INTRINSIC AND EXTRINSIC NEURAL PATHWAYS TO THE LARGE INTESTINE OF THE CAT

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ABSTRACT

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Extrinsic Afferent Pathways. The distribution of visceral afferent fibers from sacral dorsal root ganglia to the pelvic nerve, pudendal nerve, large intestine, urinary bladder and urethra was studied in the cat. Also, the relation of these fibers to neurons in parasympathetic colonic ganglia, pelvic plexus ganglia, urinary bladder ganglia and the colon-rectal myenteric plexus was determined. Wheatgerm agglutinin-horseradish peroxidase (WGA-HRP) and unconjugated wheat germ agglutinin (WGA) were injected into sacral dorsal root ganglia (S_1-S_3) bilaterally. Antibodies against neurofilament were used to label neuronal somas and processes within the myenteric plexus, pelvic plexus, urinary bladder and parasympathetic colonic ganglia. Anterogradely transported WGA-HRP and WGA were detected in peripheral afferent projections in branches of the pelvic and pudendal nerves.

Axial orad and aborad sacral visceral afferent projec-

tions to the colon and rectum-anal canal involved ascending and descending afferent fibers, respectively, within colonic and rectal fiber bundles, branch points of fiber bundles and interganglionic fiber tracts within the myenteric plexus. Dense arborization patterns of afferent fibers, some with varicosities, were detected in colon and rectal myenteric plexus ganglia. Afferent fibers were detected in approximately 9% of proximal, 16% of mid and 20% of distal colon myenteric plexus ganglia. Afferent fibers were traced from the myenteric plexus to the circular muscle layer of the colon. They were distributed around the circumference of the colon, parallel to the long axis of circular muscle fibers. Diffuse fibers, small bundles and dense arborization patterns of afferent fibers were detected on circular muscle fibers. No sacral visceral afferent fibers were detected in the longitudinal muscle layer. In submucosa, sacral visceral afferent fibers were sparse, appearing as interrupted short fragments within interganglionic fiber tracts. No afferent fibers were detected in submucosal plexus ganglia.

For urinary bladder, afferent fibers were detected as sparse fragments in serosal bundles and extrinsic muscle layers of all bladder regions. Afferent fibers were visualized in the submucosa of the neck region only. No afferent fibers were detected in mucosa.

When sacral dorsal root ganglia were injected bilaterally, afferent fibers were detected in approximately 64 % of

proximal, 68 % of mid and 77 % of distal urethral sections. They were detected in serosal bundles, longitudinal and circular smooth muscle layers, submucosa, mucosa and striated muscle of distal urethra and external urethral sphincter.

When sacral dorsal root ganglia were injected ipsilaterally and the pelvic nerve was sectioned ipsilaterally, pudendal nerve afferent fibers were detected predominantly in distal and mid urethra and external urethral sphincter. When sacral dorsal root ganglia were injected ipsilaterally and the pudendal nerve was sectioned ipsilaterally, pelvic nerve afferent fibers were detected in proximal and mid urethra and the submucosal region of distal urethra. Fibers were not detected on striated muscle fibers of distal urethra or external urethral sphincter.

Sacral afferent fibers were detected in 18 of 18 parasympathetic colonic ganglia, 60 of 61 pelvic plexus ganglia and 37 of 42 urinary bladder ganglia. They were detected in fiber bundles on the border and center of the ganglia. 100 percent of colonic and pelvic plexus ganglia and 97 percent of urinary bladder ganglia had fibers in proximity to ganglion cell bodies.

In summary, these data show that sacral visceral afferent fibers project axially both orad and aborad over relatively long distances through colonic and rectal fiber bundles, respectively, where they provide innervation to some colonic and rectal effector structures. Sacral visceral afferent fibers also project through pelvic and pudendal

nerves to innervate the urinary bladder, urethra and external urethral sphincter. Afferent fibers occurr in proximity to neurons in some myenteric plexus ganglia and nearly all sacral parasympathetic colonic ganglia, pelvic plexus ganglia and urinary bladder ganglia.

Intrinsic Neural Pathways. The morphology and projections of myenteric plexus neurons through colonic fiber bundles in cat colon were determined using in-situ retrograde transport of HRP and fast blue. Myenteric neurons were found to project from at least 5 to 59 mm orad (mean: 42 mm) or aborad (mean: 54 mm) through colonic fiber bundles. Approximately 73% of labeled cells were in ganglia within 2.8 mm of colonic fiber bundles in the axis of circular muscle fibers; none was beyond 7.7 mm. There were 2 soma morphologies. One type (Dogiel type I) had a mean soma diameter of 40.5 μm and had a rough somal surface. There were few if any short, broad dendrites, but its one long process extended to a branch point of an adjacent colonic fiber bundle. The other type (Dogiel type III) had a mean soma diameter of 26.4 μm, a smooth somal surface and few if any fine dendrites. It also projected a single long axon to colonic fiber bundles. There were twice as many Dogiel type III neurons.

In summary, these data show that myenteric neurons in the cat colon project both orad and aborad over relatively long distances through colonic fiber bundles where they form

another intrinsic neuronal pathway for the myenteric plexus.

Extrinsic Parasympathetic and Sympathetic Efferent Pathways. The distribution of neurons in parasympathetic prevertebral and sympathetic prevertebral and paravertebral ganglia that project processes to at least mid-colon through colonic fiber bundles was determined using retrograde transport of fast blue.

For parasympathetic prevertebral ganglia, a range of 44 to 477 neurons were labeled per animal (mean ± SE, 233.1 ± 49.0). This anatomical data supports previous electophysiological studies (Krier and Hartman, 1984). Together the data suggest that neurons in parasympathetic colonic ganglia provide synaptic input to myenteric and/or submucosal plexus neurons and/or directly innervate colonic effector structures.

For sympathetic prevertebral ganglia, the mean number of neurons (± SE) labeled per animal were: inferior mesenteric ganglion (IMG), 2,755 ± 660; superior mesenteric ganglion (SMG), 356 ± 131; and coeliac ganglion, 1,415 ± 874. For lumbar paravertebral chain ganglia, the mean number of neurons per experiment was 780 ± 314. These data show that a subpopulation of neurons in sympathatic prevetebral and paravertebral ganglia project long postganglionic processes through colonic fiber bundles to at least the mid-colon region. The functional significance of this anatomical arrangement is unknown. It is likely that these neurons

are noradrenergic and innervate blood vessels, myenteric plexus ganglia and intestinal smooth muscle (Furness and Costa, 1974).

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INTRODUCTION

The mammalian large intestine, which represents the terminal portion of the gastrointestinal tract, is comprised of the caecum, proximal, mid and distal colon, rectum and anal canal. It is responsible for the storage, mixing, transport and evacuation of intestinal contents. The motility of the large intestine is under the influence of both the electrical properties intrinsic to smooth muscle cells and the nervous system. The electrical properties of the smooth muscle are thought to set up a basic pattern of contractile activity which is then modulated by the autonomic nervous system.

The autonomic nervous system is classically divided into three major subdivisions; sympathetic, parasympathetic and intrinsic (Langley, 1921). The lumbar sympathetic division that innervates pelvic vicera is comprised of preganglionic and postganglionic neurons. The preganglionic neurons are cholinergic and their soma are located in the intermediolateral cell columns of the lumbar spinal cord. The postganglionic neurons are adrenrgic and their soma are located in lumbar sympathetic chain ganglia (paravertebral) and inferior mesenteric, superior mesenteric and coeliac ganglia (prevertebral). The major nerve trunks for these sympathetic postganglionic fibers include inferior splanchnic nerves, lumbar colonic nerves and hypogastric nerves (Fig. 30).

The sacral parasympathetic division that innervates pelvic viscera also includes preganglionic and postganglionic neurons. The preganglionic neurons are cholinergic and their soma are located in the intermediolateral columns of the sacral spinal cord. Sacral preganglionic fibers project through the pelvic nerves to innervate postganglionic neurons in prevertebral and possibly intrinsic ganglia. Prevertebral postganglionic neurons are located in ganglia on the serosal surface of the pelvic viscera (colonic ganglia and urinary bladder ganglia). The postganglionic pathways include colonic fiber bundles (Fig. 1) and fiber bundles on the serosal surface of the urinary bladder and urethra. Afferent fibers originate from sensory neurons located in dorsal root ganglia in both lumbar and sacral spinal regions (Figs. 1 and 4). The intrinsic division of the autonomic nervous system is located within the walls of the gastrointestinal tract and includes the myenteric (Aurebach's) plexus and the submucosal (Meissner's) plexus.

The overall objective of this dissertation is to study the intrinsic and extrinsic neural pathways to the large intestine of the cat. One major focus of this dissertation will be to determine if both intrinsic and extrinsic neurons utilize colonic fiber bundles as a peripheral nerve trunk to innervate effector structures in the colon. A second major focus of this dissertation will be to examine the peripheral distribution of sacral afferent fibers to the large intestine, prevertebral ganglia, urinary bladder and urethra.

HISTORY

EXTRINSIC NEURAL PATHWAYS

1. SACRAL AFFERENT PATHWAYS.

A. ANATOMY: DISTRIBUTION OF NEURONS IN SACRAL DORSAL ROOT GANGLIA THAT PROJECT VISCERAL AFFERENT FIBERS TO PELVIC NERVE. Primary afferent neurons in sacral dorsal root ganglia (DRG) are known to project to the pelvic nerves of the cat, monkey and rat (Morgan et al., 1981; Nadelhaft et al., 1983; Nadelhaft and Booth, 1984). In retrograde studies of cat and monkey, labeling the ipsilateral pelvic nerve with horseradish peroxidase (HRP) demonstrated sacral dorsal root ganglion neurons numbering 3,676 and 2,992, respectively (Morgan et al., 1981; Nadelhaft et al., 1983). The greatest percentage of retrogradely labeled DRG neurons was located in S_2 (80%). In similar retrograde studies of rat pelvic nerve, 95% of labeled cells were found in L_6 and S_1 (lumbosacral) DRGs (Nadelhaft and Booth, 1984). A mean of 1500 neurons was found, most having a small soma (17 x 25 μm) and central processes that entered Lissauer's tract (Nadelhaft and Booth, 1984). The visceral afferent component of dorsal root ganglia neurons is relatively small (approximately 2%) when compared to the population of spinal afferents that innervate skin and deep somatic structures (Janig and Koltzenburg, 1990).

ANATOMY: DISTRIBUTION OF NEURONS IN SACRAL DORSAL ROOT GANGLIA THAT PROJECT VISCERAL AFFERENT FIBERS TO LARGE INTESTINE. Sacral visceral afferent fibers are known to project to the distal colon of rat and cat (Keast and de Groat, 1992; Kawatani et al., 1985). In retrograde tracing studies of rat and cat, dye (Fast Blue, True Blue or Fluoro Gold) injections into distal colon labeled neurons in sacral dorsal root ganglia (Keast and de Groat, 1992; Kawatani et al., 1985). The predominant distribution of labeled neurons was S_1 for rat and S_2 for cat. The actual number of retrogradely labeled neurons was not reported in these studies.

Degeneration techniques have also been used to study the distribution of sacral afferent fibers to cat large intestine (Schofield, 1962). Degeneration of extrinsic fibers was accomplished by sectioning spinal nerves central or peripheral to dorsal root ganglia. Afferent fibers were detected as numerous degenerating fragments in myenteric plexus ganglia.

There is a paucity of information on the number and distribution of neurons in sacral dorsal root ganglia that project peripheral processes to the large intestine. The peripheral distribution of sacral visceral afferent fibers to the large intestine has also not been studied with modern tracing techniques. This dissertation will study the distribution of neurons in sacral dorsal root ganglia that project processes to at least mid colon using retrograde tracing techniques. The distribution of sacral afferent fibers to

the large intestine will also be studied using anterograde tracing techniques.

ANATOMY: DISTRIBUTION OF NEURONS IN SACRAL DORSAL ROOT GANGLIA THAT PROJECT VISCERAL AFFERENT FIBERS TO URINARY BLADDER AND URETHRA. Retrograde axonal tracing techniques have been used to determine the distribution of primary visceral afferent neurons in sacral dorsal root ganglia that project peripheral processes to urinary bladder (Downie et al., 1984; Applebaum et al., 1980) and urethra (Downie et al., 1984). One study (Downie et al., 1984) identified small populations of neurons in sacral DRG, predominantly in S_2. The mean values of retrogradely labeled afferent neurons were 81 to urethra, 159 to detrussor muscle of the urinary bladder and 178 to bladder base. The second study injected all regions of urinary bladder and identified a similar small population (mean = 365 neurons) distributed to all 3 sacral dorsal root ganglia (Applebaum et al., 1980). When considered against the estimated 7300 neurons in sacral dorsal root ganglion that project peripheral processes to cat pelvic nerves (Morgan et al., 1981), afferent innervation of the bladder and urethra represents less than 5%.

Degeneration techniques have also been used to study the distribution of sacral axon terminals in the cat urinary bladder (Uemura et al., 1973; Uemura et al., 1975). Sacral dorsal root ganglia were surgically ablated and degenerating afferent terminals were detected in the urinary bladder by

electron microscopy. These studies showed that sacral afferent fibers were distributed equally to all bladder regions (Uemura et al., 1973). Degenerating afferent terminals represented less than 5% of total axon terminals associated with smooth muscle fibers and submucosa in cat urinary bladder (Uemura et al., 1973; Uemura et al., 1975).

Projection of sacral afferent fibers across the midline of urinary bladder and urethra was demonstrated in degeneration and retrograde tracing studies. Degeneration studies of sacral afferent terminals in cat urinary bladder showed approximately one third of sacral afferent fibers cross the bladder midline to innervate the contralateral side (Uemura et al., 1973; Uemura et al., 1975). Retrograde tracing studies, in which HRP, bisbenzamide, nuclear yellow or fast blue were injected into ipsilateral urinary bladder and urethra, also demonstrated that sacral dorsal root ganglion neurons project afferent fibers across the midline in cat urethra and rat and cat urinary bladder (Downie et al., 1984; Applebaum et al., 1980). This redundant bilateral innervation by sacral afferent fibers may represent a safety feature in bladder function.

Previous studies of sacral visceral afferent pathways have relied on retrograde tracing and degeneration techniques. The former provides cell numbers, morphology and distribution while the latter provides the distribution of degenerating fiber fragments. The anterograde tracing techniques used in this study allowed me to visualize sacral

visceral afferent fibers with relative continuity over centimeter distances in proximity to neurons and colonic effector structures.

B. IMMUNOHISTOCHEMISTRY OF PRIMARY AFFERENT NEURONS AND FIBERS. Sacral dorsal root ganglion neurons in mammals are immunoreactive for substance-P (SP), calcitonin gene-related peptide (CGRP), somatostatin (SOM), galanin, opioid peptides, neurokinin A, peptide histidine isoleucine amide, vasoactive intestinal polypeptide (VIP), choleycystokinin (CCK), angiotensin II and bombesin (Buck et al., 1982; de Groat, 1987; de Groat et al, 1983; Hokfelt et al., 1975; Keast and de Groat, 1992; Klein et al., 1990). These peptides are distributed in afferent fibers of pelvic viscera, visceral afferent neurons in sacral dorsal root ganglia (lumbosacral in rats) and at sites of afferent termination in spinal cord.

In double-labeling experiments, retrograde tracers were injected into the colon and urinary bladder of rats and cats (Keast and de Groat, 1992). The retrogradely labeled dorsal root ganglia were then double-labeled for one of five peptides (SP, CGRP, VIP, enkephalin (ENK) or SOM). Neurons innervating pelvic viscera and immunoreactive for peptides had the following relative distribution; CGRP>SP>VIP>ENK>SOM (Keast and de Groat, 1992). The three most numerous peptides in sacral (lumbosacral in rat) dorsal root ganglia are summarized below.

For sacral (lumbosacral in rat) dorsal root ganglion neurons that project peripheral processes to the colon, CGRP-immunoreactive neurons had the greatest percentage of co-localization (Keast and de Groat, 1992). 70% of rat lumbosacral and 45% of cat sacral dorsal root ganglia neurons innervating the colon were immunoreactive for CGRP. SP-immunoreactivity was detected in 38% of rat and 33% of cat colon afferent neurons. VIP-immunoreactivity was detected in only 1% of rat and 18% of cat colon afferent neurons.

For rat lumbosacral dorsal root ganglion neurons that project to urinary bladder, 52% were immunoreactive for CGRP, 29% for SP and 11% for VIP (Keast and de Groat, 1992).

C. PHYSIOLOGY OF SACRAL AFFERENT FIBERS: LARGE INTESTINE.

Sensory information from the colon and rectum (sense of fullness, urge to defecate, pain impulses, chemical and mechanoreceptive input) is conveyed to the sacral spinal cord via afferent fibers in the pelvic nerve (de Groat and Krier, 1978; de Groat et al., 1981; Haupt et al., 1983; Janig and Koltzenburg, 1990; Morgan et al., 1981). When a mechanical stimulus was applied to cat anal mucosa or the colon was passively distended, afferent discharges were detected in sacral (S_1-S_2) dorsal root fibers (Bahns et al., 1987; Jani and Koltzenburg, 1991). A total of 59 S_2 afferent units were identified electrically in cat pelvic nerve (Janig and Koltzenburg, 1991). Of these, 61% responded only to passive distension of the distal colon, while 39% responded only to mechanical stimulation of the anal mucosa.

This suggests that pelvic afferent units consist of distinct populations that respond to specific stimuli.

The colonic afferents were thin myelinated and non-myelinated fibers with a median conduction velocity of 3.2 m/s (Janig and Koltzenburg, 1991). They were classified as myelinated phasic afferents, responding only transiently to colonic distension (median conduction velocity 8.0 m/s), and non-myelinated tonic afferents, discharging throughout the distension (median conduction velocity 1.7 m/s). For tonic units, increases in colonic distension resulted in increased discharge frequencies. In-situ recordings of visceral afferent fibers in cat inferior splanchnic nerves demonstrate that afferent fibers respond in a graded fashion to colon distension (Blumberg et al., 1983). These studies suggest that the colon is innervated by mechanoreceptive afferents that encode the degree of distension by increases in discharge frequency.

A relatively new class of non-myelinated afferent fibers has been described for colon. Electrophysiological studies demonstrate that approximately 95% (202/213) of non-myelinated afferent units projecting from sacral dorsal root ganglia (S_2) to pelvic nerve do not respond to mechaninical stimulation of cat colon or anal canal (Janig and Koltzenburg, 1991). The authors suggest that these silent C-fibers may be mechanoreceptors that are active only during inflamation.

REFLEXES OF SACRAL AFFERENT FIBERS TO COLON: SPINAL REFLEX.

Afferent fibers in sacral dorsal roots play a role in colonic reflexes. In in-situ studies of cat mid-distal colon (de Groat and Krier, 1978), measurements of colonic motility were performed simultaneously with electrophysiological recordings of colonic fiber bundles. Distension of the colon or rectum, or electrical stimulation of pelvic nerve afferent fibers resulted in increased efferent firing in colonic fiber bundles and sustained propulsive contractions associated with defecation. The responses were blocked by transection of the sacral dorsal roots or the pelvic nerves, indicating a spinal pathway reflex. The sacral reflexes were mediated by non-myelinated afferent and preganglionic efferent fibers (de Groat and Krier, 1978; de Groat et al., 1981).

REFLEXES OF SACRAL AFFERENT FIBERS TO COLON: AXONAL REFLEX. Afferent fibers are known to relay sensory information to the central nervous system and play a role in reflexes. It has also been suggested that afferent collaterals in the periphery may release neurotransmitter substances on enteric neurons to modulate local circuits (Delbro and Lissander, 1980; Delbro et al., 1981; de Groat et al., 1987). Stimulation of sympathetic (lumbar splanchnic nerves) and parasympathetic (vagus nerve) nerve trunks produced contractions of cat colon and stomach, respectively, that were not blocked by nicotinic (hexamethonium) or adrenergic (guanethidine) antagonists (Delbro et al., 1983; Fandriks and Delbro, 1985;

Delbro and Lisander, 1980; Fandriks et al., 1985; Delbro et. al., 1981). These contractions were greatly reduced by atropine and substance-P antagonists. This suggests that the pathway for this contraction involves cholinergic-muscarinic and SP receptors. These contractions may be mediated by antidromic activation of substance-P containing afferent fibers which in turn activate cholinergic postganglionic motor neurons (Fandriks et al., 1985).

D. PHYSIOLOGY OF SACRAL AFFERENT FIBERS: URINARY BLADDER AND URETHRA. Afferent fibers emanating from sacral dorsal root ganglia mediate sacral parasympathetic reflexes to urinary bladder and urethra (micturition)(de Groat and Booth, 1984; de Groat and Kawatani, 1989). In contrast to colon reflexes which are mediated predominantly by non-myelinated C-fibers (de Groat and Krier, 1978; de Groat et al., 1981), electrophysiological studies in cat have shown that urinary bladder reflexes involving the sacral spinal cord are mediated by both myelinated and nonmyelinated afferent fibers (de Groat et al., 1981; Habler et al., 1990).

In cat urinary bladder, electrophysiological studies have shown that myelinated afferent units have low thresholds and firing frequencies that rise with increasing intraluminal pressure during distension and isovolumetric contractions (Iggo, 1955; Floyd et al., 1976; Bahns et al., 1987). Iggo was able to demonstrate that the same afferent unit was stimulated by both passive distention and active contraction of the cat urinary bladder (Iggo, 1955). This

suggests that bladder mechanorective afferents encode the degree of intraluminal pressure by increases in discharge frequency.

SACRAL AFFERENT FIBERS TO URINARY BLADDER AND URETHRA: SENSATION. Stimulation of urinary bladder afferents in humans by gradual bladder distension elicits sensations of fullness, urge to micturate and eventually pain (Nathan, 1956). Nathan was able to distinguish several sensations associated with micturition and identify their source by studying patients that had suprapubic cystostomies and urethral ligation at the bladder neck. The "desire to micturate " was attributed to urinary bladder distention and contractions. By connecting the suprapubic catheter to a manometer and instilling water at ever increasing pressures, the urge to micturate became stronger. The sensation that "micturition is imminent" was attributed to the urethra by mechanically stimulating the urethra in the same patients.

Differences in sensation may be due in part to the type of afferent receptors in the urinary bladder. Light and electron microscopic studies have demonstrated a relative absence of specialized nerve endings in cat urinary bladder, suggesting that afferent receptors are free nerve endings (Fletcher et al., 1970; Uemura et al., 1974; Uemura et al., 1975; Uemura et al., 1973; Fletcher and Bradley, 1970; Fletcher and Bradley, 1978). These free nerve endings may act as chemoreceptors (Habler et al., 1990), thermoreceptors

(Fall et al., 1990; Nathan, 1952) and mechanoreceptors sensitive to passive distension, active contraction, mucosal deformation and bladder position (Habler et al., 1990; Fletcher and Bradley, 1978; Iggo, 1955).

A relatively new classification of visceral afferent is a high threshold mechano-chemoreceptor (Habler et al., 1990). In electrophysiological studies of cat urinary bladder, recordings of afferent fibers in sacral dorsal roots demonstrated a population of C-fibers that were not activated by innocuous or noxious increases in intravesicular pressure in normal bladder. When the bladder was inflamed by injection of mustard or turpentine oil, the previously silent C-fibers responded to both the irritant and increases in intravesicular pressure. This suggests that these silent C-fibers may be involved in the sensation of pain associated with mucosal inflamation of the urinary bladder.

SACRAL AFFERENT FIBERS TO URETHRA: MECHANO-RECEPTORS. The differences in bladder and urethra sensation may also be due to distinct populations of afferent units that respond to specific stimuli (mechanoreceptors). Urethral afferent units responded to mechanical stimulation of the urethral mucosa, but did not respond to distension or isovolumetric contraction of cat urinary bladder (Bahns et al., 1987). Light microscopic studies (haematoxylin and eosin) in cat urethra have identified specialized nerve terminals and free nerve endings (Garry and Garven, 1957). The nerve terminals were

identified as pacinian corpuscles and "cucumber" or "sausage" shaped terminals. Pacinian corpuscles were identified in mucosa and external muscle layers of the urethra, predominantly in distal urethra and external urethral sphincter. Rapidly adapting specialized nerve terminals may be responsible for the detection of mucosal mechanical stimulation associated with urinary flow in urethra (Fletcher and Bradley, 1978; Garry and Garven, 1957; Nathan, 1956).

2. POSTGANGLIONIC EFFERENT PATHWAYS

A. PARASYMPATHETIC COLONIC GANGLIA. Sacral parasympathetic preganglionic fibers provide excitatory input to the large intestine that is thought to regulate colonic motility and defecation (de Groat and Krier, 1978). Electrical stimulation of the pelvic nerve elicits contractions of the colon and action potentials in colonic fiber bundles in cats (de Groat and Krier, 1976). Both effects are markedly reduced by ganglionic blocking agents, indicating the presence of synaptic relays that may involve neurons in parasympathetic colonic ganglia and enteric ganglia.

Neurons in parasympathetic colonic ganglia may project processes to colonic effector structures through colonic fiber bundles. When colonic fiber bundles in cat distal colon are electrically stimulated, antidromic potentials are recorded in 62% of neurons tested in colonic ganglia (Krier and Hartman, 1984). This indicates that postganglionic

fibers of colonic ganglion neurons project processes through colonic fiber bundles to at least distal colon regions. The distribution of colonic ganglia neurons that project processes to mid and proximal colon via colonic fibers bundles is unknown. This dissertation will determine this distribution using retrograde tracing techniques.

B. SYMPATHETIC PREVERTEBRAL AND PARAVERTEBRAL POSTGANGLIONIC PATHWAYS. The lumbar sympathetic nervous system is comprised of cholinergic preganglionic fibers arising from lumbar spinal cord and noradrenergic postganglionic fibers arising from prevertebral and paravertebral ganglia (Baumgarten, 1982; Furness and Costa, 1974; Szurszweski and Krier, 1984). The preganglionic fibers synapse with postganglionic neurons, which in turn provide adrenergic innervation to enteric ganglia and colonic effector structures (Gabella, 1979; Jacobowitz, 1965; Manber and Gershon, 1979; Norberg, 1964; Norberg and Hamberger, 1964; Szurszewski and Krier, 1984).

In early studies of the large intestine, Langley and Anderson determined that electrical stimulation of lumbar spinal nerves in rabbits, cats and dogs decreased the motility of the large intestine and increased internal anal sphincter tone (Langley and Anderson, 1895). Electrical stimulation of sympathetic lumbar colonic nerves decreased colonic tone and depressed spontaneous and neurally evoked contractions of colon external muscle layers (Langeley and

Anderson, 1895). In these studies electrical stimulation was obtained by placing electrodes directly on isolated whole nerves and supplying a "weak tetanizing induction shock" that could be "distinctly felt" when placed on the investigaters tongue. Motility changes were qualitatively described after direct observation of the viscera.

The number, distribution and electrophysiology of neurons in cat prevertebral and paravertebral ganglia that project processes to hypogastric and lumbar colonic nerve trunks have been studied and are summarized below.

LUMBAR SYMPATHETIC CHAIN GANGLIA. Neurons in lumbar sympathetic chain ganglia project processes to cat inferior mesenteric ganglion (IMG) through inferior splanchnic nerves (Baron et al., 1985 II). When ipsilateral cat inferior splanchnic nerves were labeled with HRP, approximately 1569 neurons in lumbar sympathetic chain ganglia were labeled. These neurons were distributed predominantly to L_2-L_5 ganglia. Similar retrograde tracing studies of ipsilateral hypogastric nerve demonstrated that approximately 350 neurons in lumbar sympathetic chain ganglia had fibers that continued through the IMG to the hypogastric nerve in cat (Baron et al., 1985 I).

Postganglionic fibers from lumbar sympathetic chain ganglia are also found in the pelvic nerve. When ipsilateral cat pelvic nerve was labeled with HRP or true blue, approximately 120 cells were labeled in lumbar sympathetic chain ganglia (Kuo et al., 1984). Electrophysiological evidence

showed that stimulation of the sympathetic chain from L_3 to L_6 and occasionally as high as L_2 elicited firing in the ipsilateral pelvic nerve (Kuo et al., 1984). They proposed that these sympathetic postganglionic fibers passed through the sacral paravertebral ganglia to the pelvic nerve. The distribution of lumbar sympathetic chain ganglia neurons that project processes to mid and proximal colon through colonic fiber bundles is unknown.

Intracellular recording and injection techniques were used to describe the morphological and electrophysiological characteristics of neurons in cat lumbar paravertebral ganglia (Percy et al., 1988). Two distinct morphologies, spherical (mean soma area; 730 μm^2) and fusiform (mean soma area 540 μm^2), were detected in a 2:1 ratio, respectively. The two morphologies could not be distinguished electrophysiologically. Intracellular recordings of lumbar sympathetic chain neurons indicated that 86% had myelinated fibers and 14% had non-myelinated fibers projecting to lumbar splanchnic nerves or to the lumbar sympathetic chain (Hartman and Krier 1984).

INFERIOR MESENTERIC GANGLION (IMG). The IMG projects processes through two postganglionic trunks, the lumbar colonic nerves and the hypogastric nerves (Baron et al., 1985a and b). When cat lumbar colonic nerves were retrogradely labeled with HRP, approximately 24,000 neurons were identified in the IMG (Baron et al., 1985 III). In a similar study, cat hypogastric nerves were retrogradely labeled with HRP and

32,300 neurons were detected in the IMG (Baron et al., 1985 I). The subpopulation of IMG neurons that enter the colon via colonic fiber bundles to innervate colonic effector structures is unknown.

SUPERIOR MESENTERIC GANGLION (SMG) AND COELIAC GANGLION. The number and distribution of neurons in SMG and coeliac ganglia that provide innervation to the colon is relatively unexplored. When lumbar colonic nerves were labeled with HRP, cell bodies in SMG were detected in only 2 of 5 cats (six cells and seven cells)(Baron et al., 1985 III). The distribution of neurons in the coeliac ganglion was not reported. The distribution of neurons in these prevertebral ganglia that project processes through colonic fiber bundles to at least mid colon is not known.

The SMG and coeliac ganglia receive excitatory input from the colon. Electrophysiological studies in guinea pig demonstrated that both coeliac and SMG neurons received excitatory input from the colon that was increased by colon distension and abolished by cutting the intermesenteric nerves (Kreulen and Szurszewski, 1979). This suggests the possibility of a peripheral reflex loop between these prevertebral ganglia and enteric ganglia of the colon (Kruelen and Szurszweski, 1979). The postganglionic fibers may project to the colon via hypogastric nerves and colonic fiber bundles.

The number and distribution of neurons in sympathetic prevertebral and paravertebral ganglia that project process-

es to at least mid colon will be determined using retrograde tracing techniques.

3. INTRINSIC NEURAL PATHWAYS: MYENTERIC PLEXUS. Intrinsic nerves of the large intestine are located within interconnected ganglia of the myenteric and submucosal plexus (Costa and Furness, 1976; Gabella, 1979; Gershon, 1981). The myenteric plexus, located in the connective tissue layer between the longitudinal and circular smooth muscle layers, consists of large ganglia interconnected by numerous interganglionic fiber tracts. The myenteric plexus has been described morphologically, immunocytochemically, and electrophysiologically in an attempt to correlate these characteristics with function (Dogiel, 1899; Christensen et al., 1984; Christensen, 1988; Costa et al., 1982; de Groat, 1987; Gunn, 1959; Gunn, 1968).

MORPHOLOGY. Dogiel was the first to describe three cellular morphologies for myenteric neurons (Dogiel, 1899) and his work remains the standard for enteric neuron morphology. Using methylene blue to stain the myenteric plexus of large and small intestine in man, guinea-pig, dog, cat, rabbit and rat, he identified three distinct morphologies, Dogiel type I, II and III (Dogiel, 1899). Dogiel type I cells have a rough somal surface, short, broad dendrites and a long axon. Dogiel type II cells have a smooth somal surface, numerous fine processes, and no discernible long axon. Dogiel type III cells have a smooth somal surface, several fine dendrites and one long axon. Dogiel speculated that type I

cells were motor neurons and that type II cells were sensory neurons. The three morphological types of myenteric neurons described by Dogiel have been verified in the cat ileum (Gunn, 1959). Using silver impregnation and methylene blue techniques, Gunn was able to distinguish Dogiel types I, II and III as well as a fourth smaller cell type (mean soma diameter approximately 15-20 μm^2) not previously described. The fourth cell type was weakly stained with the silver impregnation technique, had few discernible processes and was found predominantly in the interganglionic fiber tracts. Gunn speculated that these small cells were also neurons.

The distribution of ganglia in the myenteric plexus has been described in numerous mammals (Christensen et al., 1983; Christensen et al., 1984; Santer and Baker, 1988). The distribution of myenteric neurons/cm^2 has been determined in the small and large intestine of young and aging rats using the NADH-tetrazolium reductase method (Santer and Baker, 1988). In young rats (6 months) a mean colonic value of 14,214 neurons/cm^2 was determined while aging rats (24 months) had a mean colonic value of 5,128 neurons/cm^2 (colon). This represents a 64% decrease in the number of neurons/cm^2 in the colon of aging rats.

Silver impregnation techniques were used to determine the density and distribution of myenteric plexus ganglia in the distal colon of eight mammals (Christensen et al., 1984). The range of mean ganglia/cm^2 in distal colon was 40 ganglia/cm^2 (dog) to 794 ganglia/cm^2 (rat). The cat had a

mean value of 42 ganglia/cm^2 and the opossum had 45 (America opossum) to 264 (Australian opossum) ganglia/cm^2. This is in contrast to an earlier study in which, using the same techniques, Christensen reported only 18 ganglia/cm^2 in the distal colon of the opossum (specific species not identified) (Christensen et al., 1983).

IMMUNOCYTOCHEMISTRY. In recent years, a plethora of neuropeptides have been identified immunocytochemically in neurons and/or fibers of the myenteric plexus that may play a role in neurotransmisssion: substance P (SP), somatostatin (SOM), calcitonin gene-related peptide (CGRP), cholecystokinin (CCK), dynorphin (DYN), enkephalin (ENK), galanin (GAL), gastrin releasing peptide (GRP), neuropeptide Y (NPY), neurotensin, peptide HI, neurokinin A and vasoactive intestinal polypeptide (VIP) (Bornstein et al., 1984; Schultzberg et al., 1980; Costa et al., 1980; Costa and Furness, 1983; Ekblad, 1987; Furness et al., 1983; Furness et al., 1985; Furness and Costa, 1979; Furness and Costa, 1982; Fujimori et al., 1989; Jessen et al., 1980; Llewellyn-Smith, 1987; Pearse and Polak, 1975). Immunoreactivity for SP, VIP, CGRP, CCK, NPY, ENK, and SOM was observed in cell bodies as well as fibers of the myenteric plexus of rat and guinea pig small intestine (Bornstein et al., 1984; Costa et al., 1980; Ekblad, 1987; Schultzberg et al., 1980; Furness et al., 1985). Immunoreactivity to neurotensin was observed only in fibers (Shultzberg et al., 1980). In most instances, the

myenteric plexus had a greater density of peptidergic neurons than did the submucosal plexus (Shultzberg et al., 1980). In contrast, VIP containing neurons were found in greater number in the submucosa (Shultzberg et al., 1980, Furness et al., 1981).

The role of many of these neuropeptides in gastrointestinal function remains unknown, although in recent years some progress has been made in determining some physiological functions of several peptides. Ascending contraction (contraction orad to a bolus) and descending relaxation (relaxation aborad to a bolus) was demonstrated in isolated segments of guinea pig colon (Costa and Furness, 1976). Using in-vitro preparations of guinea-pig distal colon and rectum, Costa and Furness studied enteric reflexes by applying localized distension and recording subsequent changes in circular muscle activity. Distension of the colon produced a contraction on the orad side and relaxation on the aborad side of the stimulus. These effects were abolished by interruption of the myenteric plexus but not affected by removal of the mucosa or submucosa. The ascending excitatory pathways were partly blocked by hyoscine (cholinergic-muscarinic antagonist) and methysergide (5-hydroxytryptamine (5-HT) antagonist) and by making the preparation tachyphylactic to 5-HT. The ascending excitatory pathways apparently involve cholinergic neurons as well as 5-HT-like neurons. The descending inhibitory pathway did not respond to cholinergic or adrenergic antagonists and is presumed to be nonadrener-

gic and noncholinergic in nature. Neuropeptides may play a role in these nonadrenergic, noncholinergic effects.

Vasoactive intestinal polypeptide (VIP). One peptide suggested to play a role in descending relaxation is VIP (Farhrenkrug et al., 1978; Furness and Costa, 1978). Myenteric neurons labeled with antibodies to VIP project varicose processes less than 1mm to underlying circular muscle in both orad and aborad directions, aborad to other myenteric neurons for 2-10mm, and aborad to submucosal ganglia up to 15mm (Furness et al., 1985).

VIP has been suggested to play a role in intestinal vasodilation (Costa et al., 1980; Fahrenkrug et al., 1978), intestinal wall relaxation (Costa et al., 1980; Costa and Furness, 1983; Furness et al., 1981, Grider et al., 1985a; Grider et al., 1985b; Grider and Makhlouf, 1986) and increased transepithelial transport of water and electrolytes (Costa and Furness, 1983). VIP neurons were 'S' type (fast EPSPs) and had two morphologies, Dogiel type I (Katayama, 1986) and Dogiel type III (Furness et al., 1981).

Calcitonin gene-related peptide (CGRP). CGRP is reported to be a potent vasodilator in rabbit skin (Brain et al., 1985) and rat mesentery (Han et al., 1990a; Han et al., 1990b) and inhibits smooth muscle contractor responses in mouse vas deferens (Al-Kazwini et al., 1986). In <u>in-situ</u> studies, human or rat CGRP mixed with ^{133}Xe was injected into rabbit

skin to measure ^{133}Xe clearance (Brain et al., 1985). Their results show that femtomole doses of CGRP induced microvascular dilation and increased blood flow, suggesting that local CGRP release may be involved in control of blood flow.

Rat and Human CGRP were also used to study their effects on contractor responses of mouse vas deferens (Al-Kazwini et al., 1986). Both rat and human CGRP were effective at inhibiting contractions of mouse vas deferens evoked by either electrical stimuli or acetylcholine. CGRP did not alter the uptake of [^{3}H]-noradrenaline or the fractional release of [^{3}H]-noradrenaline from preloaded vas deferens, suggesting CGRP does not function by interference with adrenergic mechanisms. These observations suggest that CGRP exerts its effects through post-junctional CGRP receptors.

Substance-P (SP). SP has been shown to be excitatory to myenteric neurons (Katayama and North, 1978). When SP is iontophoretically applied directly onto myenteric neurons of the guinea-pig (region of gastrointestinal tract not identified), SP causes a depolarization that is unaffected by hexamethonium (cholinergic-nicotinic antagonist), atropine (cholinergic-muscarinic antagonist), naloxone (opiate antagonist) or enkephalin (Katayama and North, 1978).

SP also has a contractile effect on guinea-pig ileal longitudinal smooth muscle (Matthijs et al., 1990), cat stomach and large intestine (Delbro et al., 1983; Fandriks

et al., 1985; Fandriks and Delbro, 1985) and canine small intestine (Neya et al., 1989). _In-vitro_ experiments on fura-2 loaded guinea pig ileal longitudinal smooth muscle demonstrate that intracellular calcium concentrations and contractile force increase in a dose dependent manner when exposed to SP (Matthijs et al., 1990).

4. COLONIC AND RECTAL FIBER BUNDLES.

In addition to neurons, ganglia and interganglionic fiber tracts in the myenteric plexus, portions of the gastrointestinal tract also contain large fiber bundles. The distribution of these fiber bundles has been described in human lower esophagus, stomach, small intestine, mid and distal colon and rectum using siver impregnation techniques (Kumar and Phillips, 1989).

The morphology and distribution of fiber bundles has also been described in numerous experimental mammals using silver impregnation techniques (Christensen and Rick, 1985; Christensen and Rick, 1987; Christensen, et al., 1983; Christensen et al, 1984). Fiber bundles in the large intestine originate within the pelvic plexus and pass orad to the colon (Fig. 2) and aborad to the rectum and anal canal. For the cat colon, six to eight colonic fiber bundles ascend orad beneath the serosal surface of the distal colon and between the external muscle layers. They project considerable distances to the mid and proximal regions of the colon. Silver impregnation techniques also identified rectal fiber

bundles in the cat (Figure 4 of Christensen et al., 1984), but they were not mentioned in the text of the paper.

Colonic fiber bundles in cat have been shown to contain sacral parasympathetic preganglionic fibers (de Groat and Krier, 1978), parasympathetic postganglionic fibers (Krier and Hartman, 1984; de Groat and Krier, 1978) and sympathetic postganglionic fibers (de Groat and Krier, 1979). Colonic fiber bundles also contain afferent projections from neurons in sacral dorsal root ganglia in cat and dog (de Groat and Krier, 1978; Fukai and Fukada, 1984).

Sacral parasympathetic preganglionic fibers provide excitatory input to the large intestine that is thought to regulate colonic motility and defecation (de Groat and Krier, 1978). Electrical stimulation of the pelvic nerve elicits contractions of the colon and action potentials in colonic fiber bundles in cats (de Groat and Krier, 1976). Both effects are markedly reduced by ganglionic blocking agents, indicating the presence of synaptic relays that may involve neurons in parasympathetic colonic ganglia and enteric ganglia.

Colonic ganglia were identified histologically on the surface of cat distal colon (Fig. 2)(de Groat and Krier, 1976). In a later study, electrical stimulation of cat colonic fiber bundles produced antidromic potentials in approximately 62% of the colonic ganglion neurons tested. This suggests that some neurons in parasympathetic colonic ganglia project postganglionic fibers orad in colonic fiber

bundles to at least distal colon (Krier and Hartman, 1984).

Several reflexes have been shown to be dependent on the integrity of colonic fiber bundles (Fukai and Fukada, 1984). In in-situ studies of dog colon in which the colon wall was ligated at mid-colon but colonic fiber bundles were intact, mechanical stimulation of the anus and distension of the rectum and proximal colon elicited contractions in colon and rectum and colonic fiber bundle discharges. Ligation of colonic fiber bundles at mid-colon did not diminish colonic or rectal contractions at the site of stimulation, but did abolish contractions and fiber bundle discharges beyond the ligations. This demonstrates that ano-colonic, recto-colonic and colo-colonic reflexes are dependent on the integrity of colonic fiber bundles. These experiments, however, left the pelvic nerves intact, so it is unknown if these reflexes involving colonic fiber bundles are central in origin, peripheral or both.

Peristalsis may be mediated by enteric neurons (Costa and Furness, 1976). In an in-vitro study, ascending contraction (contractions orad to a bolus) and descending relaxation (relaxation aborad to a bolus) were present in isolated segments of guinea pig colon in response to a mechanical stimulus (bolus). These peristaltic waves were abolished by interruption of the myenteric plexus (Costa and Furness, 1976). Myenteric neurons were postulated to be responsible for the peristaltic wave that propelled a bolus through an isolated guinea pig colon. Colonic fiber bundles were not

mentioned in this study, but have been demonstrated in guinea pig distal colon by silver impregnation techniques (Christensen et al., 1984).

Colonic fiber bundles have branch points interconnecting them with the myenteric plexus as they ascend the colon and are known to give off myelinated and non-myelinated processes over their entire length (Christensen and Rick, 1987). It is not known, however, if myenteric neurons utilize colonic fiber bundles as another intrinsic neural pathway, possibly having a role in these reflexes. This dissertation will utilize retrograde tracing techniques to determine if myenteric neurons project processes orad and/or aborad through colonic fiber bundles.

Specific Aims

The specific aims of this dissertation are:

1. Sacral Afferent Pathways.

a. Determine the distribution, number and diameter of neurons in sacral dorsal root ganglia (S_1 - S_3) that project peripheral processes to colonic fiber bundles.

b. Determine the distribution of sacral visceral afferent fibers to mucosa, submucosa, myenteric plexus, external muscle layers and serosa of large intestine.

c. Determine the distribution of sacral visceral afferent fibers to mucosa, submucosa, external muscle layers and serosa of urinary bladder.

d. Determine the distribution of sacral visceral afferent fibers to mucosa, submucosa, external muscle layers and serosa of urethra and external urethral sphincter.

e. Determine the distribution of sacral visceral afferent fibers to prevertebral ganglia.

2. Postganglionic Efferent Pathways to the Large Intestine.

a. Determine the number and distribution of neurons in parasympathetic colonic ganglia that project processes to colonic fiber bundles.

b. Determine the number and distribution of neurons in sympathetic prevertebral and paravertebral ganglia that project processes to colonic fiber bundles.

3. Intrinsic Neural Pathways of the Colon.

a. Determine the number, morphology and distribution of myenteric neurons which project processes to colonic fiber bundles.

METHODS

Anesthesia

Male or female cats were used in all experiments. In experiments involving neuroanatomical tracing techniques, cats were initially anesthetized with intraperitoneal injections of pentobarbital sodium, 34mg/kg. A peripheral intravenous (i.v.) line was established (0.9% NaCl) and anesthesia was maintained with dilute pentobarbital sodium (1:5 0.9% NaCl) as needed. Body temperature was maintained during surgery with a K-thermia pad (38.6^{o}C) and respirations were spontaneous. After appropriate survival periods the animals were reanesthetized with pentobarbital sodium (50mg/kg; i.p.). Tissues were removed and the animals were euthanized.

Sterile Surgical and Labeling Techniques

Experiments involved animal recovery and survival periods. Surgical procedures were performed in a surgical suite under aseptic conditions. After anesthesia, the surgi-

cal sites were shaven and scrubbed with a betadine preparation. All surgical instruments and drapes were autoclaved prior to procedures.

Anterograde Tracing

A mid-line dorsal surgical incision was made exposing the lumbar and sacral vertebral segments (L_6 - S_3). Laminectomies were performed on the sacral vertebral segments (S_1 - S_3) and the sacral dorsal root ganglia (S_1 - S_3) were identified bilaterally (Fig. 1).

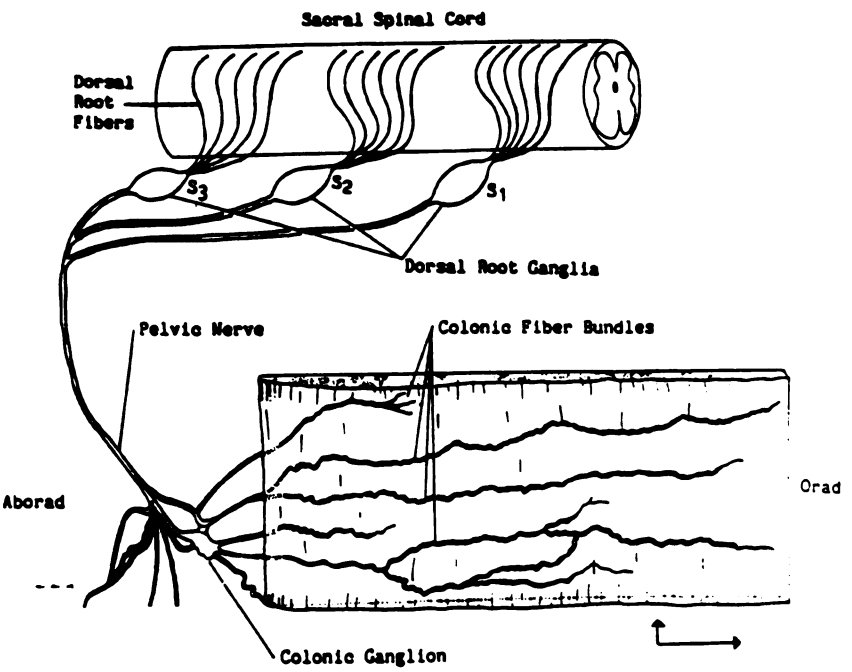


Figure 1. Drawing of cat sacral spinal cord, sacral dorsal root ganglia (S_1-S_3), pelvic nerve, colonic ganglion and colon with colonic fiber bundles. WGA-HRP was pressure injected into sacral dorsal root ganglia (S_1-S_3) bilaterally or ipsilaterally. Long arrow indicates axis of longitudinal muscle fibers. Short arrow indicates long axis of circular muscle fibers.

A 2% solution (distilled deionized water) of wheatgerm agglutinin (WGA)(Vector), either unconjugated orconjugated to horseradish peroxidase (WGA-HRP, Vector) was pressure injected into the ganglia via a glass micropipette (tip diameter range, 18-25 μm). The micropipettes were sealed to a 1 μl syringe with heated sealing wax. Pressure injections of volumes ranging from 0.5 to 1 μl were made bilaterally (n = 9) or ipsilaterally (n = 3) at two or three sites for each dorsal root ganglion ("n" refers to the number of cats unless otherwise specified). In the 3 ipsilateral injection experiments, the ipsilateral sacral ventral roots (S_1 - S_3) were sectioned to rule out incidental labeling of efferent fibers. After injection, the muscle layers and skin were closed. Following time periods ranging from 72 to 120 hours, animals were reanesthetized and perfused.

Retrograde Tracing

A midline abdominal incision was made exposing the large intestine and allowing identification of the bilateral pelvic nerves and their neural connections with parasympathetic colonic ganglia and colonic fiber bundles (DeGroat and Krier, 1976; DeGroat and Krier, 1978)(Fig. 2). Five millimeter segments of 2 to 6 colonic fiber bundles were dissected from the serosal surface of the mid colon at the level of the inferior mesenteric artery or at the distal colon one cm orad to the pelvic brim. The distal colon is defined as the region extending 1.5 cm below the pelvic brim

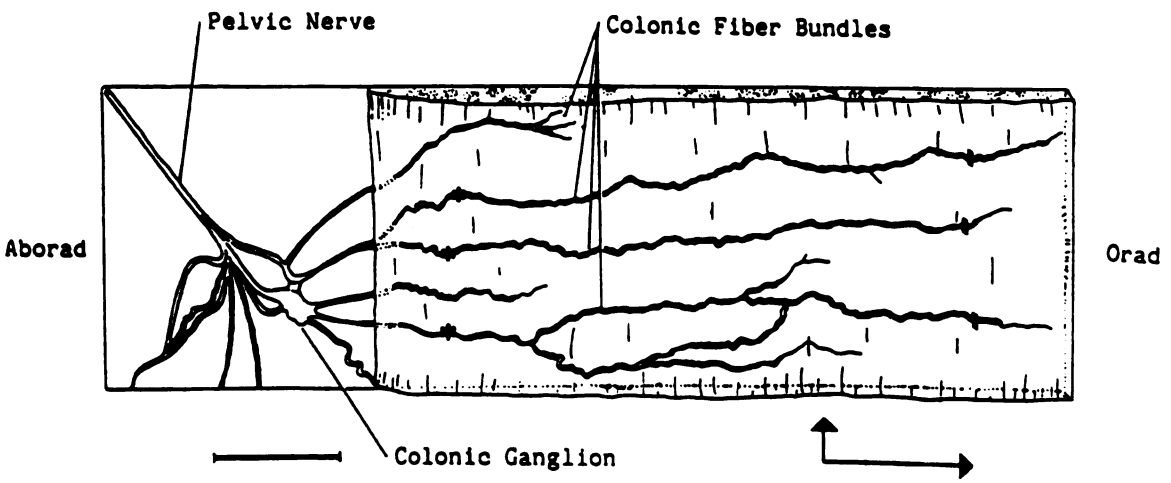


Figure 2. Drawing of one side of cat distal and mid colon with attached pelvic nerves, colonic ganglia and colonic fiber bundles. Filled bars at orad end indicate transection site of colonic fiber bundles at level of inferior mesenteric artery (IMA). Asterisks indicate transection sites of colonic fiber bundles 1cm orad to pelvic brim. HRP or Fast Blue was placed on central cut end (side continuous with pelvic nerve) or peripheral cut end (side continuous with colon) of transected colonic fiber bundles. Long arrow indicates axis of longitudinal muscle fibers and colonic fiber bundles. Short arrow indicates axis of circular muscle fibers. Bar = 10mm.

to 1.0 cm below the inferior mesenteric artery. The proximal colon is defined as the region extending 2.0 cm below the ileocecal sphincter to 4.0 cm above the inferior mesenteric artery. The mid colon is defined as the region between the proximal and distal colon. Central or peripheral cut ends of colonic fiber bundles were placed into small chambers containing 10 μl of 15% horseradish peroxidase (HRP) solution (Sigma Type VI) or 8% Fast Blue (Sigma). The open end of the chamber was sealed with Vaseline. After a 90 minute incubation period, the colonic fiber bundles were removed from the chambers and the abdomen was closed. After 36 to 96 hours, animals were reanesthetized and perfused.

Perfusion

Animals were anesthetized (pentobarbital sodium, 50 mg/kg,i.p.) and given 1000 units of heparin intravenously. The thorax was opened and the animal was transcardially perfused with ice-cold filtered 0.9% sodium chloride/0.05% sodium nitroprusside, followed by ice-cold filtered 4% paraformaldehyde (Immunocytochemical and Fast Blue experiments) or 1% paraformaldehyde/1% glutaraldehyde (HRP experiments) phosphate-buffered fixative and then ice-cold filtered 0.05 M phosphate buffer.

Dissection and Tissue Preparation

After fixation, all tissue to be sectioned was first

cryoprotected by sequential equilibration in 10%, 20% and 30% sucrose buffer. Cryoprotected tissue was then sectioned at 30 to 40 μm on a freezing microtome and processed either free floating or mounted on gel coated slides. Tissue to be processed as whole mounts was dissected free and stored free-floating in 0.1 M phosphate buffered saline (PBS).

Large Intestine. The large intestine was cut open along its mesenteric border and pinned flat with the mucosal border up. The mucosa/submucosa was first removed as a single sheet, followed by removal of the circular muscle layer. From the mucosa/submucosa layer, two to three 1 cm^2 pieces from each colonic region (proximal, mid and distal) were removed and cryoprotected before sectioning at 40 μm. Each square centimeter of mucosa/submucosa (approximately 2 mm thick) produced 25 to 50 (40 μm) sections. Using a dissecting microscope with polarized optics, successive circular muscle strips (lengths, 2 cm to 5 cm in long axis of the circular muscle fibers; widths, 1 mm to 5 mm) were dissected free from the underlying connective tissue layer. The connective tissue layer containing the myenteric plexus of the cat colon proved to be too thick in distal and mid colon regions for conventional processing techniques. For distal and mid colon, the longitudinal muscle layer was separated as a continuous sheet from the adherent connective tissue layer containing the myenteric plexus and colonic fiber bundles. Sheets of myenteric plexus were cut into rectangu-

lar sections (2 cm to 3 cm lengths in long axis of the longitudinal muscle, 4 cm to 5 cm in long axis of circular muscle fibers). For proximal colon the connective tissue layer containing the myenteric plexus was much thinner and could not be seperated as a sheet. Whole mount sections of myenteric plexus adherent to the longitudinal muscle layer were prepared for proximal colon. For the longitudinal muscle layer, successive segments (lengths, 2 cm to 5 cm in long axis of the longitudinal muscle fibers ; widths, 2 cm to 5 cm) were prepared. The myenteric plexus, circular muscle strips and longitudinal muscle were processed free floating and whole mounted.

Urinary Bladder and Urethra. The urinary bladder and urethra were removed and separated at the bladder neck-proximal urethra. The urinary bladder was first hemisected into right and left halves, then divided into dome (apex), ventral wall, dorsal wall, trigone and neck regions. One to two 1 cm^2 sections of bladder wall from each of 8 bladder regions and the entire bladder neck were sliced in horzontal section on a freezing microtome at 40 μm. Each sqaure centimeter of urinary bladder wall (approximately 1 to 2 mm thick depending on bladder distension) produced 20 to 45 (40 μm) sections. In 2 animals, sections of bladder wall (2 mm x 10 mm) were also sliced in cross section at 40 μm. The urethra was divided into equal thirds by length. For unilateral injection experiments the urethra was hemisected into right and

left halves. For 4 animals, a 2 mm length of urethra from each region was cut in cross section at 40 μm. The remainder of the 6-10 mm long sections of urethra were cut in horizontal sections at 40 μm. The external urethral sphincter was dissected from the distal urethra and cut in horizontal or cross section at 40 μm. Some slides were counterstained with neutral red to identify tissue layers.

Histochemical Processing

Peroxidase. HRP labeled neurons and fibers were visualized by incubating tissue with tetramethyl benzidine dihydrochloride (TMB) for 12 to 18 h at 4°C (Mesulam, 1978) followed by 3,3'-diaminobenzidine (DAB) for 5 min at room temperature (22-25°C) (Lemann, 1985; McRorie et al., 1991). The tissue was rinsed in PBS and dehydrated in serial dilutions of ethanol followed by xylene. All tissue was then coverslipped in Permount and allowed to dry for 24 hours.

Immunohistochemistry. Antibodies to the cytoskeletal protein neurofilament were used to label neuronal somas and processes within the myenteric plexus, pelvic plexus ganglia and colonic ganglia (Sternberger and Sternberger, 1983). The tissue was incubated overnight in neurofilament antibody (mouse anti-neurofilament, Sternberger-Meyer, SMI32 1:250-500 and SMI33 1:500 PBS/2-10% fetal calf serum/1% triton X-100 non-ionic detergent), rinsed three times with PBS/1% triton X-100 and incubated in a secondary anti-mouse antibody conjugated to fluoroscein isothiocyanate (FITC) or

tetramethylrhodamine isothiocyanate (TRITC) (Cappell, 1:100 PBS/1% triton X-100) for 2 hours at room temperature. This was followed by rinsing the tissue three times in PBS/1% triton X-100 and twice in PBS. The sheets of myenteric plexus and the pelvic plexus and colonic ganglia sections were mounted/coverslipped with a PBS/glycerol/p-Phenylene-diamine dihydrochloride buffer to retard the fading of fluorescence (Johnson et al., 1981). This technique was also combined with the anterograde neuroanatomical tracer wheat germ agglutinin (WGA). The tissue was incubated overnight in a moist chamber at 4^{o}C with goat primary anti-sera to WGA (1:200, Vector) and mouse monoclonal antibodies to neuro-filament (SMI32 and SMI33, 1:500, Sternber-ger-Meyer)(PBS/0.5-2.0 % Triton X-100/5 % fetal calf serum). The tissue was then rinsed in PBS/0.5-2.0 % Triton X-100 and incubated with biotinylated rabbit anti-goat (Vector). The tissue was next rinsed with PBS/0.5-2.0 % Triton X-100 and incubated with TRITC-conjugated avidin and FITC-conjugated rabbit anti-mouse secondary antibody (1:100; PBS/0.5% triton X-100) for 2 hours at room temperature (22-25^{o}C). The tissue was then rinsed in PBS and mounted in PBS/glycerol/p-Phenylenediamine dihydrochloride buffer to retard the fading of fluorescence (Johnson et al. '81).. TRITC labeled primary afferent fibers and FITC labeled myenteric neurons and processes were visualized by fluores-cence microscopy.

Data Analysis

Microscopy. HRP labeled neurons and fibers were viewed under bright field and fluorescent microscopy using magnifications between 100 and 1000 X. The thickness of the connective tissue layer containing the myenteric plexus frequently precluded the use of bright field optics due to increased incidence of stray light. It was determined that fluorescent optics in the 420-490 nm range (cube H_2, Leitz) offered not only less optical interference, but also assisted in visualizing glutaraldehyde induced fluorescence. Glutaraldehyde fixation causes all tissue to fluoresce. The intensity of this fluorescence is optimal in the 420-490 nm range (green or FITC). In the connective tissue layer containing the myenteric plexus, the colonic fiber bundles, ganglia and interganglionic fiber tracts have a greater density than surrounding connective tissue layers, causing them to fluoresce bright green against a pale green/dark background. This allowed for photomicrographs and camera lucida drawings of both the black HRP labeled neurons and fibers as well as the fluorescent ganglia and fiber tracts surrounding them. This same phenomenon was observed in other sliced and whole-mounted tissues fixed with glutaraldehyde. Attempts to counterstain HRP-labeled tissue frequently resulted in loss of HRP reaction product. The glutaraldehyde induced background fluorescence was used to visualize soma and tissue layers that were not readily discernible under bright field optics in non-counterstained tissues. Fluorescently labeled

neurons and fibers were visualized by fluorescent optics (Leitz) using magnifications between 100 and 1000X. Permanent records were made by both 35 mm photomicroscopy and camera-lucida drawings (Leitz).

Cell Counts. When neuronal soma were counted in sectioned tissue (freezing microtome), the raw numbers were corrected for double counting by the method of Abercombie (Abercombie, 1946). The formula is as follows: corrected number = raw number X (slice thickness/mean soma diameter + slice thickness). The mean soma diameter was computed as being one half of the sum of the long and short axis. Abercombie's formula yields a number that accounts for soma size and slice thickness in correcting for potential double counting in sliced tissue.

CHAPTER 1

DISTRIBUTION OF PRIMARY SACRAL AFFERENT FIBERS TO LARGE INTESTINE, URINARY BLADDER, URETHRA,EXTERNAL URETHRAL SPHINCTER, AND PREVERTEBRAL GANGLIA

Visceral afferent fibers emanating from sensory neurons in sacral dorsal root ganglia project to pelvic viscera via the pelvic nerve (Langley and Anderson, 1895; 1896; de Groat and Krier, 1978). Sacral afferent fibers transmit mechanoreceptive and nociceptive stimuli from the colon (Floyd and Lawrenson, 1979; Floyd et al., 1976; Haupt et al., 1983; Bahns et al. 1985; Janig and Koltzenberg, 1990). Some of the afferent fibers in pelvic nerve and colonic fiber bundles mediate spinal reflexes during defecation (de Groat and Krier, 1978).

Sacral afferent fibers also transmit mechanoreceptive and noiceceptive stimuli from the urinary bladder (de Groat et al., 1981; Habler et al., 1990; Iggo, 1955; Floyd et al., 1976; Bahns et al., 1987) and urethra (Bahns et al., 1987). Afferent fibers emanating from sacral dorsal root ganglia mediate sacral parasympathetic reflexes to urinary bladder and urethra (micturition)(de Groat and Booth, 1984; de Groat and Kawatani, 1989).

The central distribution of sacral visceral afferents from the pelvic nerve to sacral dorsal root ganglia, dorsal horn spinal gray matter and to the sacral parasympathetic nucleus is well known for several species (Nadelhaft

et al., 1980; Nadelhaft et al., 1983; Nadelhaft and Booth, 1984; Mawe et al., 1986; Morgan et al., 1981; Vera and Nadelhaft, 1990). The subpopulation of sacral afferent neurons that provide innervation to mid and proximal colon through colonic fiber bundles is unknown.

The distribution of sacral afferent fibers in guinea-pig and cat colon external muscle layers and myenteric plexus ganglia has been described using degeneration techniques (Schofield, 1962). Degeneration techniques have also been used to describe the distribution of sacral afferent fibers to the cat urinary bladder (Uemura et al., 1973). The distribution of sacral afferent fibers to the large intestine, urinary bladder, urethra and prevertebral ganglia has not been studied using anterograde tracing techniques.

This chapter identifies the origin of neurons in sacral dorsal root ganglia that project their peripheral processes to mid colon using techniques of retrograde axonal transport. It also identifies the distribution of sacral visceral afferent fibers in the pelvic and pudendal nerves, large intestine, urinary bladder, urethra, external urethral sphincter and prevertebral ganglia using techniques of anterograde axonal transport of WGA-HRP and unconjugated WGA.

MATERIALS AND METHODS

Retrograde Tracing

Experiments were performed on 7 anesthetized cats. A

midline abdominal incision was made 5 to 6 colonic fiber bundles were labeled with 4% fast blue at the level of the inferior mesenteric artery (Fig. 3). The abdomen was closed and after 90 to 100 hours, animals were reanesthetized and perfused.

Sacral dorsal root ganglia (S_1 - S_3), lumbar dorsal root ganglia (L_7) and coccygeal dorsal root ganglia (Cg_1) were removed bilaterally and sectioned in the horizontal axis at 40 μm. Fast blue-containing neurons were visualized by fluorescence microscopy at 100 to 400X. In each horizontal section, counts of neurons were corrected for double counting (Abercombie, 1946).

Anterograde Tracing

Experiments were performed on 12 anesthetized cats. A surgical incision was made exposing the lumbar and sacral vertebral segments (L_5-S_3). A 2% solution (distilled deionized water) of wheat germ agglutinin (WGA, Vector), either unconjugated or conjugated to horseradish peroxidase (WGA-HRP, Sigma) was pressure injected into sacral dorsal root ganglia (S_1-S_3) either bilaterally (n = 9) or unilaterally (n = 3) via a glass micropipette (tip diameter 18-25 μm) (Fig. 1). In all three unilateral experiments the ipsilateral sacral ventral roots (S_1-S_3) were chronically sectioned (96-120 hours) to eliminate labeling of preganglionic efferent fibers. In 2 of the unilateral experiments, either the ipsilateral pelvic or pudendal nerve was sectioned (96-120 hours) to determine the relative contributions of the

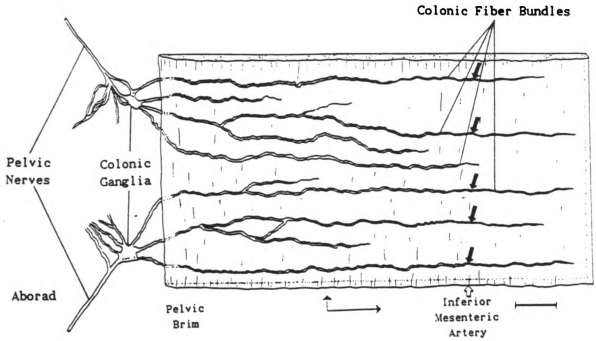


Figure 3. Drawing of cat colon with attached pelvic nerves, colonic ganglia and colonic fiber bundles. Fast blue was placed on central cut end (end continuous with pelvic plexus and pelvic nerve) of transected colonic fiber bundles at level of inferior mesenteric artery (arrows). Long arrow indicates long axis of longitudinal muscle fibers and colonic fiber bundles. Short arrow indicates long axis of circular muscle fibers. Horizontal bar is equal to 10 mm.

pudendal and pelvic nerves, respectively. After injection, the muscle layers and skin were closed. Following time periods ranging between 72 to 120 hours, animals were reanesthetized and perfused.

Large Intestine. The mucosa/submucosa layer was cut into one square centimeter segments and 3 to 4 segments were examined from each intestinal region. For circular muscle layer, successive circular muscle strips (lengths, 2 cm to 5 cm in long axis of the circular muscle fibers; widths, 1 mm to 5 mm) were dissected 1.0 cm orad from the pelvic brim to 6 cm orad to the inferior mesenteric artery (Fig. 3). For the longitudinal muscle layer, successive segments (lengths, 2 cm to 5 cm in long axis of the longitudinal muscle fibers; widths, 2 cm to 5 cm) were prepared. Sheets of myenteric plexus were cut into rectangular sections (2 to 3 cm lengths in long axis of the longitudinal muscle, 4 to 5 cm in long axis of circular muscle fibers). For proximal colon, whole mounts sections of myenteric plexus and colonic fiber bundles, adherent to the longitudinal muscle layer were prepared. The myenteric plexus, circular and longitudinal muscle strips were processed free floating and whole mounted.

Urethra. The long axis of the urethra (3.5 to 5.5 cm), extending from the bladder neck to the external urethral sphincter (Fig. 4), was divided into equal thirds by length

and defined as proximal (continuous with bladder neck), distal (continuous with the external meatus) and mid (region between proximal and distal). For each region of urethra (proximal, mid and distal), a 2mm length was cut in cross-section and an 6-9 mm length was cut in horizontal section. The external urethral sphincter was removed and sliced in horizontal section.

Urinary Bladder. The urinary bladder was first hemi-sected then surgically divided into neck, trigone, dorsal wall, ventral wall and dome (apex). The bladder neck was cut in horizontal sections. For the remaining bladder, one or two 1 cm^2 sections of bladder wall (with mucosa attached) were removed from each region and sliced in the horizontal plane.

Prevertebral Ganglia. The colonic ganglia, urinary bladder ganglia and pelvic plexus ganglia were sliced in the horizontal plane. Tissue was processed for TMB/DAB (WGA-HRP) or immunocytochemistry (unconjugated WGA) and visualized by fluorescence microscopy using magnifications between 100 and 400 X.

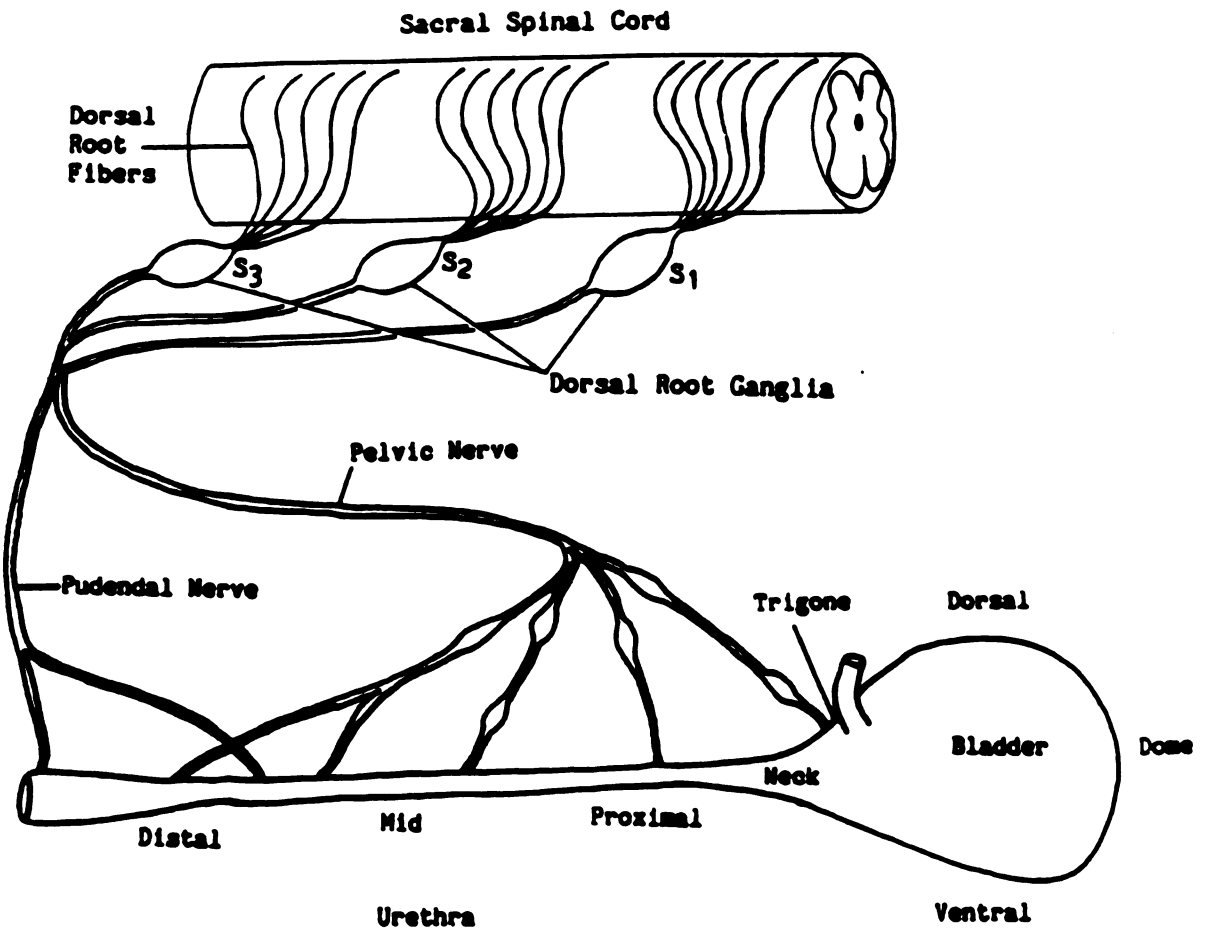


Figure 4. Drawing of cat sacral spinal cord, sacral dorsal root ganglia, pelvic and pudendal nerves, urinary bladder and urethra. Wheat-germ agglutinin conjugated to horseradish peroxidase was pressure injected into sacral dorsal root ganglia (S_1-S_3) and transported anterogradely via pelvic and pudendal nerves to urinary bladder, urethra, external urethral sphincter and prevertebral ganglia.

RESULTS

Retrograde Labeling of Neurons in Sacral and Lumbar Dorsal Root Ganglia

The majority of colonic fiber bundles emanating from both sides of the pelvic plexus were cut at the mid-colon region (level of the inferior mesenteric artery) (Fig. 3). When central cut ends of colonic fiber bundles were placed in a fast blue solution, labeled cell bodies were found in sacral dorsal root ganglia (Figure 5). The somas had a blank nucleus, no processes and were filled with fluorescent blue granules (mean soma diameter ± SE; 29.1 ± 0.6 μm, n = 423).

Sacral Dorsal Root Ganglia. The distribution of neurons in sacral dorsal root ganglia which project to at least mid colon of four cats is shown in Table 1. Neurons were distributed bilaterally in sacral dorsal root ganglia (S_1-S_3)(Fig. 6) with a peak concentration in S_2 dorsal root ganglia. The range of neurons per animal was 660 to 1193 (mean ± SE = 946 ± 114). The mean percentage of neurons (± SE) in bilateral sacral dorsal root ganglia was the following: S_1; 2.5 ± 1.3 ; S_2, 77.3 ± 9.8; S_3, 20.1 ± 10.3. No neurons were detected in the first coccygeal dorsal root ganglia.

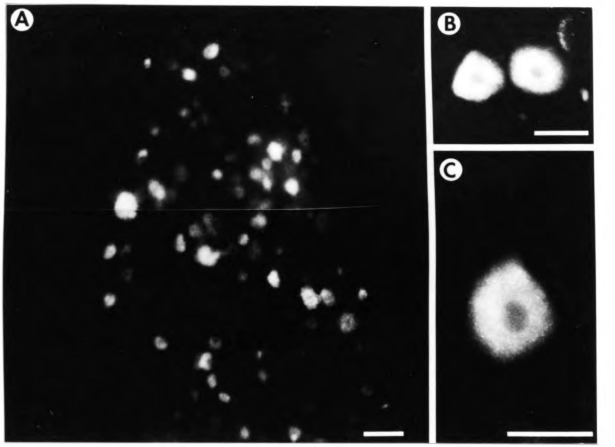


Figure 5. Fluorescent photomicrographs of neurons in sacral dorsal root ganglion (DRG). Colonic fiber bundles were retrogradely labeled at the level of the IMA with fast blue. A: Low magnification view of neurons in sacral (S_2) DRG section. B,C: Higher magnification views of DRG neurons. Somas were partially filled with a blue granular reaction product. Horizontal bar equals 80 μm in A; 40 μm in B and C.

Table 1

Distribution of Sacral Dorsal Root Ganglia Neurons That Project to Colonic Fiber Bundles

	LEFT S_1	RIGHT S_1	LEFT S_2	RIGHT S_2	LEFT S_3	RIGHT S_3
1)	3	3	688	145	31	13
2)	12	1	327	273	325	255
3)	24	17	264	320	17	18
4)	15	8	405	391	57	170

(n = 4, corrected for double counting by method of Abercombe)

Anterograde Labeling of Soma in Sacral Dorsal Root Ganglia

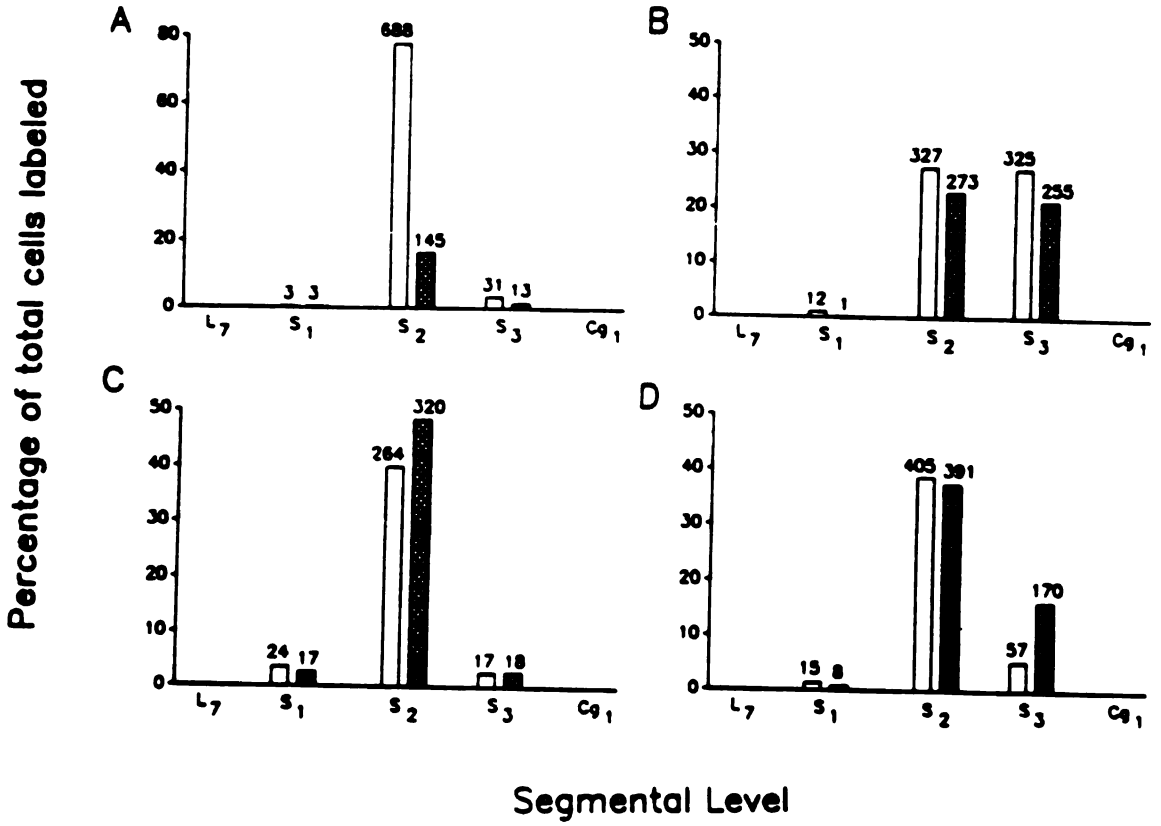
Sacral dorsal root ganglia (S_1-S_3) were pressure injected bilaterally with wheatgerm agglutinin conjugated to horseradish peroxidase (WGA-HRP). Dorsal root ganglia were nearly completely filled with dense reaction product, obscuring individual soma and their processes. In peripheral regions of the ganglia labeled somas were identified.

Anterograde Labeling of Sacral Spinal Cord

Sacral afferent fibers were anterogradely labeled bilaterally in the three sacral segments (S_1-S_3) of the spinal cord. The greatest density of reaction product occurred in Lissauer's tract (LT), lateral collateral pathway (LCP), medial collateral pathway (MCP), and the

Figure 6 Distribution of neurons in sacral dorsal root ganglia that project peripheral afferent processes to colonic fiber bundles to at least mid colon region. Colonic fiber bundles were retrogradely labeled with fast blue. Ordinate; percentage of total neurons labeled. Abscissa; lumbar (L_7), sacral (S_1-S_3) and coccygeal (Cg_1) dorsal root ganglia. A-D; each panel represents data obtained from one animal. Open bars represent left ganglia, hatched bars represent right ganglia. Numbers above horizontal bars indicate total number of cells.

Figure 6


A
B
C
D
Percentage of total cells labeled
Segmental Level
L7
S1
S2
S3
Cg1
3 3
688
145
31 13
12 1
327
273
325
255
24 17
264
320
17 18
15 8
405
381
37
170

dorsal gray commissure (DCM). The dorsal horn (Laminae I - VI) also contained a diffuse reaction product giving the granules a random appearance. When ipsilateral sacral dorsal root ganglia (S_1-S_3) were injected with WGA-HRP, afferent fibers and reaction product were identified only in the ipsilateral LT, LCP, MCP, and dorsal horn (Fig. 7).

For the majority of experiments (7 of 9), no somas were detected bilaterally in the intermediolateral regions or ventral horns of the spinal cord. In two experiments some neuronal soma were identified in the ventral horn of the spinal cord.

Sacral Afferent fibers: Peripheral Destinations

PREVERTEBRAL GANGLIA

Colonic Ganglia. Nonvaricose sacral afferent fibers were detected bilaterally in pelvic nerve (Fig. 8A) and sacral parasympathetic colonic ganglia (Fig. 8 B-I)(18 of 18 ganglia, n= 9). When sacral dorsal root ganglia (S_1-S_3) were pressure injected ipsilaterally, afferent fibers were detected only ipsilaterally (n = 2). No afferent fibers were detected in colonic ganglia after the pelvic nerve was sectioned (n = 1). For the majority of ganglia, fibers were detected in bundles along the borders and center of the ganglia (Fig. 8 B,C). Small bundles and dispersed fibers branched from large fiber bundles in proximity to neuronal somas in 100% of colonic ganglia (Figs. 8 D-I; 17G,H).

Figure 7. Brightfield photomicrograph of cross section of sacral spinal cord (S_2 spinal cord segment). Ipsilateral sacral dorsal root ganglia (S_1 - S_3) were pressure injected with wheat germ agglutinin conjugated to horseradish peroxidase. Ipsilateral sacral spinal cord (S_1 - S_3) contained sacral afferent fibers in Lissauers' tract (LT), lateral collateral pathway (LCP) and medial collateral pathway (MCP). Diffuse reaction product was also observed in the dorsal gray commissure (DCM). No labeled somas were detected. Horizontal bar is equal to 500 μm.

Figure 7

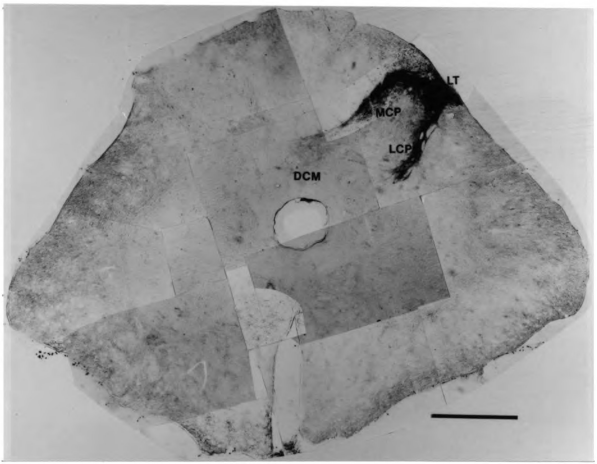
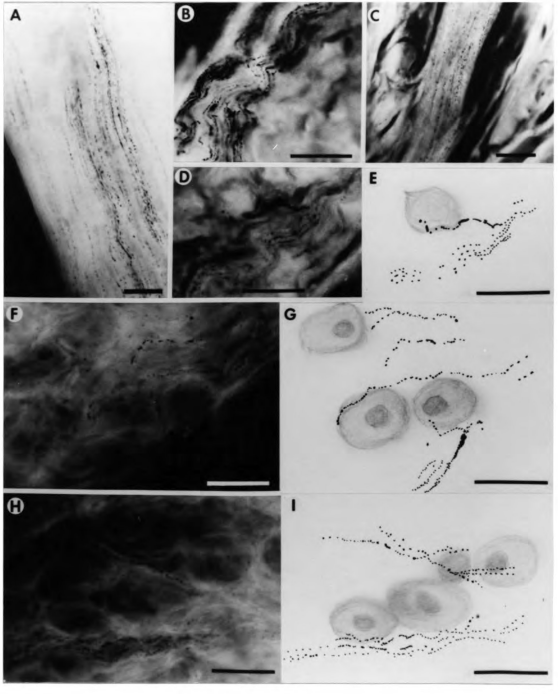


Figure 8. Photomicrographs and camera lucida drawing of sacral afferent fibers in pelvic nerve (A) and sacral parasympathetic colonic ganglia (B-I). A; horizontal section (40 μm) of pelvic nerve with afferent fibers. Horizontal section of colonic ganglion (40 μm) showing afferent fibers in fiber tracts at border (B) and center (C) of ganglion. D-I; afferent fibers in proximity to soma of ganglion cells. Drawings in E, G and I correspond, respectively to photomicrographs D, F and H. Horizontal bars in A-I are equal to 50 μm.

Figure 8



Pelvic plexus ganglia. In 6 animals, 61 ganglia were removed bilaterally from the pelvic plexus. Nonvaricose sacral afferent fibers were detected in 60 of 61 ganglia. A similar bilateral distribution of sacral afferent fibers was detected in experiments where sacral dorsal root ganglia (S_1-S_3) were pressure injected ipsilaterally.

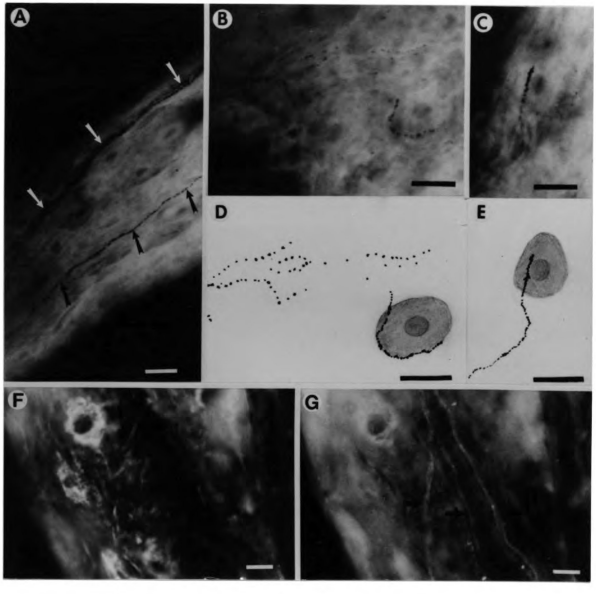
Similar to colonic ganglia, afferent fibers were detected within fiber bundles along the border of ganglia and in fiber bundles which course through ganglia (Fig. 19A). 100% (60/60) of pelvic plexus ganglia had sacral afferent fibers in proximity to neuronal somas (Figs. 19 B-G; 23E-F). No sacral afferent fibers were detected in pelvic plexus ganglia after the pelvic nerve was sectioned (n = 1).

Large Intestine

Colonic Fiber Bundles. When sacral dorsal root ganglia (S_1-S_3) were pressure injected bilaterally with wheatgerm agglutinin conjugated to horseradish peroxidase (WGA-HRP) or unconjugated wheatgerm agglutinin (WGA), afferent fibers were detected bilaterally within pelvic nerves (Figs. 8A; 19A), colonic branches of the pelvic nerve, colonic fiber bundles and branch points of colonic fiber bundles (Figs. 10; 13A,B; 16A). When sacral dorsal root ganglia (S_1-S_3) were pressure injected ipsilaterally sacral afferent fibers were detected only ipsilaterally. No sacral afferent fibers

Figure 9. Photomicrographs and camera lucida drawings of sacral afferent fibers in pelvic plexus ganglia. Sacral dorsal root ganglia (S_1-S_3) were injected with wheat germ agglutinin conjugated to horseradish peroxidase (A-E) or unconjugated wheat germ agglutinin (F-G). A; horizontal section of pelvic plexus ganglion (40 μm) showing afferent fibers in fiber tracts at border (white arrows) and center (black arrows) of ganglion. B-E; afferent fibers in proximity to soma of ganglion cells. Drawings in D and E correspond, respectively to photomicrographs B and C. F; fluorescent photomicrographs of neurofilament labeled somas and processes. G; same frame showing afferent fibers (arrows) through center of ganglion in proximity to ganglion cells. Horizontal bars in A-G are equal to 30 μm.

Figure 9



were detected in colonic fiber bundles after the pelvic nerve was sectioned (n = 1).

Afferent fibers were detected continuously or discontinuously in 4 to 8 colonic fiber bundles emanating from bilateral pelvic plexuses (n = 5). Their density varied within colonic fiber bundles. In different regions of the colon sacral afferent fibers either completely (Fig. 10A,B) or partially filled (Fig. 13A) the colonic fiber bundle.

Afferent fibers in colonic fiber bundles could be traced orad from the distal colon at the level of the pelvic brim to the mid and proximal colon at maximal orad axial distances ranging from 2.6 to 11.3 cm. The maximal axial orad distances where sacral afferent fibers were detected within colonic fiber bundles are shown in Figure 11.

Rectal Fiber Bundles. The distribution of sacral afferent fibers to the rectum and anal canal was also studied (n = 3). The rectum/anal canal region of the cat contains four to six axially orientated fiber bundles which can be traced one cm orad to the external anal sphincter (Langley and Anderson, 1895; Christensen et al., 1984). There are also branches of rectal fiber bundles that enter the myenteric plexus.

Afferent fibers were visualized intermittently in rectal fiber bundles and their branch points (Fig. 12A-C). Some were visualized in rectal fiber bundles one cm orad to the striated musculature of the external anal sphincter.

Figure 10. Camera lucida drawing and photomicrographs of sacral afferent fibers in a branched colonic fiber bundle. Wheat germ agglutinin conjugated to horseradish peroxidase was pressure injected into sacral dorsal root ganglia (S_1-S_3) bilaterally. A; camera lucida drawings of afferent fibers in branched colonic fiber bundle. B; bright-field photomicrograph of afferent fibers in panel A. C; fluorescent photomicrograph of afferent fibers leaving colonic fiber bundle to enter plane of myenteric plexus. Horizontal bar in A is equal to 2 mm. Horizontal bars in B and C are equal to 50 μm.

Figure 10

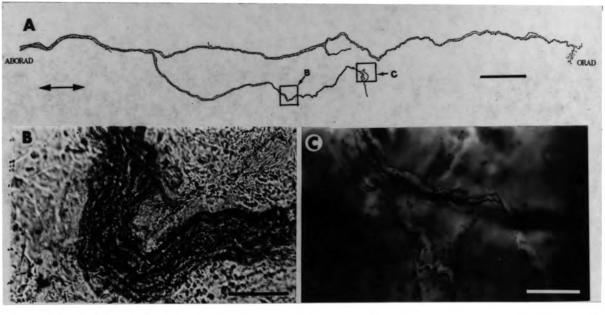


Figure 11. Distribution of sacral afferent fibers in colonic fiber bundles. Abscissa; experiment number. Ordinate; maximal orad distances sacral afferent fibers were visualized in colonic fiber bundles. Orad distances are expressed in millimeters from the pelvic brim. Vertical bars represent individual colonic fiber bundles.

Figure 11

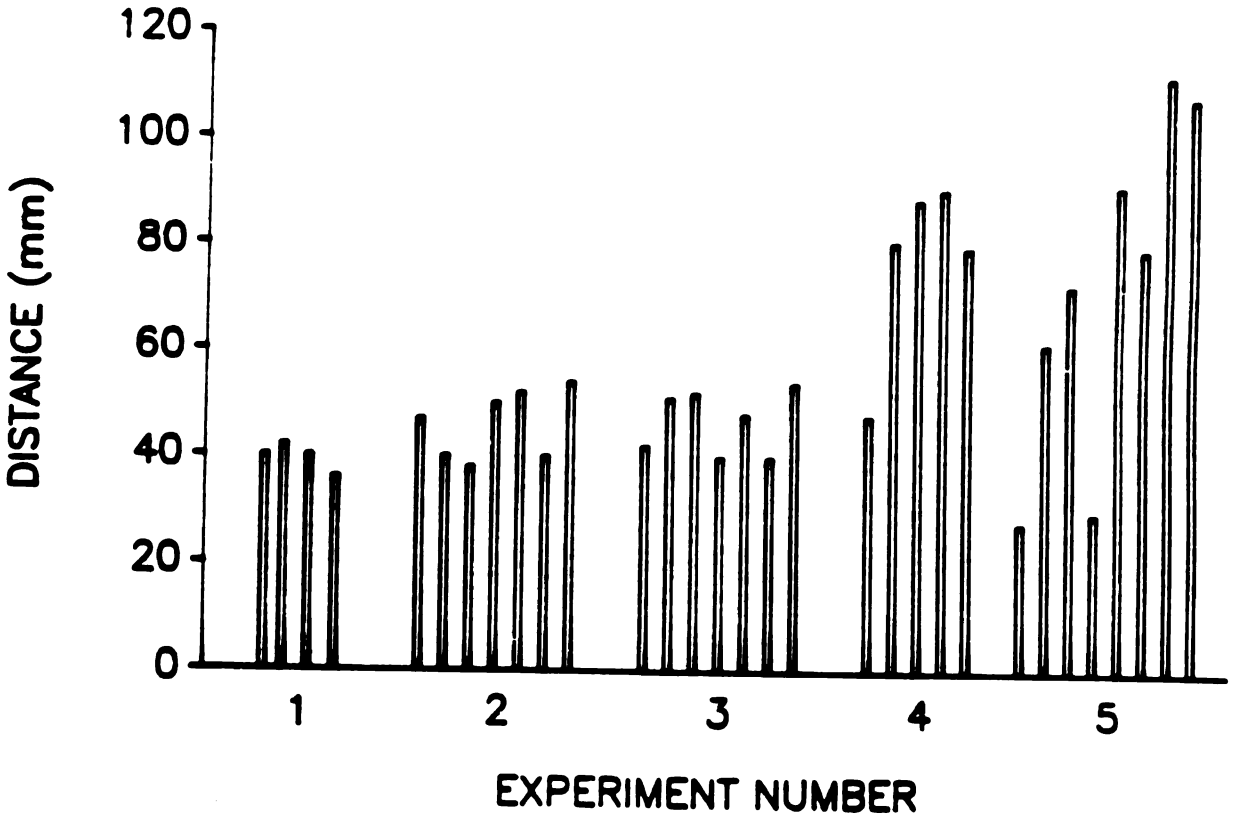
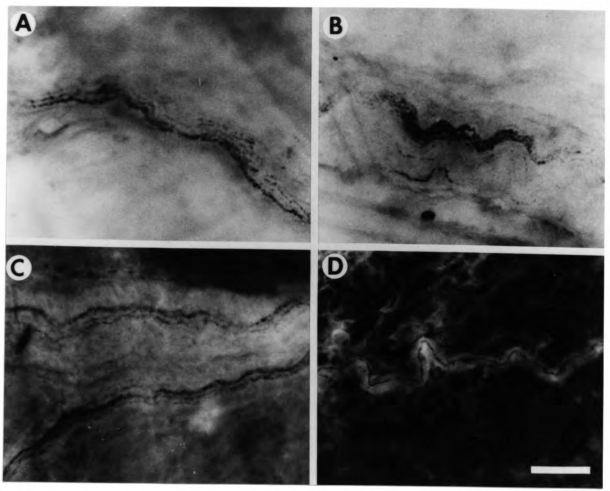


Figure 12. A-C; Fluorescent photomicrographs of sacral afferent fibers in fiber bundles located in myenteric plexus of rectum. A; afferent fibers in fiber bundle 1.5 cm orad of external anal sphincter. B; afferent fibers in fiber bundle 1 cm aborad from pelvic brim. C; afferent fibers in branching fiber bundle 2 cm aborad from pelvic brim. D; Fluorescent photomicrograph of sacral afferent fiber in interganglionic fiber tract of distal colon submucosal plexus. Horizontal bar in D equal to 50 μm, and refers also to A-C.

Figure 12



The maximal axial aborad distances where afferent fibers were detected ranged from 3 to 4 cm. No afferent fibers were detected in rectal fiber bundles after the pelvic nerve was sectioned (n = 1).

Colonic-Rectal Myenteric Plexus. After leaving branch points of colonic or rectal fiber bundles, sacral afferent fibers projected bilaterally to interganglionic fiber tracts and predominantly to myenteric ganglia that were adjacent to colonic fiber bundles. When sacral dorsal root ganglia (S_1-S_3) were pressure injected ipsilaterally, afferent fibers were detected only ipsilaterally in colonic fiber bundles and in myenteric ganglia that were adjacent to colonic fiber bundles. No afferent fibers were detected in myenteric plexus ganglia after the pelvic nerve was sectioned (n = 1).

In the colon, afferent fibers projected either lateral or orad to a fiber bundle or its branch point (Figs. 13A,B; 15A). No afferent fibers were detected projecting aborad to interganglionic fiber tracts or to myenteric ganglia. For the rectum-anal canal, afferent fibers projected either lateral or aborad to a fiber bundle or its branch point. No afferent fibers were detected projecting orad to interganglionic fiber tracts or to myenteric ganglia.

In colon myenteric plexus, some afferent fibers remained as small bundles within interganglionic fiber tracts and coursed in an axial orad direction through 1 to 2 ganglia (Fig. 13B) or numerous ganglia (Fig. 14). Others left

the afferent bundles and ended as fibers within the ganglia (Figs. 13B; 14; 15; 16).

Both varicose and nonvaricose sacral afferent fibers were detected as isolated fibers and arborizations within myenteric ganglia of the distal, mid and proximal colon (Figs.13B; 14; 15; 16A-E) or rectum (Fig. 16F,G). Varicose fibers were visualized in 12 percent of myenteric ganglia (38 of 313, n = 3) that contained afferent fibers. The remainder of ganglia contained nonvaricose fibers (275 of 313). The patterns of arborization included some nonvaricose fibers with 'basket-like' arrangements (Fig. 16 D,E). Other arborization patterns showed varicose afferent fibers (Fig. 15B-D).

Figure 17 A-F shows sacral afferent fibers and neurons in myenteric plexus ganglia. Some neurons were in proximity to branching afferent fibers (Fig. 17A,B). In other ganglia, branching afferent fibers (Fig 17D, arrows) followed the contour of myenteric neurons (Fig. 17C, arrow). Other sacral afferent fibers (Fig. 17F, arrow) were in proximity to myenteric neurons located at the border of the ganglion (Fig. 17E).

Figure 13. A; Camera lucida drawings of sacral afferent fibers in a bifurcating colonic fiber bundle with adjacent myenteric plexus ganglia. B; higher magnification of segment of colonic fiber bundle with connection to myenteric plexus ganglia. Afferent fibers were identified intermittently through colonic fiber bundle up to a maximal orad axial distance of 6.5 cm from distal colon. Note, afferent fibers enter branch point of colonic fiber bundle to enter one myenteric ganglion. Afferent fibers course through interganglionic fiber tract to enter and exit another myenteric ganglion. C; afferent fiber coursed through myenteric ganglion and interganglionic fiber tract to circular smooth muscle. Horizontal bar in A is equal to 5 mm. Horizontal bars in B and C are equal to 400 μm and 200 μm, respectively.

Figure 13

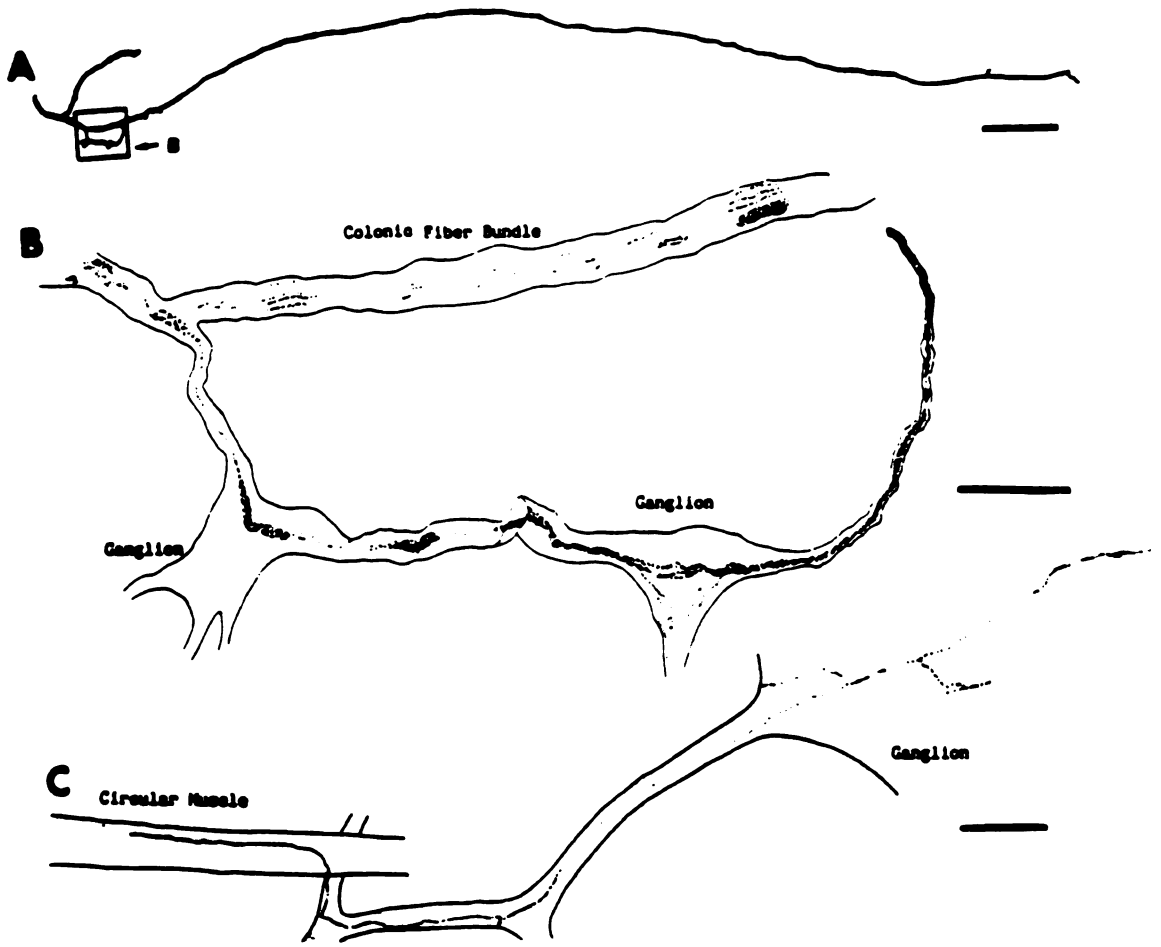

A
B
Colonic Fiber Bundle
Ganglion
Ganglion
C
Circular Muscle
Ganglion

Figure 14. A; camera lucida drawing of orad projections of sacral afferent fibers in interganglionic fiber tracts and ganglia of mid colon myenteric plexus. Afferent fiber bundles projected orad continuously for 1.5 cm through interganglionic fiber tracts and ganglia. Asterisks indicate the location of myenteric ganglia. The most orad projection of afferent fibers left plane of myenteric plexus to branch onto circular smooth muscle (arrow). B; higher magnification of afferent fibers within one myenteric plexus ganglion indicated in panel A. Note, afferent fibers enter ganglion as one bundle, branch into an arborization of fibers and exit as two bundles. C,D; higher magnification views of afferent fibers in myenteric plexus ganglia and interganglionic fiber tracts indicated in panel A. Horizontal bar equals 1mm for panel A, 200 μm for panels B-D.

Figure 14

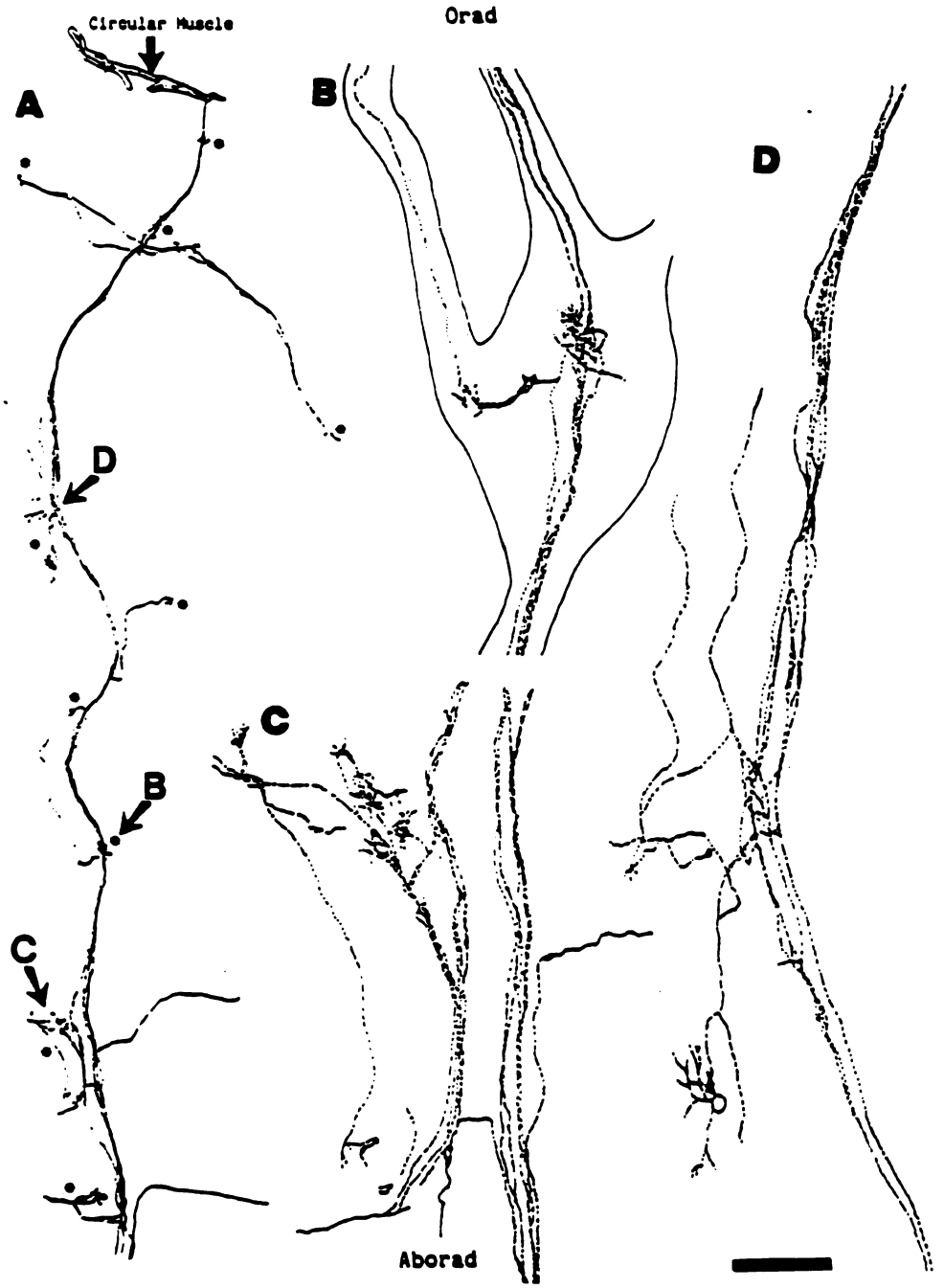


Figure 15. Camera lucida drawings (A, C) and fluorescent photomicrograph (B) of sacral afferent fibers in a myenteric plexus ganglion. A; myenteric plexus ganglion with afferent fibers. B; fluorescent photomicrograph of afferent fibers in panel A. C; Higher magnification of afferent fibers in A and B. Note, multiple intermittent increases in fiber diameter, suggestive of varicosities. Horizontal bar equals 200 μm for A, 50 μm for B and 30 μm for C.

Figure 15

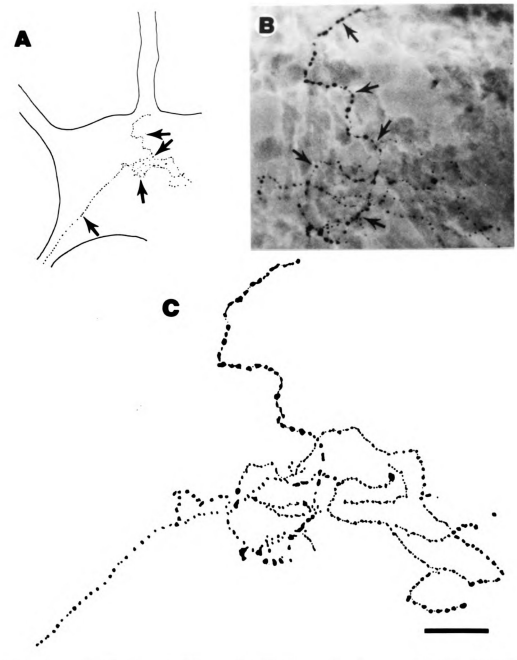


Figure 16. Camera lucida drawings of sacral afferent fibers in ganglia of myenteric plexus in distal colon (A,B,C), mid colon (D,E) and rectum (F,G). A; afferent fibers project in an orad axial direction in colonic fiber bundle and exit colonic fiber bundle at branch point oriented nearly perpendicular to colonic fiber bundle to enter myenteric ganglia. B, D and F; location of afferent fibers within other myenteric plexus ganglia. C, E and G; higher magnification of afferent fibers in B,D and F, respectively. Arborizations of afferent fibers were unique to each ganglion. Horizontal bars in B and D are equal to 200 μm. Horizontal bars in A, C, E and F are equal to 100 μm. G; horizontal bar is equal to 50 μm.

Figure 16

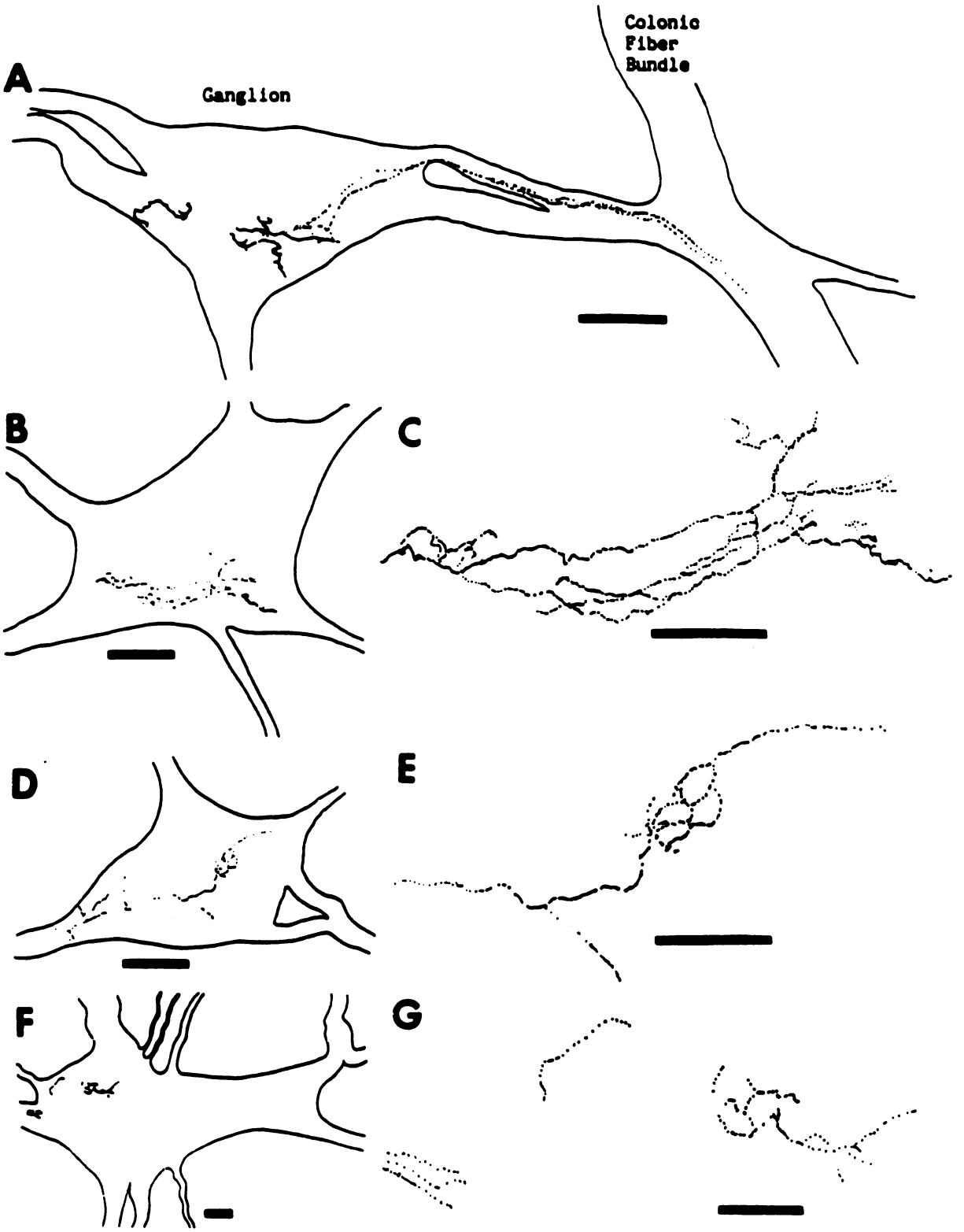


Figure 17. Fluorescent photomicrographs of sacral afferent fibers in proximity to neurons in myenteric (A-F) and colonic ganglia (G-H). Sacral dorsal root ganglia (S_1 -S_3) were pressure injected with unconjugated WGA. Neuronal somas were visualized by indirect immunofluorescence of neurofilament (A, C, E and G). Corresponding photographs of same frame (B, D, F and H, respectively) show afferent fibers. G-H; afferent fibers (H, open arrows) course through colonic ganglion in proximity to neuronal somas (G). Note, large afferent fiber ending (H, closed arrow) in proximity to neuronal soma (G). Bar equals 50 μm and refers to A-H.

Figure 17

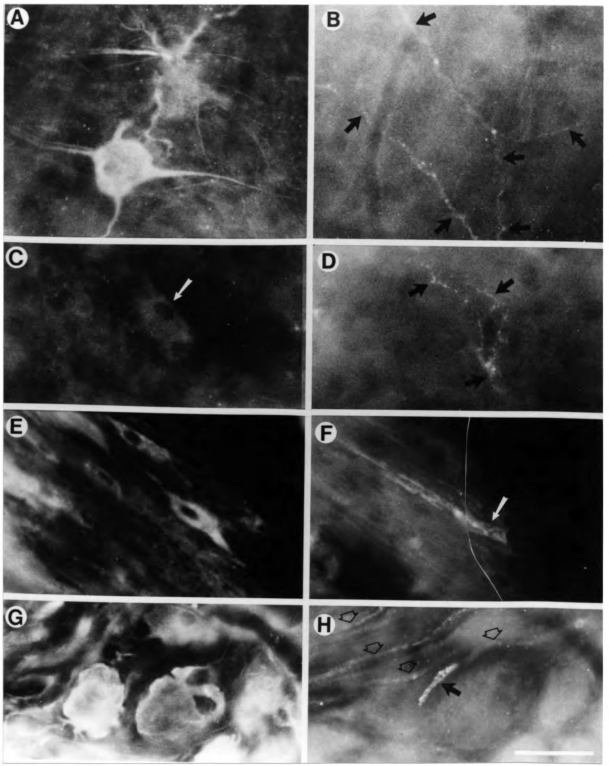


Table 2 summarizes the distribution of neurons and ganglia in the myenteric plexus of the colon (n = 6). It also shows the distribution of ganglia that contained sacral afferent fiber bundles, arborizations and fibers (n = 3). Data are expressed as the mean (± SE) ganglia per square centimeter and mean number of neurons (± SE) per ganglia for each colonic region.

Table 2. Profiles of Neurons and Ganglia in Myenteric Plexus of Cat Colon

Parameter	Distal Colon	Mid Colon	Proximal Colon
Neurons/ganglion	66.7 ± 9.1	71.6 ± 4.6	81.1 ± 13.2
Ganglia/cm^2	13.1 ± 2.3	11.4 ± 1.3	10.3 ± 0.7
*Ganglia/cm^2	2.5 ± 0.7	1.8 ± 0.2	0.9 ± 0.5

Values are mean ±SE
* Ganglia containing sacral afferent fibers

The mean percentage of myenteric ganglia containing sacral afferent fibers decreased from distal colon to proximal colon. The mean percentage of ganglia (± SE) was the following: proximal colon, 8.6 ± 5.1; mid-colon, 15.5 ± 1.9; distal colon, 20.2 ± 4.5.

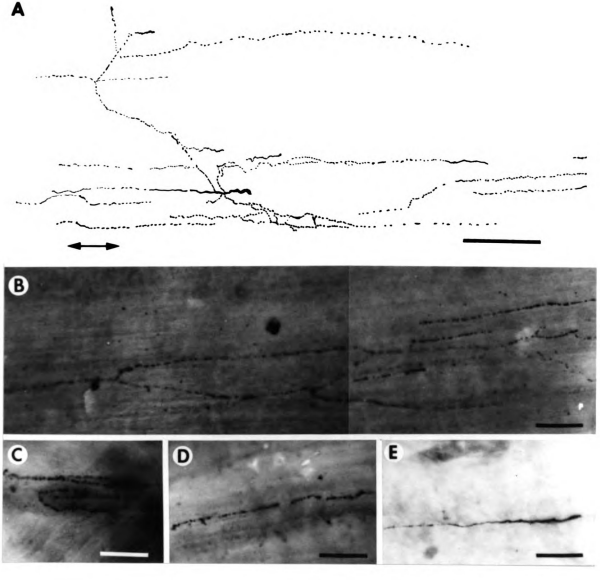
Colon Circular Muscle. Sacral afferent fibers were traced from the myenteric plexus to the circular muscle layer (Fig. 13C; 14A). They were also detected on circular muscle strips obtained from the entire circumference of proximal, mid and distal colon regions (n = 9). A similar distribution of afferent fibers occurred in experiments where sacral dorsal root ganglia (S_1-S_3) were pressure injected ipsilaterally.

Afferent fibers were routinely visualized as interrupted small fiber bundles and nonvaricose fibers. They were orientated parallel to the long axis of the circular muscle fibers over the entire length (range 2 to 5 cm) of individual circular muscle strips (Fig. 18 D,E). Some of the fibers had a club-like appearance (Fig. 18E).

Dense arborizations of afferent fibers were also detected on circular muscle (Fig. 18 A-C). Some fibers were perpendicular or oblique to the long axis of circular muscle fibers (Fig. 15 A). Others coursed parallel to the long axis of circular muscle fibers (Fig. 15A,B). No afferent fibers were detected on smooth muscle fibers of the extrinsic longitudinal muscle layer.

Figure 18. Camera lucida drawing and photomicrographs of sacral afferent fibers on circular muscle of mid colon (A), distal colon (B-D) and proximal colon (E). A-C; arborization patterns of afferent fibers on circular muscle. Double arrow indicates long axis of circular muscle fibers. Note, arrangement of fibers oriented perpendicular, oblique and parallel to long axis of circular muscle fibers. D; afferent fiber parallel to long axis of circular muscle fibers. E; afferent fiber with club-like appearance. Horizontal bar in A is equal to 100 μm. Horizontal bars in B-E are equal to 50 μm.

Figure 18



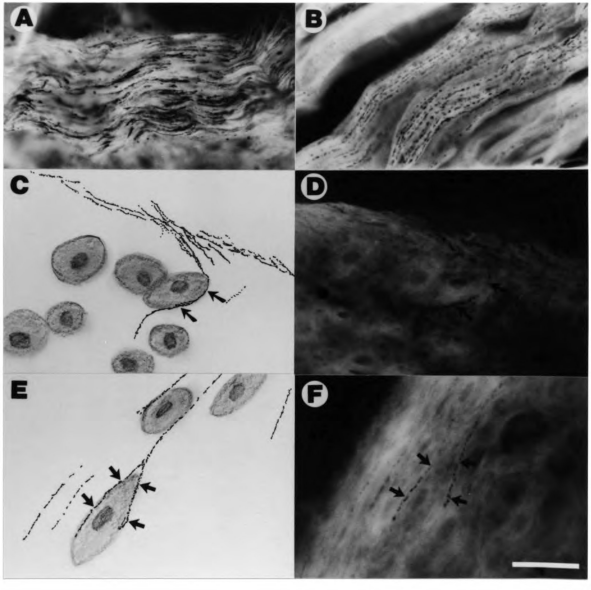
Submucosal Plexus. Few sacral afferent fibers were detected in colon submucosal plexus. Non-varicose fibers were identified exclusively within interganglionic fiber tracts (n = 5)(Fig. 12D). No afferent fibers were detected in mucosa.

URINARY BLADDER GANGLIA, URINARY BLADDER AND URETHRA

Pelvic Nerve, Pudendal Nerve and Urinary bladder ganglia. When sacral dorsal root ganglia (S_1 -S_3) were injected bilaterally with wheatgerm agglutinin conjugated to horseradish peroxidase (WGA-HRP), non-varicose sacral afferent fibers were detected bilaterally within pelvic and pudendal nerves (Fig. 19A-B) and in 37 of 42 urinary bladder ganglia (n = 4). They were in bundles along the borders of ganglia (Fig. 19C), through central regions (Fig. 19C-F) and in proximity to neuronal soma (97% (36/37)) (Fig. 19C-F). No fibers were detected in ganglia after chronic ipsilateral section of pelvic nerve (n = 1). This indicates that afferent fibers pass through pelvic nerve and pelvic plexus to enter urinary bladder ganglia.

Figure 19. Fluorescent photomicrographs and camera lucida drawings of sacral afferent fibers (arrows) in 40 μm horizontal sections of pelvic nerve (A), pudendal nerve (B), urinary bladder ganglion (C-D) and pelvic ganglion (E-F). Note afferent fibers in proximity to neuron in bladder (C; arrows) and pelvic plexus ganglion (E; arrows). Horizontal bar equals 50 μm in A, B, D and F and 60 μm in C and E.

Figure 19



Urinary Bladder. There was a paucity of sacral afferent fibers detected in urinary bladder. Table 3 shows their regional distribution in tissues of urinary bladder. There was an abscence of fibers in mucosa and submucosa of bladder wall and a paucity of fibers in submucosa of bladder neck (Fig. 20A). Afferent fibers occured in extrinsic muscle layers of all bladder regions (Fig. 20A-E). No fibers were detected in urinary bladder after chronic ipsilateral section of pelvic nerve (n = 1).

TABLE 3

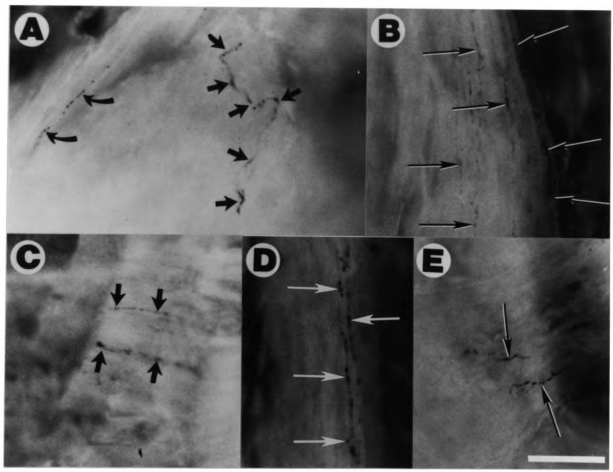
RELATIVE DISTRIBUTION OF SACRAL AFFERENT FIBERS TO URINARY BLADDER

Urinary Bladder	Serosa	Extrinsic Muscle	Submucosa	Mucosa
Apex (n=5*)	+	+	-	-
Dorsal Wall (n=6)	+	+	-	-
Ventral Wall (n=6)	+	+	-	-
Trigone (n=6)	+	++	-	-
Neck (n=5)	+	++	+	-

("n" indicates number of animals with afferent fibers, "+" indicates afferent fibers, "+-" indicates relative increase in number of afferent fibers, "-" indicates no afferent fibers detected, "*" n = 2 of 5 for serosa; 3 of 5 for extrinsic muscle layers)

Figure 20. A; Fluorescent photomicrograph of sacral afferent fibers on urinary bladder smooth muscle (curved arrows) and submucosa (straight arrows) of bladder neck. Afferent fibers on smooth muscle of bladder neck (B), dorsal wall (C), trigone (D) and ventral wall (E). Horizontal bar in E equals 50 μm and also refers to A-D.

Figure 20



Urethra. Sacral afferent fibers were detected in all regions and layers (Fig. 21A) of the urethra. Their distribution is shown in table 4. The mean percentage of histological sections containing afferent fibers was 69.8%.

Table 4

DISTRIBUTION OF SACRAL AFFERENT FIBERS IN CAT URETHRA

	PROXIMAL	MID	DISTAL
Total number of sections	106	144	122
Horizontal sections	74	114	80
Cross sections	32	30	42
Number of sections with afferent fibers	79	93	101
Percentage of total (mean ± SE)	64.1 ± 16.1	68.4 ± 6.2	76.8 ± 5.4

(Horizontal and cross sections = 40 μm, n = 4)

Afferent fiber distribution in urethral tissue is shown in table 5. For urethral serosa, afferent fibers occured in bundles (n = 4)(Fig. 21B). In longitudinal and

circular smooth muscle layers, afferent fibers occurred as bundles and fibers oriented parallel to the long axis smooth muscle fibers (Fig. 21 C-E). In striated muscle of distal urethra they were oriented parallel, oblique and perpendicular to the long axis of muscle fibers (n = 5)(Fig. 22A-D). In external urethral sphincter, they were parallel to the long axis of striated muscle fibers (n = 4)(Fig. 23E-F).

Sacral afferent fibers were relatively more numerous in urethral submucosa. They occurred as fibers (Fig. 23D), fiber bundles (Fig.23C and E) and arborizations (Fig. 23F)(n = 5). Some fiber bundles entered the mucosa (Fig. 23A). In mucosa, afferent fibers occurred as short fragments along the entire length of the urethra (Fig. 23A-C)(n = 5).

Table 5

DISTRIBUTION OF SACRAL AFFERENT FIBERS IN TISSUE LAYERS OF CAT URETHRA

TISSUE	PROXIMAL	MID	DISTAL
Serosa	46.4 ± 9.7 (32)	36.9 ± 13.4 (32)	22.5 ± 0.2 (23)
Longitudinal muscle	51.1 ± 17.3 (33)	45.9 ± 7.1 (42)	19.8 ± 2.4 (18)
Circular muscle	61.4 ± 15.4 (38)	59.9 ± 5.7 (58)	15.9 ± 4.8 (20)
Submucosa	84.4 ± 5.3 (64)	88.0 ± 7.0 (87)	85.7 ± 5.6 (93)
Mucosa	41.7 ± 21.0 (24)	48.1 ± 4.6 (47)	50.5 ± 6.1 (56)
Striated muscle	**	**	46.8 ± 8.9 (43)

(n = 4, mean percentage ± S.E. of labeled sections, (#) number of sections labeled, ** absence of striated muscle fibers)

Figure 21. Camera lucida drawings and photomicrographs of sacral afferent fibers in horizontal sections (40 μm) of proximal (D,E), mid (A,B) and distal (C) urethra. A; camera lucida drawing of mid urethra. Serosa (S), serosal bundle (SB), longitudinal smooth muscle (LM), circular smooth muscle (CM), submucosa (SM), mucosa (M) and lumen (L). B; fluorescent photomicrograph of fibers (arrows) in serosal fiber bundle. C; fluorescent photomicrograph of fibers (arrows) parallel to long axis of longitudinal smooth muscle fibers D; camera lucida drawing of fibers (arrows) parallel to long axis of circular smooth muscle fibers. E; bright-field photomicrograph of panel D. Horizontal bar equals 400 μm in A, 75 μm in B and E, 50 μm in C and 220 μm in D.

Figure 21

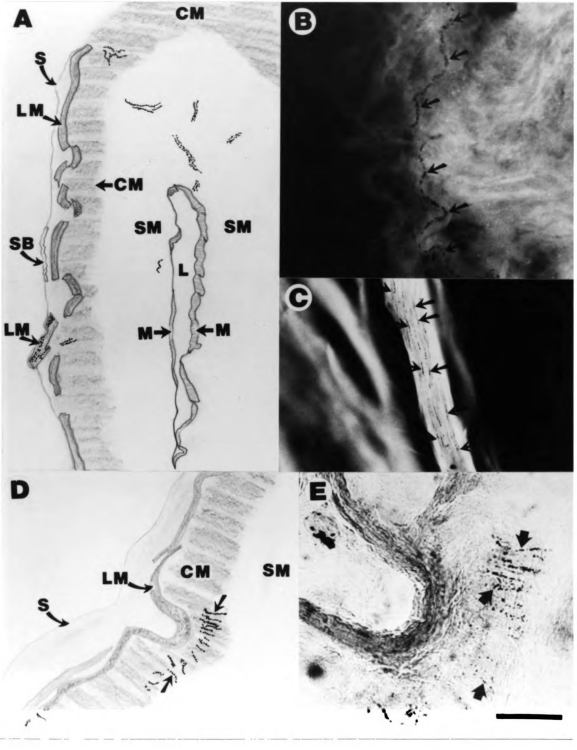
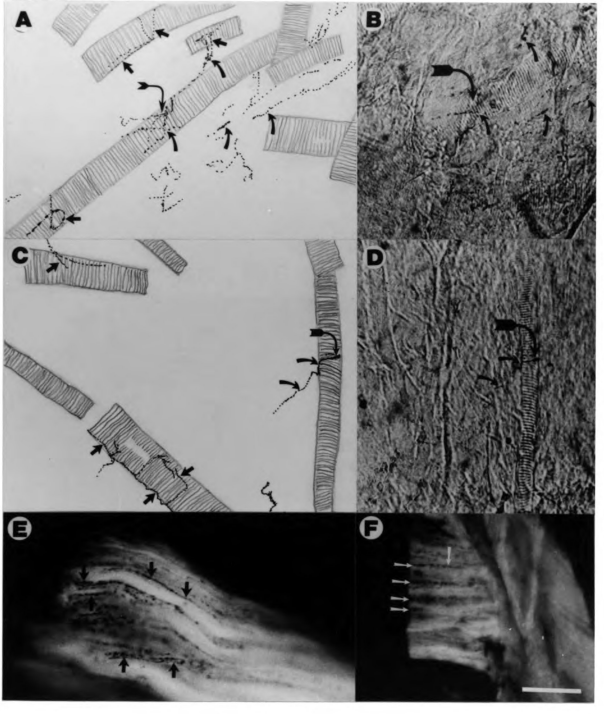


Figure 22. Camera lucida drawings (A,C) and photomicrographs (B, D, E, F) of sacral afferent fibers on striated muscle fibers of distal urethra (A-D) and external urethral sphincter (E-F). A, C; camera lucida drawings of afferent fibers (arrows) oriented parallel, perpendicular and oblique to long axis of striated muscle fibers. B, D; brightfield photomicrographs corresponding to panels A and C, respectively. E,F; fluorescent photomicrographs of afferent fibers (arrows) oriented parallel to long axis of striated muscle fibers. Panels A-D and F obtained from preparations with pelvic and pudendal nerves intact. Panel E obtained from preparation where ipsilateral pelvic nerve was chronically sectioned. Horizontal bar equals 60 μm in A and C and 75 μm in B, D, E and F.

Figure 22



IPSILATERAL INJECTIONS

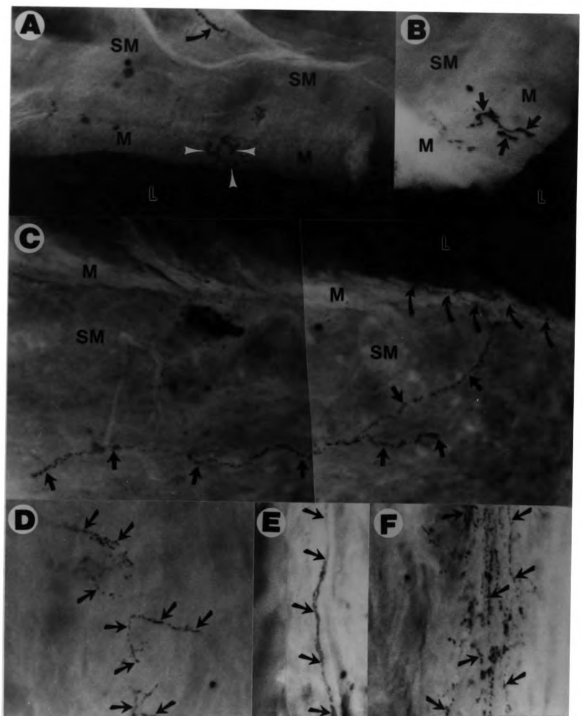
The concept that sacral afferent fibers cross the midline in the periphery was tested. Sacral dorsal root ganglia (S_1 -S_3) were injected unilaterally with WGA-HRP (n = 3). Afferent fibers were detected in ipsilateral pelvic (Fig. 19A) and pudendal (Fig. 19B) nerves but were absent in contralateral pelvic and pudendal nerves. They occurred bilaterally in urinary bladder (n = 2), urethra (n = 3) and external urethral sphincter (n = 2). They were detected in 11 of 17 pelvic plexus ganglia (64.7%)(8 of 8 ipsilateral, 3 of 9 contralateral)(n = 2) and in 17 of 19 urinary bladder ganglia (89.5%)(10 of 10 ipsilateral, 7 of 9 contralateral) (n = 2).

The distribution of sacral afferent fibers that pass through pelvic and pudendal nerves was determined. Sacral dorsal root ganglia (S_1 -S_3) were injected ipsilaterally with WGA-HRP and either the ipsilateral pelvic or pudendal nerve was chronically sectioned.

Pelvic Nerve afferents. When sacral dorsal root ganglia (S_1 -S_3) were pressure injected unilaterally with WGA-HRP and the ipsilateral pudendal nerve was chronically

Figure 23. Fluorescent photomicrographs of sacral afferent fibers in proximal (D), mid (A,C) and distal (B, E and F) urethra mucosa (M) and submucosa (SM). A, B; Afferent fibers (arrows) in horzontal section (40 μm) cross section (40 μm), respectively. C; fibers in submucosa (straight arrows) branch within mucosa (curved arrows). D-F; fibers (arrows) in submucosa. For panels A, C, D and E both pelvic and pudendal nerves are intact. Panel F, afferent fibers in urethra submucosa. Ipsilateral pudendal nerve was chronically sectioned. Panel B, mucosal afferent fibers. Ipsilateral pelvic nerve was chronically sectioned. Horizontal bar in F equals 50 μm and also refers to A-E.

Figure 23



sectioned, afferent fibers were detected in ipsilateral pelvic nerve but were absent in ipsilateral pudendal nerve and the contralateral pelvic and pudendal nerves (n = 1). They occurred bilaterally in urinary bladder, proximal and mid urethra and in submucosa of distal urethra (approximately 5 mm rostral to EUS)(Fig. 23F). Afferent fibers were detected in 3 of 7 pelvic plexus ganglia (3/3 ipsilateral and 0/4 contralateral) and 7 of 8 urinary bladder ganglia (4/4 ipsilateral and 3/4 contralateral). These observations show that sacral afferent fibers pass through ipsilateral pelvic nerve to bilaterally innervate urinary bladder, urethra and urinary bladder ganglia.

Pudendal Nerve Afferents. When the ipsilateral pelvic nerve was chronically sectioned, afferent fibers were detected in ipsilateral pudendal nerve (Fig. 19B), but were absent in ipsilateral pelvic and contralateral pudendal and pelvic nerves (n = 1). Fibers occurred bilaterally in EUS (Fig. 22E) and distal urethra (serosa, striated and smooth muscle layers, submucosa and mucosa) (Fig. 23B) and in submucosa of mid and proximal urethra. Afferent fibers were not detected in urinary bladder, pelvic plexus ganglia or

bladder ganglia. These results demonstrate that sacral afferent fibers pass through ipsilateral pudendal nerve to innervate effector structures in urethra and EUS.

DISCUSSION

This study shows that some neurons in sacral dorsal root ganglia project afferent fibers to pelvic and pudendal nerves, large intestine, urinary bladder, urethra and external urethral sphincter. Afferent fibers also occur in parasympathetic colonic ganglia, urinary bladder ganglia and pelvic plexus ganglia.

Some neurons in bilateral sacral dorsal root ganglia project their peripheral sensory processes to at least mid colon through pelvic nerves and colonic fiber bundles. Axial orad and aborad sacral afferent fiber projections to effector structures in the large intestine occur over relatively long distances through colonic and rectal fiber bundles, respectively. In the colon, maximal axial orad distances from the pelvic brim ranged between 2.6 and 11.3 cm. For rectum, maximal axial aborad distances from the pelvic brim ranged between 3.0 and 4.0 cm. Neurons in ipsilateral

sacral dorsal root ganglia projected their peripheral sensory processes only to the ipsilateral pelvic nerve, parasympathetic colonic ganglia and colonic and rectal fiber bundles.

Sacral afferent fibers projected orad through colonic fiber bundles to enter a minority of myenteric plexus ganglia of the colon through branch points of colonic fiber bundles and interganglionic fiber tracts. The majority of afferent fibers were located in myenteric ganglia and interganglionic fiber tracts that were adjacent to and obliquely orad to branch points of colonic fiber bundles. Some fibers could be traced continuously from branch points of colonic fiber bundles to one adjacent myenteric ganglion. Others could be identified continuously in two or three myenteric ganglia and in their interganglionic fiber tracts. In rare instances, afferent fibers formed a continuous axial orad projection in interganglionic fiber tracts and to numerous adjacent myenteric ganglia. These results indicate that sacral afferent fibers emanating from neurons in sacral dorsal root ganglia project orad through colonic fiber bundles and through myenteric ganglia and interganglionic fiber tracts.

The data show that sacral afferent fibers were detected in approximately 15% of myenteric ganglia but in nearly 100% of colonic, pelvic plexus and bladder ganglia. The data also show sacral afferent fibers present in myenteric ganglia that are adjacent to colonic fiber bundles and connected to them by their branch points. It appears unlikely that the differences in afferent labeling we report in colonic-pelvic plexus and myenteric ganglia are related to the anterograde tracers WGA-HRP and unconjugated WGA. For rat stomach and duodenum, vagal efferents were detected in a minority of myenteric ganglia when Phaseolus vulgaris leucoagglutin was used as the anterograde tracer (Kirschgessner and Gershon, 1989), but a majority of ganglia appeared to contain both vagal afferent and efferent fibers when DiI was used as the anterograde tracer (Berthould et al., 1990).

Sacral afferent fibers which coursed through the myenteric plexus were traced to colonic circular muscle. Within circular muscle, there were two morphological arrangements. One had several intramuscular afferent fiber bundles and dispersed fibers that ran parallel to the long axis of the smooth muscle fibers. Afferent fibers were observed on both

mucosal and myenteric borders of circular muscle and were present around the entire circumference of the colon. The other occurred as a dense arborization pattern with afferent fiber bundles and dispersed fibers either parallel, oblique or perpendicular to the long axis of circular muscle fibers.

It is likely that the circumferentially oriented afferent fibers identified anatomically in the present study within circular muscle correspond to afferents activated by passive distension of cat distal colon (Janig and Koltzenburg, 1991). In their electrophysiological study, a total of 59 afferent units were defined in sacral dorsal roots (S_2) that projected to pelvic nerve. Of these, 61% responded only to passive distension of the distal colon, while 39% responded only to mechanical stimulation of the anal mucosa.

Colonic afferents, responding only to passive distension, were identified as thin myelinated and non-myelinated fibers with a median conduction velocity of 3.2 m/s (Janig and Koltzenburg, 1991). Myelinated afferent fibers responded only transiently to colonic distension (median conduction velocity 8.0 m/s), and were classified as phasic. Unmyelinated afferent fibers discharged throughout the distension (median conduction velocity 1.7 m/s), and were classified as

tonic. For tonic units, increases in colonic distension resulted in increased discharge frequencies.

Other afferent fibers identified anatomically within circular muscle may correspond to another population of non-myelinated sacral afferent units. Approximately 95% (202/213) of non-myelinated sacral (S_2) afferent units projecting to the pelvic nerve did not respond to mechanical stimulation of the colon or anal canal. These silent C-fibers may only be active when sensitized by inflamation (Janig and Koltzenburg, 1991; Habler et al., 1990).

In circular muscle, sacral afferent fibers with a perpendicular and oblique orientation may course through the circular layer to innervate mucosa or submucosa. Vagal afferent fibers which penetrate circular muscle to enter submucosal and mucosa regions have been described for cat esophagus (Clerc and Condamin, 1987).

Sacral afferent fibers were not traced from myenteric plexus to the longitudinal muscle layer nor were they visualized as intramuscular fiber bundles. Also, a paucity of sacral afferent fibers was detected in interganglionic fiber tracts within the submucosal plexus and none were detected in submucosal ganglia and colonic mucosa. An absence of

vagal afferent fibers in longitudinal muscle has been reported for rabbit stomach and duodenum (Sato and Kayono, 1987). For rat stomach and duodenum, few vagal afferent fibers were visualized in submucosa and none in mucosa (Berthoud et al., 1990).

For most regions of urinary bladder (dome, trigone and dorsal, ventral and lateral walls), a paucity of afferent fibers was detected in smooth muscle layers and they were absent in mucosa and submucosa. For urinary bladder neck, afferent fibers were detected in the smooth muscle layer of bladder wall and submucosa.

The abscence or paucity of sacral afferent fibers to mucosa and submucosa, respectively, of colon and urinary bladder and the paucity detected in smooth muscle layers of urinary bladder is not understood. It may be related to difficulty in visualizing a paucity of small diameter fibers with the anterograde transport of WGA-HRP or unconjugated HRP. This appears unlikely because afferent fibers were detected at a greater density in urethral mucosa/submucosa and were visualized in the proximal colon at long conduction distances (14 to 15 cm).

Our data show that sacral visceral afferent fibers

contained within colonic fiber bundles at the level of the mid colon enter the sacral spinal cord primarily through bilateral second (77%) and third (20%) sacral dorsal roots. An average of 945 sacral sensory neurons with small soma diameters were visualized. These results confirm earlier data that show that a majority of cat afferent fibers in bilateral pelvic nerves (Morgan et al. 1981) and distal colon (Kawatani et al., 1985; de Groat, 1987) have their cell bodies in the S_2 and S_2 - S_3 dorsal root ganglia, respectively.

The total number of neurons located in sacral dorsal root ganglia which provide sensory innervation to effector structures in the large intestine is yet to be determined. However, it is possible to estimate this number. The total number of neurons in bilateral pelvic nerves of the cat is approximately 7300 (Morgan et al., 1981). We speculate that one-third provide innervation to the large intestine (approximately 2400 neurons) and that the remainder innervate urinary bladder, urethra and sexual organs. If this assumption is correct, one thousand neurons innervate the distal colon (Kawatani et al., 1985; de Groat, 1987) and nearly another thousand innervate mid and proximal colon (present

study). The remainder, we surmise, innervate the rectum-anal canal.

Within parasympathetic colonic ganglia, pelvic plexus ganglia, urinary bladder ganglia and ganglia of the myenteric plexus, sacral afferent fibers were nonvaricose and visualized as a linear arrangement of reaction product granules of uniform diameter. Some fibers in myenteric plexus ganglia were varicose. Afferent fibers were detected as bundles to ganglion borders and central regions and as dispersed fibers in proximity to neuronal soma. 100% of labeled parasympathetic colonic ganglia and pelvic plexus ganglia and 97% of labeled urinary bladder ganglia had sacral afferent fibers in proximity to neuronal soma. Also, in myenteric plexus there were dense arborization patterns present. We speculate that some may be mechanoreceptors or collaterals of sacral afferents which mediate axon reflexes. A similar anatomical proposal has been made for vagal afferent fibers in cat esophagus (Rodrigo et al., 1982), rabbit stomach and duodenum (Sato and Kayono, 1987) and rat stomach, small and large intestine (Berthoud et al., 1990). Data from recent electron microscopic studies suggest that vagal afferent fibers form synaptic contacts with neurons in

rat esophageal myenteric plexus (Neuhuber, 1987).

Afferent collaterals may release neurotransmitter substances on enteric neurons to modulate local circuits (Delbro and Lissander, 1980; Delbro et al., 1981; de Groat et al., 1987). Electrical stimulation of afferent fibers in sympathetic (lumbar splanchnic nerves) and parasympathetic (vagus nerve) nerve trunks cause non-nicotinic, non-adrenergic contractions of cat colon and stomach, respectively (Delbro et al., 1983; Fandriks and Delbro, 1985; Delbro and Lisander, 1980; Fandriks et al., 1985; Delbro et. al., 1981). These contractions were unaffected by ganglionic and adrenergic antagonists, but were reduced by substance-P antagonists and atropine. This suggests that the contractions involved substance-P containing afferent fibers and cholinergic fibers. It is likely that afferent fibers detected in proximity to neurons in myenteric plexus and prevertebral ganglia in the present study may be afferent collaterals involved in local reflex mechanisms.

The data show that some neurons in sacral dorsal root ganglia also project peripheral afferent fibers to the urinary bladder, urethra and external urethral sphincter through pelvic and pudendal nerves. Neurons in unilateral

sacral dorsal root ganglia project peripheral sensory fibers to the ipsilateral pelvic and pudendal nerves, but bilaterally to urinary bladder, urethra and external urethral sphincter.

The data show that sacral afferent fibers were sparse to all regions of the urinary bladder. They passed through the pelvic nerve and were located predominantly on smooth muscle fibers. A paucity of sacral afferent fibers were previously detected when degeneration (Uemura et al., 1973) and retrograde tracing (Downie et al., 1984) techniques were used. Degenerating terminals comprised less than 5% of total axon terminals detected in cat urinary bladder (Uemura et al., 1973). In a retrograde tracing study of cat urinary bladder and urethra, a small number of neurons was located in sacral dorsal root ganglia. The mean number of afferent neurons was: urethra, 81; detrussor smooth muscle, 159; and bladder base, 178 (Downie et al., 1984). This represents less than 6% of the estimated 7300 sacral dorsal root ganglion neurons that project peripheral processes to cat pelvic nerve (Morgan et al., 1981).

Afferent fibers in the present study may correpond to mechanoreceptive afferent fibers described in cat urinary

bladder. Mechanoreceptive afferent fibers are both myelinated (mean conduction velocity = 10 m/s) and non-myelinated (mean conduction velocity = 1.0 m/s) fibers (Habler et al., 1990; de Groat et al., 1981; Bahns et al., 1987). Myelinated fibers are predominantly small diameter fibers with low thresholds and firing frequencies that rise with increasing intraluminal pressure during passive distension and isovolumetric active contractions (Bahns et al., 1987; Iggo, 1955).

Approximately 98% of non-myelinated afferent fibers in pelvic nerve did not respond to innocuous or noxious increases in intravesicular pressure (Habler et al., 1990). However, when the bladder was inflamed by injection of mustard oil, previously silent non-myelinated fibers initiated an action potential discharge to both the irritant and increases in intravesicular pressure. This suggests that these silent non-myelinated fibers may be chemoreceptors and mechanoreceptors (Habler et al., 1990). They may also be involved in mediating the sensation of pain (Habler et al., 1990).

Sacral afferent fibers to urethra occurred in serosal fiber bundles, external smooth muscle layers, submucosa, mucosa and striated muscle fibers of distal urethra and

external urethral sphincter. Afferent fibers which projected through pelvic nerves were detected in all effector structures of mid and proximal urethra and submucosa of distal urethra (approximately 5 mm rostral to external urethral sphincter). Afferent fibers which projected through pudendal nerves innervate effector structures of distal urethra and submucosa of distal, mid and proximal urethra. This represents a partial overlap of innervation by sacral afferent fibers via pelvic and pudendal nerves to the distal, mid and proximal urethra.

For external smooth muscle layers, sacral afferent fibers were detected as small bundles and fibers oriented in the long axis of smooth muscle fibers. The mean percentage of labeled sections was approximately 39% and 46%, respectively, for longitudinal and circular smooth muscle.

The greatest number of afferent fibers in urethra occurred in the submucosa. Approximately 86% of labeled urethral sections had afferent fibers in submucosa. They were detected as dispersed fibers and arborizations in all regions of submucosa. In addition, approximately 47% of labeled urethral sections had afferent fibers in all regions of mucosa.

The distribution of sacral afferent fibers to urethral mucosa/submucosa may have functional significance. Electrophysiological studies demonstrate that urethral afferent units in sacral dorsal roots respond to mechanical stimulation of the urethral mucosa, but do not respond to passive distension or active contraction of cat urinary bladder (Bahns et al., 1987). These afferents may be responsible for the sensations of impending micturition and urinary flow attributed to human urethra (Nathan, 1956).

Afferent fibers to urinary bladder and urethra may also be thermoreceptors. When cold water was introduced into cat urinary bladder or urethra, a reflex contraction of urinary bladder occurred (Fall et al., 1990). The bladder to bladder cooling reflex was unaffected by acute sectioning of hypogastric nerves but abolished by acute sectioning of pelvic nerves. The urethra to bladder cooling reflex was abolished by sectioning pudendal nerves. It is possible that sacral afferent fibers, identified anatomically in urinary bladder and urethra in the present study, may be thermoreceptors involved in reflex contraction of urinary bladder.

The present study confirms that sacral afferent fibers

cross the midline to innervate contralateral urinary bladder, urethra and pelvic plexus and bladder ganglia. Afferent fibers which project through pudendal nerve to external urethral sphincter and urethra also show peripheral crossover. In cat urinary bladder, one third of degenerating sacral afferent fibers cross the midline to innervate the contralateral side (Uemura et al., 1973; Uemura et al., 1975). In retrograde tracing studies, neurons in sacral dorsal root ganglia project afferent fibers across the midline in cat urethra and rat and cat urinary bladder (Downie et al., 1984; Applebaum et al., 1980). The functional significance of this bilateral innervation is unknown.

This report describes the distribution of sacral afferent fibers through pelvic nerves to the large intestine, colonic ganglia, pelvic ganglia, urinary bladder ganglia, urinary bladder, urethra and external urethral sphincter. It also describes the distribution of sacral afferent fibers through pudendal nerves to urethra and external urethral sphincter. A dense innervation of afferent fibers occurred in urethral submucosa, colon circular muscle, and a small percentage of myenteric ganglia. These afferent fibers may correspond in part to the substance-P (SP), calcitonin gene-

related peptide (CGRP) and vasoactive intestinal polypeptide (VIP) containing somas previously demonstrated with immunohistochemical techniques in sacral dorsal root ganglia (de Groat et al., 1983; Kawatani et al. 1985; de Groat, 1987, Keast and de Groat, 1992).

CHAPTER 2

INTRINSIC NEURAL PATHWAYS OF THE COLON

Introduction

Colonic fiber bundles originate within the pelvic plexus and pass to the colon, rectum and anal canal. Their distribution has been reported for humans (Kumar and Phillips, 1989) and experimental animals (Christensen and Rick, 1985; Christensen and Rick, 1987; Christensen et al., 1983; Christensen et al., 1984). For the cat, usually 6 to 8 fiber bundles ascend orad beneath the serosal surface of the distal colon and between the external muscle layers (de Groat and Krier, 1976; de Groat and Krier, 1978; Krier and Hartman, 1984; Langley and Anderson, 1895). They project considerable distances to the mid and proximal regions of the colon. Branches of the pelvic plexus also descend as rectal fiber bundles to the rectum and anal canal. There are branch points of both myelinated and non myelinated colonic fiber bundles in relation to neurons and cell pro-

cesses of the myenteric plexus (Christensen and Rick, 1987).

Colonic fiber bundles contain sacral parasympathetic as well as lumbar and sacral sympathetic pathways. The sacral parasympathetic pathway regulates colonic (de Groat and Krier, 1976; de Groat and Krier, 1978; Fukai and Fukada, 1984) and rectal motility. It originates in the sacral autonomic nucleus and extramural colonic ganglia and projects through the pelvic nerve and colonic fiber bundles (de Groat and Krier, 1976; de Groat and Krier, 1978; Kennedy and Krier, 1987; Krier and Hartman, 1984). The sacral sympathetic pathway originates from neurons in sacral paravertebral ganglia and projects through the pelvic nerve and colonic fiber bundles (Kuo et al., 1984). The lumbar sympathetic pathway originates from neurons in the inferior mesenteric ganglia and distributes fibers through the hypogastric nerve and colonic fiber bundles (de Groat and Krier, 1976).

Although the anatomical organization of these fiber bundles is well known for several species, it remains to be determined whether they function as another intrinsic connection for myenteric neurons. Some neurons in the distal colon may project orad to mid-colon whereas others in the

proximal and mid-colon may project aborad to mid and distal colon, respectively. This dissertation identifies the origin of myenteric fibers in colonic fiber bundles using techniques for retrograde axonal transport.

METHODS

Retrograde tracing techniques

Experiments were performed on 10 anesthetized cats. A midline abdominal incision was made exposing the large intestine and five millimeter segments of 2 to 6 colonic fiber bundles were dissected from the serosal surface of the mid colon at the level of the inferior mesenteric artery or at the distal colon 1.0 cm orad to the pelvic brim (Fig.2). Colonic fiber bundles were cut at the level of the inferior mesenteric artery and the central or peripheral ends were labeled to determine orad and aborad projections, respectively, of myenteric neurons. Colonic fiber bundles were cut at the level of the distal colon, 1 cm orad to pelvic brim and peripheral ends were labeled to determine the aborad projections of myenteric neurons. Central and

peripheral cut ends of colonic fiber bundles were placed into small chambers containing 10 μl of 15% horseradish peroxidase (HRP) solution (Sigma Type VI)(6 s) or 10 μl of 8% fast blue (Sigma)(4 s). After a 90 minute incubation period, the nerves were removed from the chambers and the abdomen was closed. After 36 to 96 hours, animals were re-anesthetized and perfused. HRP-labeled neurons were visualized by incubating the tissue with TMB/DAB. Whole-mount sections were viewed under light- and dark-field microscopy using magnifications between 100 and 400 X. Fast blue-labeled neurons were visualized by fluorescence microscopy at 100 and 250X. For each whole-mount section, the distribution of myenteric neurons in relation to colonic fiber bundles was determined by counting neurons in the axis of both circular and longitudinal muscle fiber layers. Neurons were counted in 700 μm wide serial rows oriented in the axis of longitudinal muscle fibers.

RESULTS

Ascending axial projections of myenteric neurons

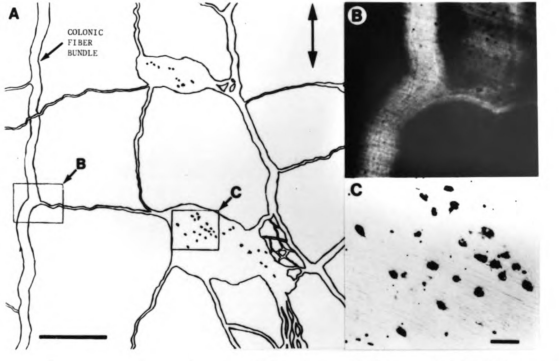
When central cut ends of 2 or 3 colonic fiber bundles were placed in an HRP solution, cell bodies and

processes were traced to the myenteric plexus of the mid and distal colon. As shown in Figure 24, somas were grouped within ganglia and their cell processes extended within interganglionic fiber tracts to branch points of colonic fiber bundles (Fig.24B). No somas or processes were labeled when axonal transport was interrupted by damaging colonic fiber bundles.

The axial distribution (distribution along the axis of colonic fiber bundles) of myenteric neurons is shown in Figure 25. Cell bodies were in the mid and distal colon at axial distances from 10 to 45 mm (maximum distance: 34 to 45 mm; mean $\pm$ SE, 42 $\pm$ 1; n = 5). The total labeled cells were from 82 to 393 (mean $\pm$ SE, 192 $\pm$ 48; n = 5). These data show that myenteric neurons project orad through colonic fiber bundles.

Figure 24. A: Camera lucida drawing of colonic fiber bundle with fiber connections to ganglia in the myenteric plexus of distal colon. Stippled areas in ganglia show location of myenteric neurons retrogradely labeled with HRP. B: fluorescent photomicrograph of colonic fiber bundle with branch point indicated in A. C: Bright-field photomicrograph of myenteric neurons in ganglion indicated in A. Double arrow indicates long axis of longitudinal muscle fibers. Horizontal bar in A equals 1mm. Horizontal bar in C equals 100 μm and also refers to B.

Figure 24



Descending axial projections of myenteric neurons

Other colonic fiber bundles were cut 1 cm orad to the pelvic brim (Figure 2). When peripheral cut ends of 4 or 5 colonic fiber bundles were placed in an HRP solution, cell bodies and processes were detected in the myenteric plexus of the mid and distal colon (Fig.26A, 26B) at axial distances ranging from 10 to 59 mm (maximum: 49 to 59 mm; mean $\pm$ SE, 54.0 $\pm$ 1.5). The total labeled cells were from 147 to 375 (mean $\pm$ SE, 220 $\pm$ 33; n = 4). In one animal, the peripheral cut ends of three colonic fiber bundles in distal colon (1 cm orad to pelvic brim) and two in mid-colon (1 cm orad to inferior mesenteric artery) were placed in a fast blue solution. There were cell bodies in the distal and mid-colon (Fig. 26C) and mid and proximal colon (Fig. 26D) at maximal distances of 49 and 44 mm, respectively. The total number of neurons labeled was 194 and 175, respectively. These data show that a population of myenteric neurons in both proximal and mid colon project aborad through colonic fiber bundles.

Figure 25. Distribution of myenteric neurons in distal colon in relation to axial distance from labeled colonic fiber bundles. Bars in each panel show consecutive wholemount segments. Each panel shows data from a different animal. Axial distance reported is limited to labeling site of colonic fiber bundle.

Figure 25

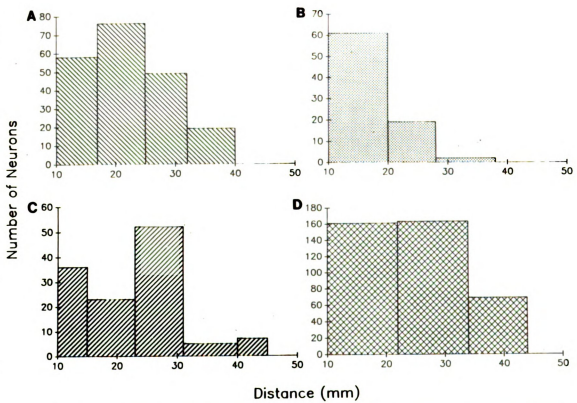
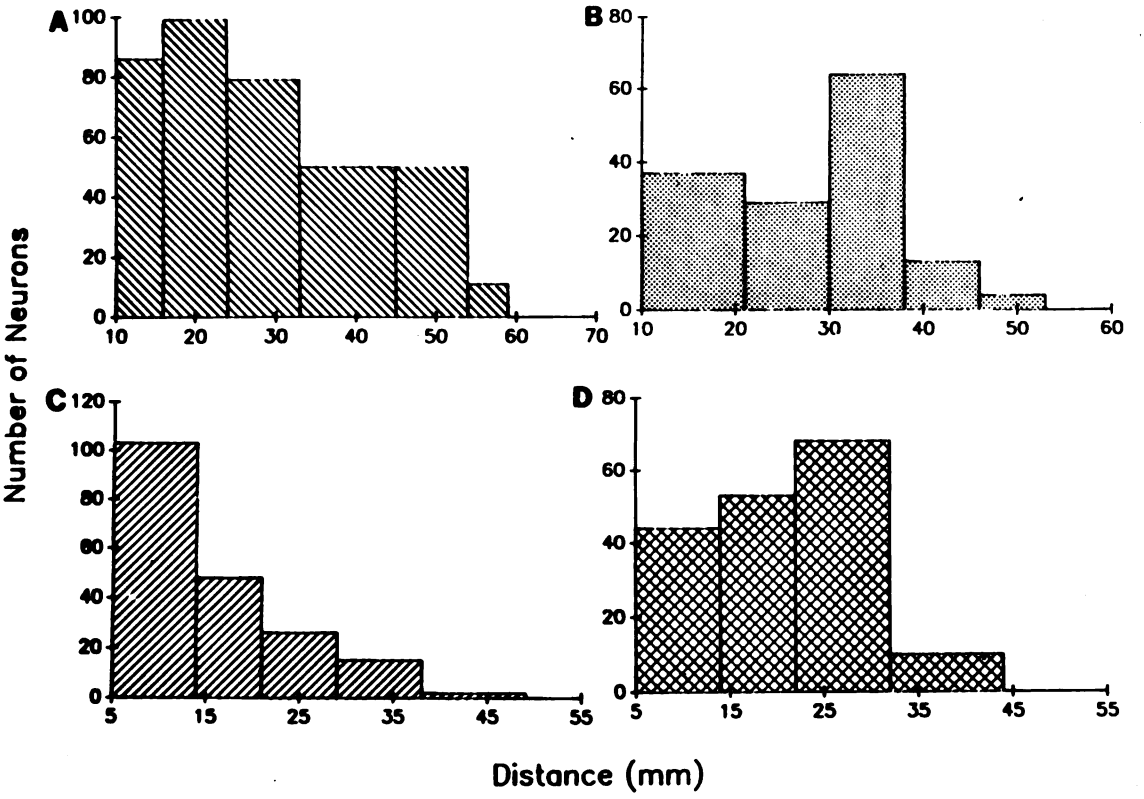


Figure 26. Distance of myenteric neurons in mid-distal colon (A-C) and mid-proximal colon (D) in relation to axial distance from labeled colonic fiber bundles. Bars in each panel show consecutive wholemount segments. A,B: Neurons labeled using retrograde transport of HRP. C,D: Neurons labeled using retrograde transport of Fast Blue. A and B show data from different animals. C and D show data from the same animal. Axial distance reported is limited to labeling site of colonic fiber bundle.

Figure 26



Lateral distribution of myenteric neurons

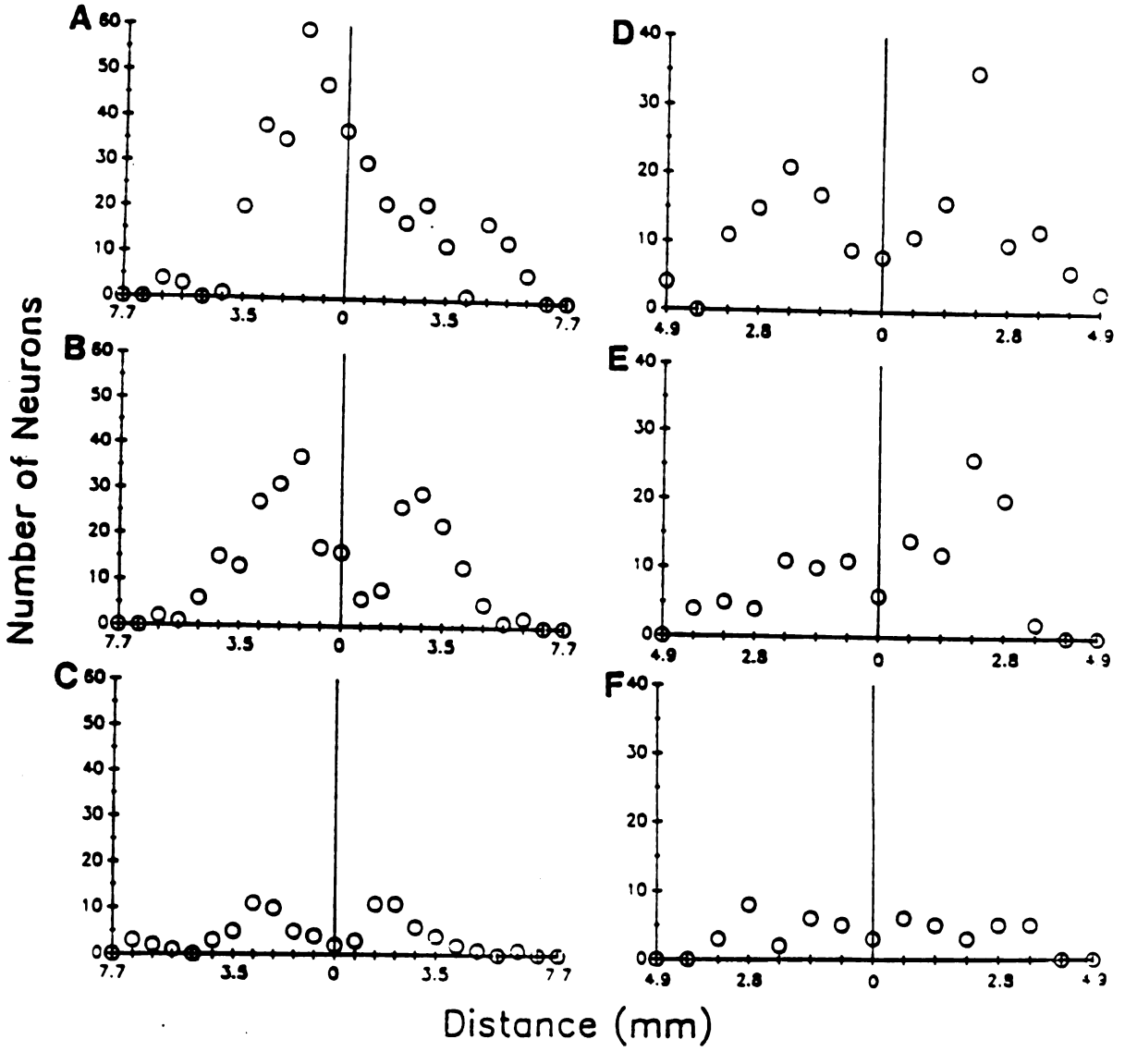
Myenteric neurons not only project fibers along the axis of colonic fiber bundles, they also lie at different distances beside them (axis of circular muscle fibers, Figure 28). For neurons in mid and distal colon which project orad (Fig.27 A-C), 65% (577 of 756) were within 2.8 mm of a labeled colonic fiber bundle. The remainder (179 of 756) were between 2.8 mm and 7.0 mm of a labeled colonic fiber bundle. For neurons in mid and distal colon which project aborad (Fig.27D-F), 85% (329 of 385) were within 2.8 mm of a labeled colonic fiber bundle. The remainder (56 of 385) were between 2.8 and 4.9 mm of a labeled colonic fiber bundle. These data indicate that the majority of neurons which project axons in either an orad or aborad axial direction have soma which lie within 2.8 mm of a colonic fiber bundle.

Morphological characteristics of myenteric neurons

Neurons were divided into two types using morphological criteria. One type had a rough somal surface (mean soma

Figure 27. Distribution of myenteric neurons lateral to labeled colonic fiber bundles. Center vertical line is position of colonic fiber bundle. Wholemount consecutive sections obtained at axial distances from labeling site. A, 10-21mm; B, 21-32mm; C, 32-43mm; D, 10-24mm; E, 24-45mm; F, 45-70mm. A-C, histograms obtained from four animals; D-F, histograms obtained from one animal.

Figure 27



diameter ± SE, 40.5 ± 0.6 μm) and was filled with a brown, agranular reaction product. It also had multiple short, broad processes which radiated from the soma and it had one long process (Figure 28). It was similar to myenteric cells in guinea-pig ileum (Furness et al., 1988) and opossum rectum (Christensen, 1988)(Dogiel type I cells; Dogiel, 1899). The other type had a smooth oval or rectangular soma (mean soma diameter ± SE, 26.4 ± 0.3 μm) which was filled with a black granular reaction product. It had a blank nucleus and few if any short fine processes (Figure 29). It was similar to neuronal somas in the myenteric plexus (Dogiel type II or Dogiel type III)(Dogiel, 1899; Furness et al., 1988; Furness et al., 1985). Because of the long axonal projection to colonic fiber bundles, these cells are considered to be Dogiel type III. For neurons which project orad, 62% (369 of 598) were classified as Dogiel type III, the remainder (229 of 598) were classified as Dogiel type I. For neurons which project aborad 70% (653 of 939) were Dogiel type III and the remainder (286 of 939) were Dogiel type I.

Figure 28. Bright-field photomicrograph of Dogiel type I neurons which show short, broad processes and one long process. Long processes of neurons in B, C, D, F and G project to colonic fiber bundle at distances ranging from 2.9 to 6.0 mm. Long process of neuron in A was traced to an interganglionic fiber tract at a distance of 1.1 mm. Long process of neuron in E remained within ganglion at a distance of 0.14 mm. Bar equals 50 μm.

Figure 28

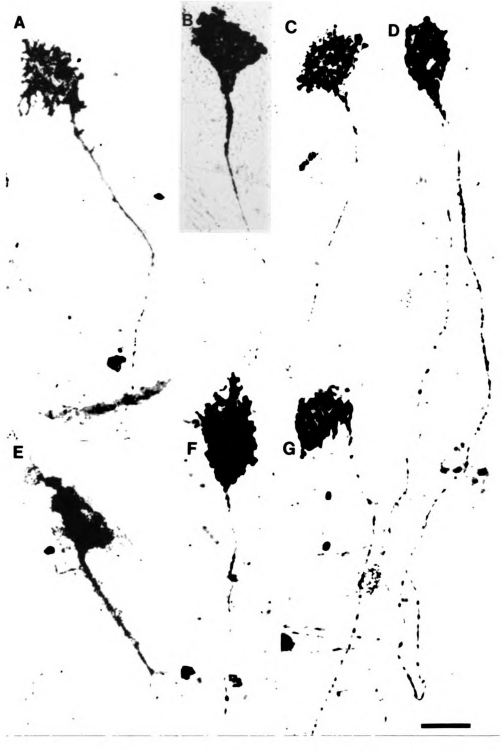
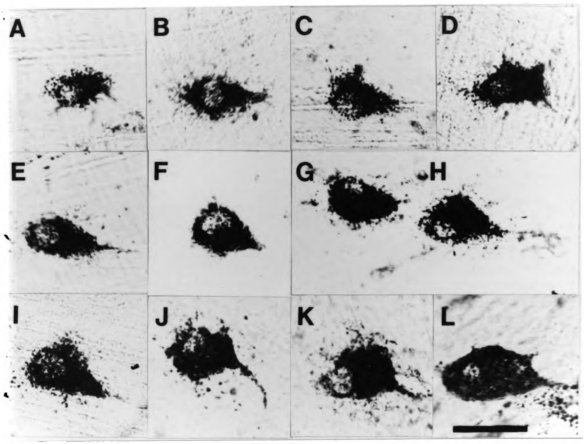


Figure 29. A-L: Bright-field photomicrographs of Dogiel type III neurons which show a nucleus and a soma partially filled with black granular reaction product. Bar equals 50 μm.

Figure 29



DISCUSSION

This study shows that some myenteric neurons in the cat colon project processes orad or aborad through colonic fiber bundles from at least 5 to 59 mm. Neurons in the distal colon project to mid-colon at the level of the inferior mesenteric artery and those in proximal colon and mid-colon extend to mid-colon and distal colon, respectively. Neurons were in ganglia adjacent to colonic fiber bundles, about 73% of which were within 2.8 mm and none was beyond 7.7 mm. The inference is that colonic fiber bundles are another intrinsic fiber connection for neurons in the myenteric plexus.

The distribution of neurons that project through colonic fiber bundles described here may have functional significance. They may innervate other myenteric or submucosal neurons, mucosa-submucosa or extrinsic smooth muscle layers and blood vessels to regulate reflexly trans-epithelial fluid transport, blood flow and muscle contractions.

Several reflexes have been shown to be dependent on the integrity of colonic fiber bundles (Fukai and Fukada, 1984).

Colonic contractions induced by rectal distension (recto-colonic) or mechanical stimulation of the anus (ano-colonic) remain after ligation of the myenteric plexus/muscularis externa, but are abolished by colonic fiber bundle transection (Fukai and Fukada, 1984). Ascending contractions and descending relaxations demonstrated in response to a bolus in an isolated guinea-pig distal colon were abolished by interuption of the myenteric plexus, presumably also interupting the colonic fiber bundles in the same plane (Costa and Furness, 1976; Christensen et al., 1984). This would suggest that myenteric neurons projecting processes orad and aborad over long axial distances in colonic fiber bundles may play a role in the propagation of reflex contraction and/or relaxation of intestinal smooth muscle.

Myenteric neurons which project orad and aborad through colonic fiber bundles are Dogiel type I and type III. Dogiel type I (Christensen, 1988; Furness et al., 1988) had a rough somal surface, few if any short, broad dendrites and one long process which often extended to a branch point of an adjacent colonic fiber bundle. The reaction product in this cell type was an agranular brown precipitate that filled the soma and obscured the nucleus. Dogiel type III

neurons had a smooth somal surface and few if any fine dendrites. They had one long process which projected through colonic fiber bundles. The reaction product in this cell type was a black granular precipitate that left the nucleus blank.

Neurons in the cat colon may be similar to myenteric (Bornstein et al., 1984; Furness et al., 1988; Furness et al., 1985; Iyer et al., 1988) and submucosal neurons (Furness et al., 1985) of guinea-pig small intestine and taenia coli (Furness et al., 1981). Dogiel type I cells in the present study may be primarily "S" type (cholinergic, fast EPSP's which discharge repetitively during depolarizing current pulses; Hirst et al., 1974; Iyer et al., 1988; Tamura and Wood, 1989) and are perhaps immunoreactive for enkephalin (Bornstein et al., 1984; Krier and Hartman, 1984), 5-hydroxytryptamine (Costa et al., 1982) or VIP (Furness et al., 1981; Katayama et al., 1986).

Neurons similar to Dogiel I morphology and immunoreactive for VIP (Furness et al., 1981) projected axons to the external longitudinal smooth muscle layer of guinea-pig taenia coli. VIP immunoreactive neurons also project processes aborad 2 to 10 mm to other myenteric neurons and up

to 15mm aborad to submucoal neurons.

Neurons similar in morphology to Dogiel I and immunoreactive for enkephalin were also "S" type neurons, recieved a cholinergic input from other enteric neurons and were predicted to project to the circular muscle layer and orally to other myenteric ganglia (Bornstein et al., 1984; Furness and Costa, 1980). These results suggest that some Dogiel type I cells in the small intestine which are immunoreactive for enkephalins and VIP may have a motor function.

Dogiel type III neurons identified in the present study compare to those in guinea pig taenia coli that are immunoreactive for VIP (Furness et al., 1981). VIP has been suggested to play a role in descending smooth muscle relaxation associated with peristalsis (Farhrenkrug et al., 1978; Furness and Costa, 1979). VIP may also play a role in intestinal vasodilation (Costa et al., 1980; Fahrenkrug et al., 1978) and increased transepithelial transport of water and electrolytes (Costa and Furness, 1983).

Cat Dogiel type III neurons are also comparable to a population of Dogiel type III myenteric and submucosal neurons in guinea pig small intestine that project to the underlying mucosa (Furness et al., 1985). These neurons are

immunoreactive for several peptides, including CGRP. CGRP is a potent vasodilator in rabbit skin (Brain et al., 1985) and rat mesentery (Han et al., 1990a; Han et al., 1990b) and inhibits smooth muscle contraction in mouse vas deferens (Al-Kazwini et al., 1986). Some of the small intestinal neurons in the myenteric and submucosal plexuses with Dogiel type III morphology innervate mucosa, suggesting a secretory and/or vasomotor function.

In summary, this chapter describes observations that show that myenteric neurons in cat colon project axons both orad and aborad over relatively long distances through colonic fiber bundles and have cell bodies within only a few millimeters of them. There might also be a similar organization of myenteric neurons in the stomach and esophagus for which serosal fiber bundles have been described (Christensen and Rick, 1985; Kumar and Phillips, 1989).

Chapter 3

Sacral Parasympathetic and Lumbar Sympathetic Postganglionic Pathways

Introduction

Preganglionic fibers which originate from neurons in sacral spinal cord provide input to neurons in parasympathetic colonic ganglia (de Groat and Krier, 1976; Krier and Hartman, 1984; Kennedy and Krier, 1987). Neurons in parasympathetic colonic ganglia project postganglionic fibers through colonic fiber bundles to distal colon (Krier and Hartman, 1984). In the present study I used retrograde tracing techniques to estimate the number of neurons in parasympathetic colonic ganglia that project postganglionic fibers to at least mid colon regions.

The distribution of neurons in lumbar sympathetic chain ganglia (paravertebral) and inferior mesenteric ganglia (prevertebral) that project processes to hypogastric and lumbar colonic nerve trunks has been studied with retrograde tracing technique (Baron et al., 1985, I; Baron et al, 1985

II; Baron et al., 1985 III). The subpopulation of sympathetic neurons within prevertebral and paravertebral ganglia that project processes to colon via hypogastric nerves and colonic fiber bundles is unknown. I determined a subpopulation of sympathetic prevertebral and paravertebral neurons that project processes through hypogastric nerves and colonic fiber bundles to at least the mid-colon region utilizing retrograde tracing techniques.

Methods

Retograde Labeling techniques

Experiments were performed on 10 anesthetized cats. A midline abdominal incision was made exposing the large intestine and 2 to 6 colonic fiber bundles were isolated and labeled at the level of the inferior mesenteric artery (arrows)(Fig. 30) with either 15% HRP or 4% fast blue. Lumbar colonic nerves were chronically sectioned (n = 4). This procedure interupted the postganglionic fiber projections of sympathetic neurons located in inferior mesenteric ganglion (IMG), superior mesenteric ganglion (SMG), coeliac

ganglion, and lumbar sympathetic chain ganglia (Baron et al., 1985 III). After 36 to 48 hours (HRP) or 72 to 96 hours (fast blue), animals were reanesthetized and perfused. The parasympathetic colonic ganglia, inferior mesenteric ganglia (IMG), superior mesenteric ganglia (SMG), coeliac ganglia and lumbar sympathetic chain ganglia were removed and processed for HRP or fast blue as previously described. The IMG has four lobes distributed around the inferior mesenteric artery: left caudal (LC), right caudal (RC), left rostral (LR), and right rostral (RR). HRP labeled sections were viewed under light- and dark-field microscopy using magnifications between 100 and 400 X. Fast blue labeled neurons were visualized by fluorescence microscopy at 100 and 250X. In each horizontal section, counts of neurons were corrected for double counting (Abercombie, 1946). The mean soma diameter was computed as being one half of the sum of the long and short axis.

Results

Parasympathetic colonic ganglia: retrograde axonal tracing with HRP and Fast Blue

Neurons in colonic ganglia that project processes orad

within colonic fiber bundles to at least mid-colon regions (range 70 - 90 mm; mean ± SE = 83 ± 3.7 mm; n = 9) were retrogradely labeled. Horseradish peroxidase (HRP)(n = 5) or fast blue (n = 4) was placed on the central cut ends of 2 to 6 colonic fiber bundles at mid-colon (level of the inferior mesenteric artery)(Fig. 30). The retrogradely labeled soma were completely filled with black granular reaction product (HRP) with few or no processes (Fig. 31) or partially filled with blue fluorescent granules (fast blue), with no processes and a blank nucleus. Their mean soma diameter (± SE) was 32.4 ± 0.2 μm (n = 253 cells). The number of soma per experiment (mean ± SE = 233.1 ± 49.0; n = 9) is shown in table 6.

Sympathetic Prevertebral and Paravertebral Ganglia: Retrograde Tracing with Fast Blue

When fast blue was placed on central cut ends of 5 to 6 colonic fiber bundles (n=4) at mid-colon (level of inferior mesenteric artery)(Fig. 30), somas were detected in inferior mesenteric ganglion, superior mesenteric ganglion, coeliac ganglion and lumbar sympathetic chain ganglia (Fig. 32). They were partially filled with blue granular reaction product with a blank nucleus and no processes.

Figure 30. Drawing of cat colon, pelvic nerve, colonic ganglion (CG), paravertebral sympathetic chain ganglia, inferior mesenteric ganglion (IMG), superior mesenteric ganglion (SMG) and coeliac ganglion. Colonic fiber bundles were labeled with either HRP or Fast Blue at the level of the inferior mesenteric artery (arrows).

Figure 30

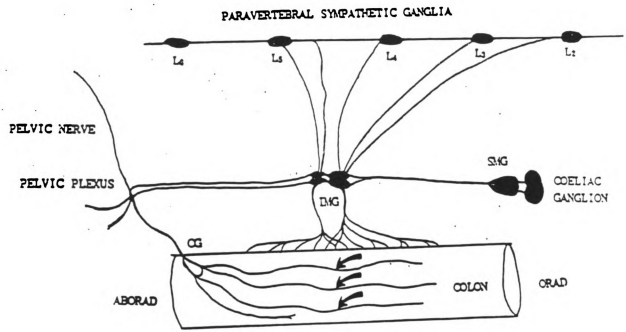

PARAVERTEBRAL SYMPATHETIC GANGLIA
L4
L3
L4
L5
L6
PELVIC NERVE
PELVIC PLEXUS
SMG
CELIAC GANGLION
IMG
CG
ABORAD
COLON
ORAD

Figure 31. Bright-field photomicrograph of horizontal section of colonic ganglion at low (A) and high (B) magnification. Colonic fiber bundles were labeled with HRP solution. Somas are completely filled and short processes are occasionally observed. Neurons projected long processes orad through colonic fiber bundles at least 60 to 90 mm. Bar in A = 80 μm, in B = 40 μm.

Figure 31

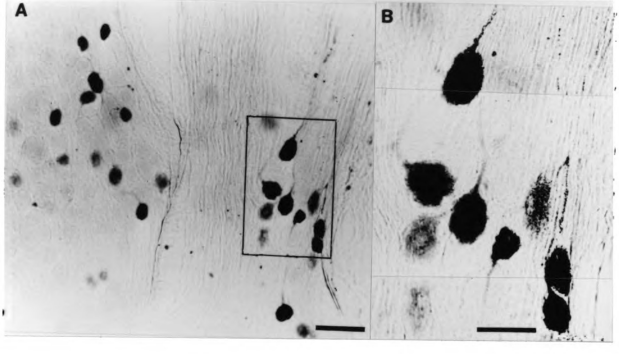


Table 6

Distribution of Neurons in Parasympathetic Colonic Ganglia that Project to Colonic Fiber Bundles

	Right Colonic Ganglia	Left Colonic Ganglia	Total
1)	31	24	55
2)	95	11	106
3)	132	51	183
4)	27	17	44
5)	168	133	301
6)	47	272	309
7)	152	106	258
8)	228	249	477
9)	104	252	356

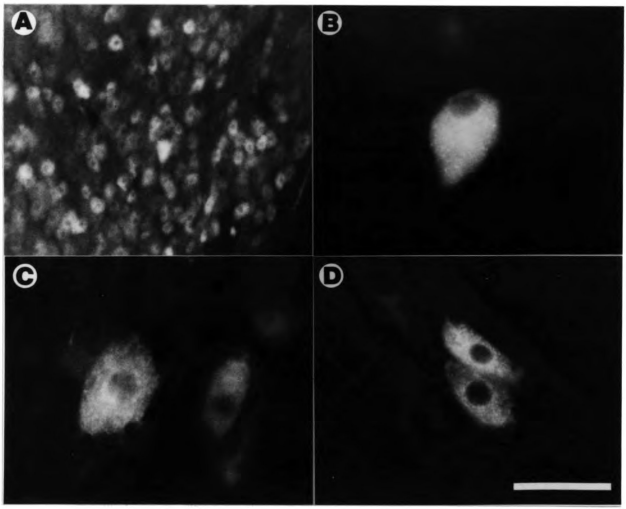
(n = 9, corrected for double counting by method of Abercombe, 1 - 5 = HRP, 6 - 9 = fast blue).

Inferior mesenteric ganglion

Somas were detected in the four lobes of the inferior mesenteric ganglion (IMG)(Fig. 32A). The mean soma diameter (± SE) was 29.2 ± 1.0 μm (n = 252 cells). Their distribution is shown in table 7. The mean (± SE) number of neurons retrogradely labeled per experiment was 2755 ± 660 (n = 4). Their distribution within individual lobes was: LC, 36.6% (3473/9481); RC, 45% (4267/9481); LR, 9.2% (874/9481); and RR, 9.1% (867/9481).

Figure 32. Fluorescent photomicrographs of horizontal sections of IMG (A), SMG (B), coeliac ganglion (C) and lumbar paravertebral chain ganglion (L_3)(D). Colonic fiber bundles were labeled with fast blue at the level of the IMA. Horizontal bar in D equals 300 μm for A and 50 μm for B-D.

Figure 32



Superior Mesenteric Ganglion

Somas were detected in the superior mesenteric ganglion (Fig. 32B). The mean soma diameter (± SE) was 31.3 ± 1.6 μm (n = 163 cells). Their distribution is shown in table 7. The mean (± SE) number of soma per experiment was 356 ± 130.7 (n = 4).

Coeliac Ganglion

Soma were detected in both right and left lobes of the coeliac ganglion. The mean soma diameter (± SE) is 33.6 ± 1.3 μm (n = 201 cells). Their distribution is shown in table 7.

The mean number of soma (± SE) per animal was 1415 ± 874.3 (n = 4). The distribution of soma per lobe was: left lobe, 52.5% (2976/5663); and right lobe, 47.4% (2687/5663).

Sympathetic Lumbar Chain Ganglia

Somas were detected in lumbar sympathetic chain ganglia (Fig. 32D). The mean soma diameter (± SE) was 25.9 ± 1.1 μm (n = 193 cells). Their distribution is shown in table 8. The mean number (± SE) of soma labeled per experiment was 779.8 ± 313.9 (n = 4). The distribution of neurons in lumbar sympathetic chain gangia is: L_1, 13% (409/3119); L_2, 29.7%

(926/3119); L_3, 31.6% (986/3119); L_4, 24.8% (773/3119); and L_5, 0.8% (25/3119).

TABLE 7

DISTRIBUTION OF NEURONS IN SYMPATHETIC PREVERTEBRAL GANGLIA THAT PROJECT TO COLONIC FIBER BUNDLES

	IMG				SMG	Coeliac Ganglia	
	Left Caudal	Right Caudal	Left Rostral	Right Rostral		Left	Right
1)	1142	1235	329	320	502	508	457
2)	922	526	200	309	593	320	408
3)	288	1021	109	121	0	3	1
4)	1121	1485	236	117	329	2145	1821

(n = 4; IMG, inferior mesenteric ganglia; SMG, superior mesenteric ganglia; corrected for double counting by the method of Abercombie)

Table 8

DISTRIBUTION OF NEURONS IN LUMBAR SYMPATHETIC CHAIN GANGLIA THAT PROJECT TO COLONIC FIBER BUNDLES

	Left L_1	Right L_1	Left L_2	Right L_2	Left L_3	Right L_3	Left L_4	Right L_4	Left L_5	Right L_5
1)	0	18	0	0	279	270	268	313	-	-
2)	51	74	71	122	94	91	13	27	5	20
3)	0	0	0	2	0	0	0	0	-	-
4)	201	65	397	334	125	127	26	126	-	-

(n = 4; corrected for double counting by the method of Abercombie)

Discussion

The present study shows that some neurons in parasympathetic prevertebral and sympathetic prevertebral and paravertebral ganglia project processes through colonic fiber bundles to at least the mid colon region. Neurons in parasympathetic colonic ganglia projected processes approx-

imately 83 mm orad in colonic fiber bundles. Neurons in sympathetic prevertebral and paravertebral ganglia projected processes over considerably longer distances to reach the same mid colon regions via hypogastric nerves and colonic fiber bundles.

For parasympathetic colonic gangia, approximately 233 neurons were retrogradely labeled per animal. This anatomical data supports a previous electrophysiological study (Krier and Hartman, 1984). Electrical stimulation of colonic fiber bundles elicited antidromic potentials in 62% of neurons tested in parasympathetic colonic ganglia. Approximately 95% of these postganglionic fibers were nonmyelinated (conduction velocity range; 0.4 to 2.0 m/s)(Krier and Hartman, 1984). The previous electrophysiological study and the present study show that neurons in parasympathetic colonic ganglia project postganglionic processes through colonic fiber bundles to at least distal colon and at least mid colon, respectively.

My data shows that approximately 2,755 neurons in IMG send processes through bilateral hypogastric nerves and colonic fiber bundles to at least mid-colon. Approximately 82% of neurons were located in caudal lobes of the IMG. The

remainder were in rostral lobes. When the ipsilateral hypogastric nerve was retrogradely labeled, approximately 16,150 neurons were detected primarily in the ipsilateral caudal lobe of the IMG (Baron et al., 1985 I). After correction for double counting (Abercombie, 1946), it was estimated that approximately 21,544 neurons in IMG project processes through bilateral hypogastric nerves. The subpopulation of neurons in IMG that project processes to at least mid colon through colonic fiber bundles (present study) represents approximately 13% (2,755/21,544).

The data show that approximately 780 neurons in lumbar sympathetic chain ganglia project processes through bilateral hypogastric nerves and colonic fiber bundles to at least mid-colon. The majority of these neurons (86%) were in bilateral L_2-L_4 ganglia. The distribution of neurons in the present study is similar to a previous study (Baron et al., 1985 I) where the ipsilateral hypogastric nerve was retrogradely labeled. Approximately 350 neurons were detected in lumbar sympathetic chain ganglia, predominantly in the ipsilateral L_4 ganglion (Baron et al., 1985 I). After correction for double counting (Abercombie, 1946), I estimated that approximately 400 neurons in lumbar sympathetic chain

ganglia project processes through bilateral hypogastric nerves. These anatomical studies suggest that almost twice as many neurons (780/400) in lumbar sympathetic chain ganglia project processes to colonic fiber bundles compared to those with processes in hypogastric nerves. This may be due to a difference in the percentage of fibers labeled by fast blue (present study) and HRP (previous study).

Our data shows that neurons in SMG (mean number ± SE, 356 ± 130) and bilateral coeliac ganglia (mean number ± SE, 1415 ± 874) project processes to at least mid colon through colonic fiber bundles. For neurons in the SMG, this appears to be the major projection pathway to colonic effector structures in view of the paucity of neurons that project their processes through lumbar colonic nerves (Baron et al., 1985 I). A sympathetic pathway originating from neurons in coeliac ganglia and projecting through hypogastric nerves and colonic fiber bundles, described in the present study, was not previously identified.

This report shows that some neurons in parasympathetic colonic ganglia project postganglionic processes over long axial distances to at least mid colon. They may provide synaptic input to myenteric and/or submucosal plexus neu-

rons and/or directly innervate colonic effector structures (de Groat and Krier, 1976). This report also identifies a subpopulation of neurons in sympathatic prevetebral and paravertebral ganglia that project long postganglionic processes through hypogastric nerves and colonic fiber bundles to at least mid-colon. For neurons in SMG and coeliac ganglia, this represents a previously unidentified projection pathway to colonic effector structures.

The functional significance of this anatomical arrangement is unknown. It is likely these neurons are noradrenergic and innervate blood vessels, myenteric plexus ganglia and intestinal smooth muscle (Furness and Costa, 1974). In myenteric plexus ganglia they may modulate cholinergic and non-adrenergic, non-cholinergic transmission by activating prejunctional adrenoceptors.

SUMMARY

In summary, this disssertation has described the distribution of sacral afferent fibers in effector structures of the colon, urinary bladder and urethra using anterograde tracing techniques. Previous anatomical studies of this distribution were limited to degeneration techniques, which visualize short fragments of degenerating fibers. The anterograde tracing techniques used in the present study demonstrated continuous long fibers and fiber arborizations within ganglia and tissue layers that were not seen with degeneration.

These sacral afferent fibers may serve as mechanoreceptors, chemoreceptors and/or thermoreceptors. They may also be activated by inflamation and be involved in sensations of pain. Sacral afferent fibers have also been described in proximity to neurons in colon myenteric plexus, urinary bladder, pelvic plexus and colonic ganglia. These afferent fibers may be collateral branches of fibers innervating effector structures. Orthodromic activation of the sensory fiber on the effector structure could initiate antidromic activation of these afferent collaterals, causing the release neurotransmitter substances in proximity to neurons.

This could represent a feedback mechanism involving sacral afferent fibers for modulating local neuronal circuits.

This dissertation has also described a population of neurons in the colon myenteric plexus that project processes over long axial distances orad and aborad through colonic fiber bundles. This suggests that intrinsic neurons utilize colonic fiber bundles to communicate with distant colonic regions. These long ascending and descending pathways may be involved in the ano-colonic, recto-colonic and colo-colonic reflex contractions demonstrated in response to mechanical stimulation of anal mucosa and passive distension of the rectum and colon, respectively.

Finally, this dissertation has described the distribution of a subpopulation of neurons in parasympathetic prevertebral and sympathetic prevertebral and paravertebral ganglia that project postganglionic processes through colonic fiber bundles to at least mid-colon. For the superior mesenteric and coeliac ganglia, this represented a peripheral pathway to the colon that had not been previously identified. The functional significance of this projection pathway is yet to be determined.

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