholinergic response of type II/AH cells.

The marker used in the current study to define serotonergic elements, [^{3}H]-5-HT, is an exogenous one. Its specificity depends on the underlying premises that nonserotonergic elements do not take up the label, whereas serotonergic neurons and neurites do so. There is considerable evidence to support both premises. Two types of peripheral neurons are known to take up [^{3}H]-5-HT, serotonergic and noradrenergic (Bertler et al., 1964; Jaim-Etcheverry and Zieher, 1969; Thoa et al., 1969; Zieher and Jaim-Etcheverry, 1971; Drakontides and Gershon, 1972); however, the affinity of noradrenergic axons for [^{3}H]-5-HT is much less than that of serotonergic neurons and their processes (Thoa et al., 1969; Gershon and Altman, 1971; Gershon et al., 1976). Moreover, the nonspecific uptake of [^{3}H]-5-HT by noradrenergic axons can be eliminated by inhibiting the norepinephrine uptake mechanism without affecting uptake of [^{3}H-5-HT by serotonergic elements (Gershon et al., 1976; Gershon and Jonakait, 1979). Critically, it has been demonstrated that exogenous 5-HT does not enter noradrenergic or other nonserotonergic axons under the conditions (low 5-HT concentration, desmethylimipramine present) used in the current investigation (Gershon and Sherman, 1982a, b). When endogenous 5-HT is depleted from the myenteric plexus with reserpine, for example, incubation with exogenous 5-HT restores the control pattern of 5-HT fibers detected immunocytochemically, indicating that only the fibers that originally contained endogenous 5-HT took up that amine (Costa et al., 1982). Moreover, treatment of mice with the indolic neurotoxin, 5,7-dihydroxytryptam-

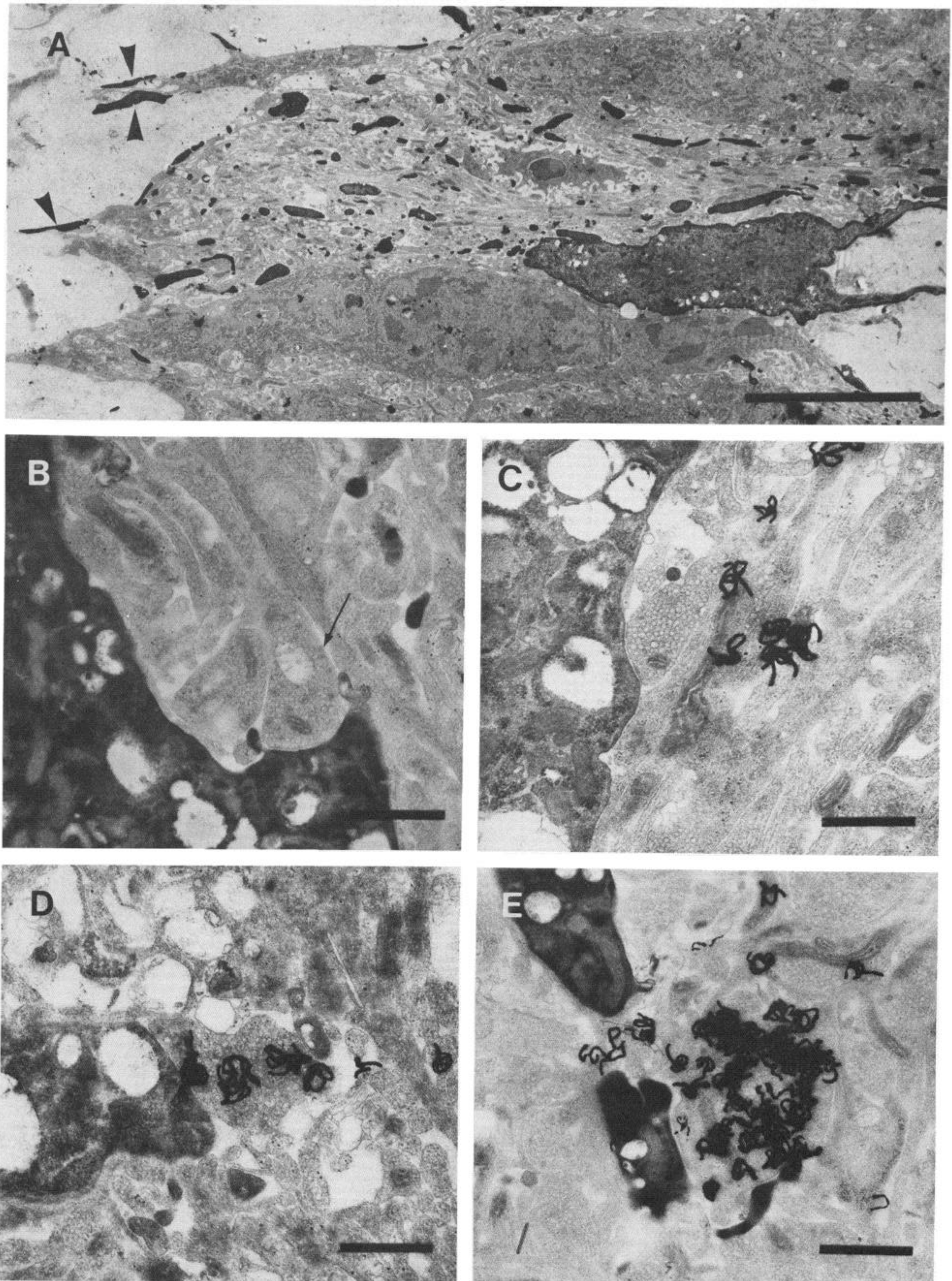

Figure 11. Electron micrographs of a type I/S cell filled with HRP and serotonergic neurites labeled with [^{3}H]-5-HT. *A*, The soma of the type I/S cell and the numerous HRP-filled profiles of its intraganglionic processes can be seen. Note that many processes contact the periganglionic connective tissue space (*arrowheads*). *B*, A varicosity not labeled by [^{3}H]-5-HT contacts a somal projection of the type I/S cell (*arrow*). Physiological records of this cell are shown in Figure 2. *C*, An unlabeled axosomatic synapse is close to [^{3}H]-5-HT-labeled neurites. *D*, A [^{3}H]-5-HT-labeled varicosity contacts in HRP-filled somal projection of the type I/S cell. *E*, varicosity heavily labeled with [^{3}H]-5-HT makes an axodendritic contact with an HRP-filled type I/S cell. *Bars: A* 20 μm; *B* to *E* = 1.0 μm.

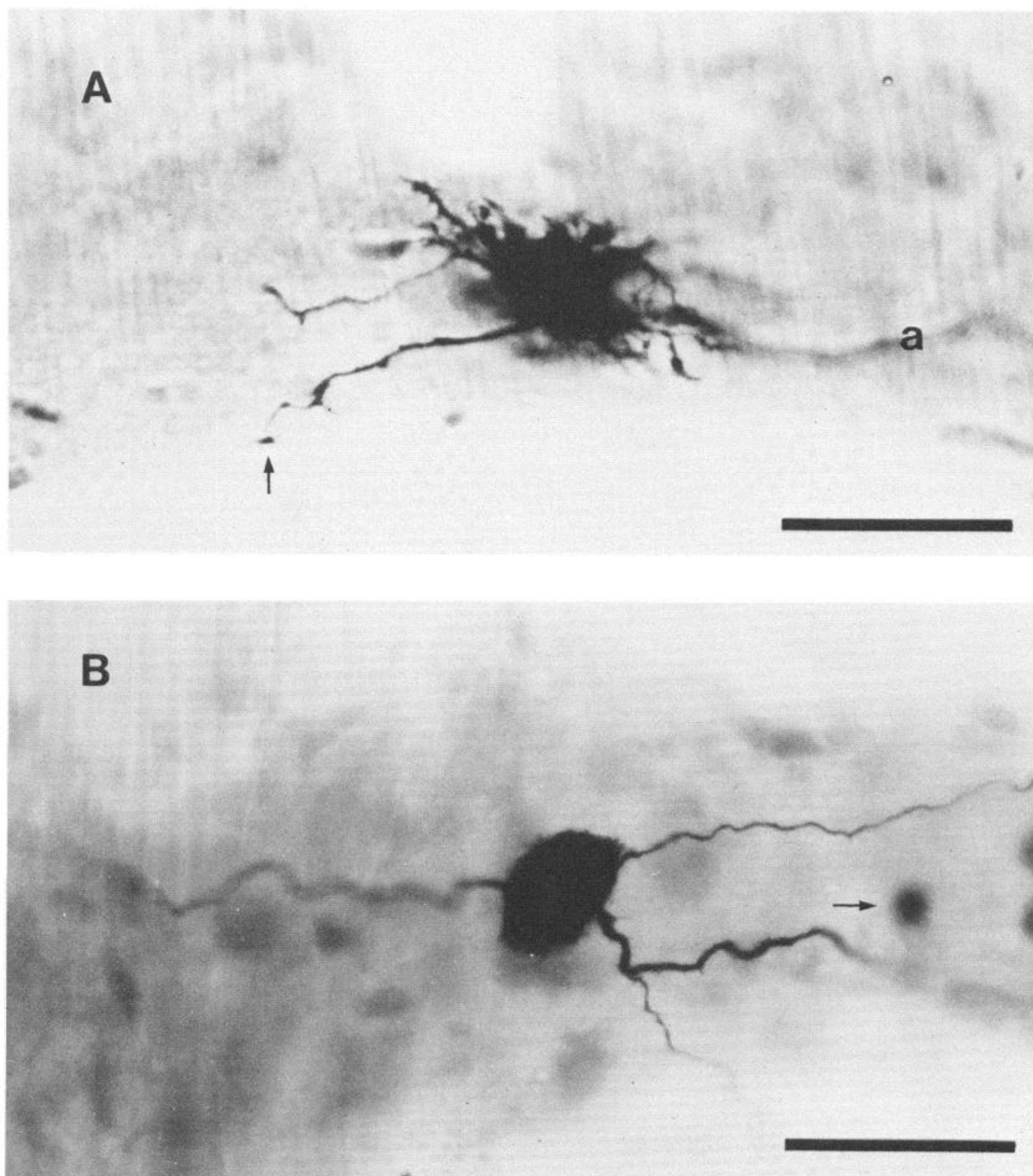

Figure 12. Identified NS cells injected with HRP through the recording micropipette. *A,* This cell has an irregular outline and its perikaryon bristles with many short processes. Longer processes end in varicose expansions at the boundary of the myenteric plexus (*arrow*). The axon (*a*) projects circumferentially and out of the plane of focus before leaving the ganglion. *B,* This NS neuron has a smooth soma and relatively few long, thin processes; one of these ends in a retraction bulb (*arrow;* out of the plane of focus). *Bars* = 50 μm.

ine, lesions just those neurites in the plexus that contain 5-HT, and reduces uptake of [^{3}H]-5-HT (Gershon et al., 1980). Finally, electron microscopic radioautographs reveal that [^{3}H]-5-HT labels only a single population of axon terminals (Gershon and Sherman, 1982a, b) recognizable by their content of many small, clear vesicles (average diameter, 62.4 $\pm$ 1.0 nm) and a much smaller number (50/mm^2) of large, dense-cored vesicles (average diameter, 122.8 $\pm$ 6.5 nm). This is the same population of terminals that can be labeled by 5,7-dihydroxytryptamine and visualized by fixation with $NaMnO_4$ and that is different in appearance from identified noradrenergic and presumed cholinergic terminals which have different vesicle diameters. The small, dense-cored vesicles in noradrenergic terminals are smaller (mean diameter, 53.6 $\pm$ 0.5 nm) and there are fewer large dense-cored vesicles (6/mm^2) than in the serotonergic axons. The small lucent vesicles in presumptive cholinergic neurons are also smaller (mean diameter, 45.0 $\pm$ 2.4 nm) than those in terminals labeled by [^{3}H]-5-HT (Manber and Gershon, 1979). Therefore, the use of [^{3}H]-5-HT for electron microscopic radioautography is probably a valid marker for serotonergic neurons under conditions such as those used in the current investigation to prevent nonspecific noradrenergic uptake of the amine; the radioautographic method for the electron microscopic identification of serotonergic elements has been widely used and validated in both brain and periphery (see Beaudet and Descarries, 1981; Gershon, 1981).

The observations made in the current investigation are consistent with, and thus supportive of, the initial hypothesis that 5-HT is a mediator of slow EPSPs in type II/AH cells. The observations do not indicate that 5-HT is the only such transmitter. The finding that all of the cells that were found to show a slow EPSP received serotonergic terminals does not in any way exclude the possibility that these cells might also receive synapses that contain SP. In fact, evidence favoring the coexistence of 5-HT and SP in the cell bodies of some myenteric neurons has been found (Legay et al., 1984). The neurons that contain both substances appear to be small in number in

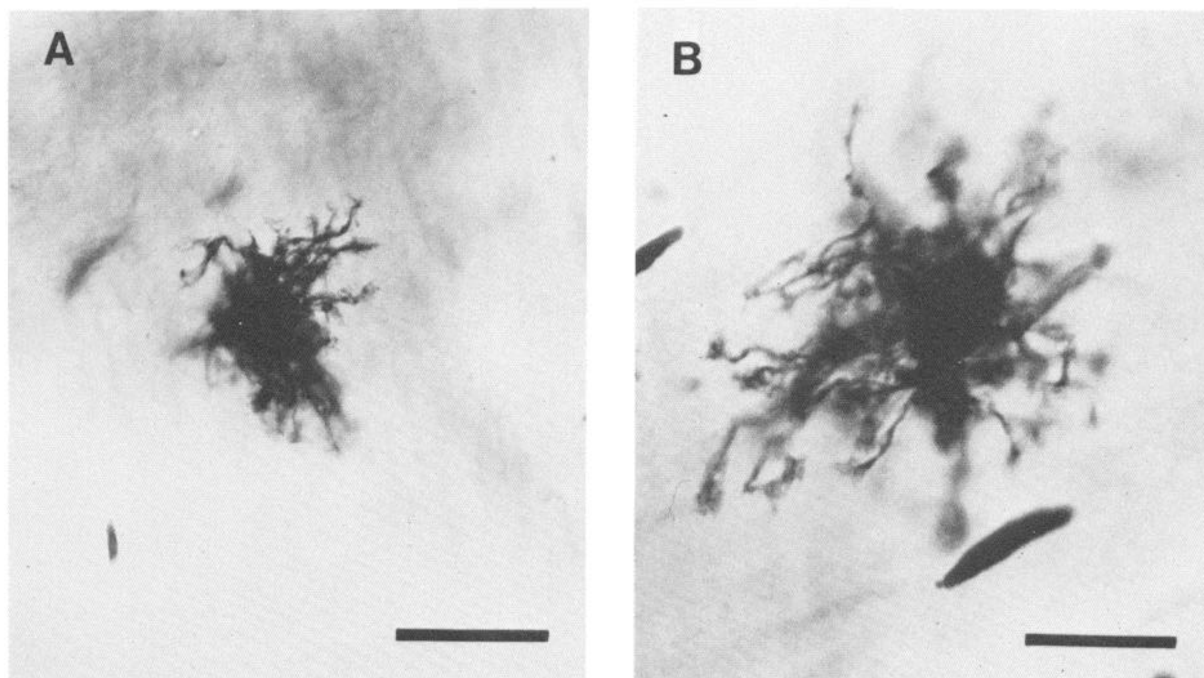

Figure 13. NS cells injected with HRP through the recording micropipette. These cells appear astrocytic in contour; all processes are local. They do not show EPSPs and are identified as glia. *Bars: A* = 50 μm; *B*, 20 μm.

comparison to those that contain either 5-HT or SP alone. Moreover, the current experiments were designed to preferentially stimulate long tracts and fibers in the myenteric plexus so as to decrease potential interference by SP. It is conceivable, therefore, that both 5-HT and SP are physiological mediators of slow EPSPs in type II/AH cells. For example, SP might mediate the response elicited by driving neurons within the same ganglion as the cell manifesting the slow EPSP, and 5-HT may be the mediator of the response elicited by neurons some distance away in other ganglia. In favor of this interpretation is the observation that a slow EPSP can still be evoked by intraganglionic stimulation, although not as readily, when lesions are made in descending enteric pathways, indicating that both local (possibly SP) and distant (possibly 5-HT) neurons mediate the response (Bornstein et al., 1984).

Analysis of the morphology of physiologically identified myenteric neurons made it clear that cell shape did not correlate reliably with the physiological classification of neurons. Variation and overlap in the morphologies of type I/S, type II/AH, and NS cells are sufficient that it is not possible to devise a simple, reliable classification, although one subset of cells with short stubby dendrites was consistently type I/S cells. A similar conclusion was reached by Hodgkiss and Lees (1983), although there were substantial differences between that thorough and pioneering study and this one. Hodgkiss and Lees (1983) first impaled cells with electrodes filled with 3 M KCl for physiological classification. This KCl electrode was then withdrawn and an attempt was made to penetrate the originally impaled neurons for a second time in order to inject them with Procion Yellow. That technique relies on a subjective visual determination (using positional cues) that the dye-filled neurons are the same neurons that were classified physiologically. Hodgkiss and Lees (1983) did not record from the Procion Yellow-filled electrodes and thus could not verify objectively that the same cell had been impaled twice. In the present study the marker, HRP, was injected through the same electrode as used for intracellular recording; therefore, there can be no doubt that the HRP-filled cells were the ones from which the intracellular records were obtained. Electron microscopic observations were also made possible by the use of HRP, revealing additional details of cell outline and synaptic input that could not be obtained in earlier studies using fluorescent dye injection (Erde et al., 1980; Hodgkiss and Lees, 1983 Bornstein et al., 1984). This additional detail added to the morphological overlap between physiologically identified cell types but also clarified some aspects of previous studies that remained enigmatic. For example, the large bulbous expansions that were found at the ends of some neurites in the present study were described earlier and interpreted to be "button-like endings" (Hodgkiss and Lees, 1983). It now seems likely that these structures are not specialized nerve terminals but retraction bulbs formed by the resealing of the cut ends of damaged fibers. In electron micrographs of these structures filled with HRP, an accumulation of organelles and smooth membranous elements is found and is identical in appearance to that of the proximal stumps of axons constricted in order to interrupt axonal transport (Kapellar and Mayor, 1969). In fact, electron microscopy made it evident that considerable damage is done to the input to myenteric ganglia in the preparation and the immobilization of the myenteric plexus for intracellular recording. The pressure feet used in the current investigation do leave some fiber tracts running between ganglia intact (and do not damage axons between the pressure feet and the ganglion); but many inputs to ganglia are cut off. Electrophysiological studies, therefore, must minimize the possible effects of distant ganglia and of long pathways on the ganglion under study. On the other hand, it is reassuring in terms of the validity of the physiological data obtained from this preparation that the ultrastructure of neurons, synapses, and glia is well maintained for up to 7 or 8 hr of recording *in vitro*.

One problem that arises in interpreting the physiology of the myenteric plexus is that there are clearly more types of neurons in the tissue than is apparent in the physiological classification of cells. There are more than a dozen established or putative neurotransmitters (Furness and Costa, 1982b), and electron microscopy has been used to identify at least eight different neuronal classes (Cook and Burnstock, 1976). Extracellular recording has led to the description of six patterns of neuronal activity (Wood, 1981). Only three or four types of neurons have been identified on the basis of characteristics derived from intracellular recording. It thus seems likely that the types of cells identified with intracellular criteria must each encompass a variety of transmitter, patterns of activity, and cell morphology. Conceivably, more variety in the activity of myenteric neurons and

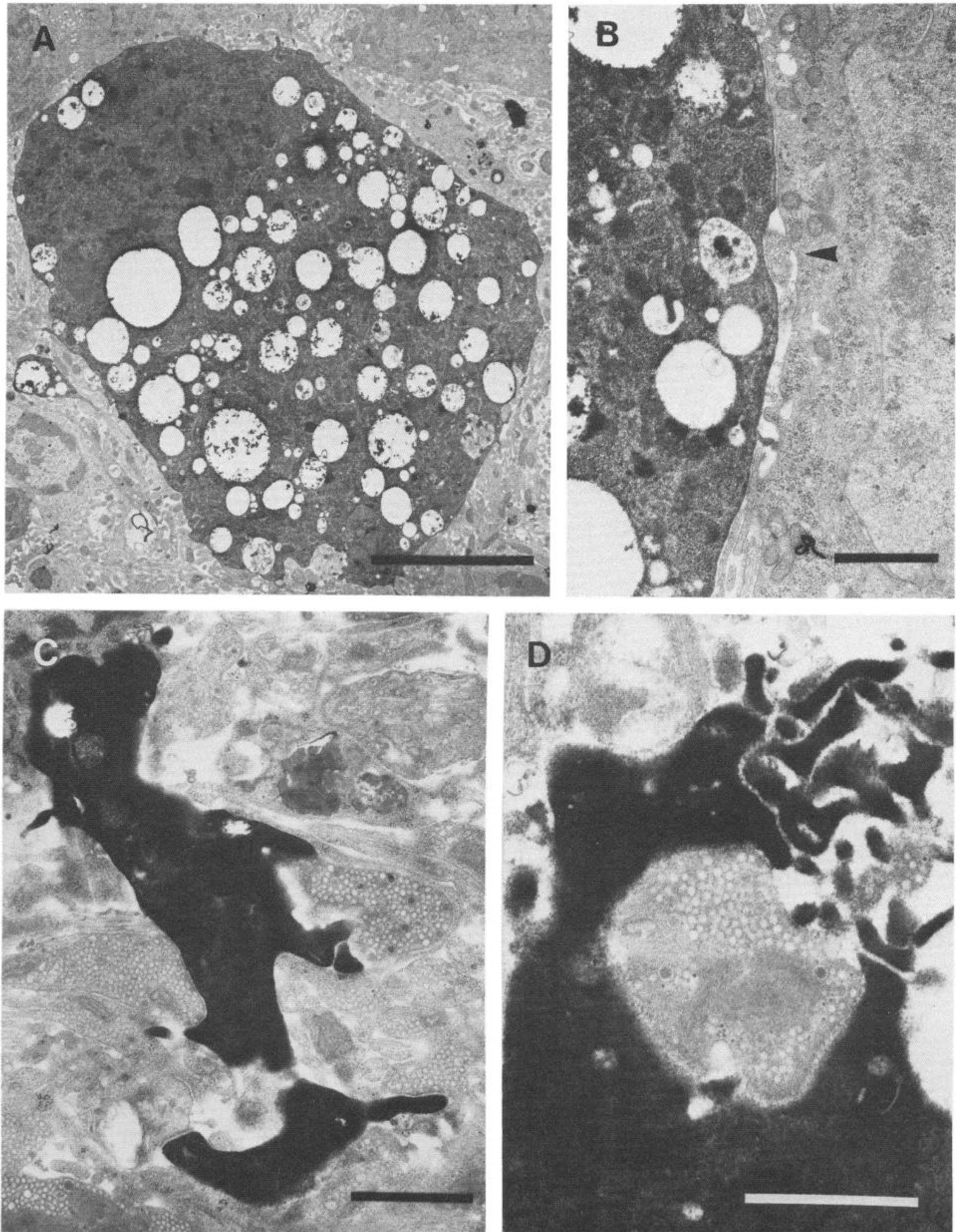

Figure 14. Electron micrographs of an NS neuron injected with HRP through the recording micropipette. *A*, The soma of the cell illustrated light microscopically in Figure 12*B* is shown. Note the smooth contour of its profile. *B*, A small varicosity contacts the soma (*arrowhead*) but is not labeled by [^{3}H]-5-HT. *C*, Axodendritic contacts of varicosities not labeled by [^{3}H]-5-HT with the NS cell. *D*, An axosomatic contact between an axonal varicosity not labeled by [^{3}H]-5-HT and the NS cell. *Bars: A* = 5.0 μm; *B* to *D* = 1.0 μm.

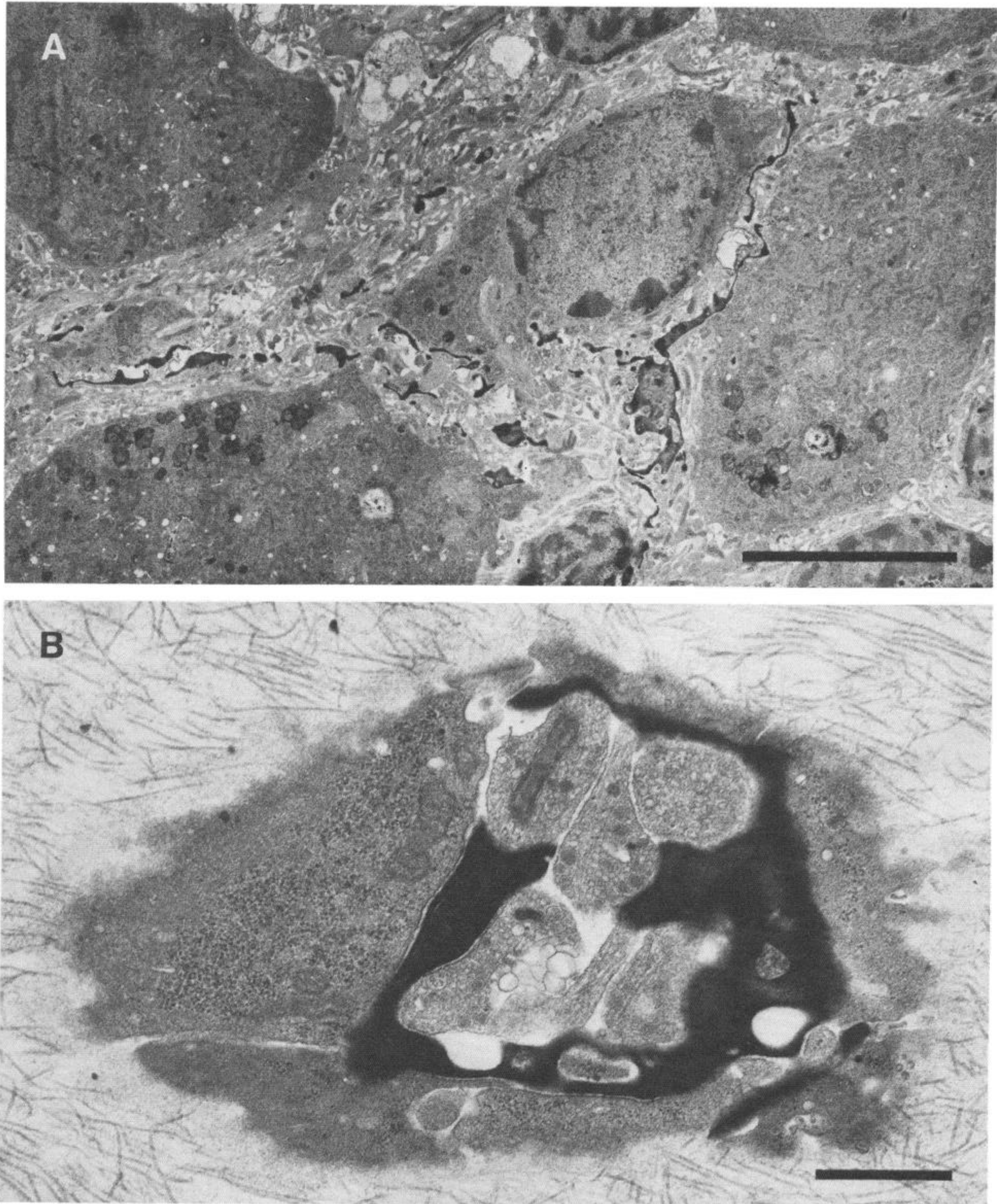

Figure 15. Electron micrographs of NS cells identified as glia injected with HRP through the recording microelectrode. *A*, HRP-filled processes are extremely thin and weave among the neurites of the myenteric neuropil. *B*, In an interganglionic connective surrounded by collagen, HRP-filled glial processes loosely support neurites. *Bars: A* = 10 μm; *B* = 1.0 μm.

a better physiological classification would be developed if connections between ganglia within the myenteric plexus and between the plexus and other layers of the gut were not interrupted whenever intracellular recording is done. Mucosal sensory receptors (Bulbring and Crema, 1958; Bulbring and Lin, 1958) and the submucosal plexus, as well as descending tracts within the myenteric plexus, clearly influence the activity of myenteric ganglion cells *in vivo*. These effects are lost or minimized in the *in vitro* preparations of the myenteric plexus.

The classification of neurons and enteric glia on the basis of intracellular criteria has led to some confusion in the past. It was, at first, thought that type I/S cells were synaptically driven whereas type II/AH cells were not (Nishi and North, 1973). The name "S" cell was used to signify synaptic input. Clearly, type II/AH cells also

TABLE I
[^{3}H]-5-HT contacts by physiological classification of cell type

Cell Type	Total	Number with [^{3}H]-5-HT Contacts
I/S	6	2
II/AH		
with slow EPSP	(5)	(5)
without slow EPSP	(5)	(2)
	10	7
NS	6	0
	22	9

receive synaptic input. This input can be identified physiologically (Grafe et al., 1979b) and in the present study was verified morphologically. Moreover, type II/AH cells that were examined did not notably differ from type I/S cells in the extent of their coverage by morphologically identifiable synaptic contacts. Synaptic input, therefore, cannot be used as it has been in the past to distinguish between these cell types. In addition, NS cells have often been classified as type III and called glia (Nishi and North, 1973; Hodgkiss and Lees, 1983). This practice, too, was found in the present study to be unfortunate. Some of these NS cells had the appearance of neurons and received synapses. Some even displayed EPSPs. Others had astrocytic contours and were probably glia; see also Gabella, 1971; however, the NS cells probably constitute a heterogeneous group that cannot be sorted out on physiological grounds alone.

In conclusion, the present study has established that type II/AH cells of the myenteric plexus receive a serotonergic innervation to a greater extent than do other cells of the plexus. The subset of type II/AH cells that manifested a slow EPSP was found to be serotonergically innervated and probably accounted for the higher incidence of serotonergic contacts found in the whole type II/AH group. These data thus strongly support the hypothesis that 5-HT is a mediator of the slow EPSP, although it may not be the only such mediator. A minority of type I/S cells also are serotonergically innervated. Type I/S cells did not manifest slow EPSPs; therefore, 5-HT probably has other roles in the myenteric plexus in addition to its function as a transmitter of the slow EPSP. This idea of multiple 5-HT-mediated effects is consistent with the numerous demonstrated actions of 5-HT in the myenteric plexus (Johnson et al., 1980a, b).

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