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The Anatomy of Spinal Sympathetic Structures in the Cat

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THE ANATOMY OF SPINAL SYMPATHETIC STRUCTURES IN THE CAT

by

Kyungsoo Chung

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillemnt of the Requirements for the Degree of Doctor of Philosophy

April 1978
Dedicated to my parents,
Mom and Dad
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A word of thanks is expressed to other departmental personnel, including graduate students, the secretarial staff and technicians, who never hesitated in helping me to develop this work...
VITA

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Her doctoral research program on spinal sympathetic structures has been carried out at Loyola under the guidance of Dr. Robert D. Wurster, Department of Physiology, and Dr. Faith W. LaVelle, Department of Anatomy.

Since October, 1977, she has been a research associate in the Marine Biomedical Institute, Galveston, Texas.
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INTRODUCTION

A more comprehensive description of spinal autonomic nuclei is required as an aid to understanding the relationship of the architecture of the central nervous system to the autonomic functions of the body. Although a number of reports on spinal autonomic structures can be found in the literature, the descriptions and nomenclature of the cell groups vary among investigators. Previous histological investigators, using either silver impregnation or Nissl stain methods, have described spinal preganglionic groups in seven distinct locations within the cord: (a) intermediolateral cell column (Rexed's lamina VII), (b) intercalated nucleus (VII), (c) central autonomic area (X), (d) lateral funiculus, (e) intermediomedial cell column (VII) and (f) subpial nucleus (Poljak, 1924; Bok, 1928; Laruelle, 1937; Rexed, 1954). On the other hand, the study of chromatolysis following sympathectomy demonstrated that the majority of preganglionic fibers originated from neurons in the intermediolateral cell column and, to a lesser extent, from the cells in the lateral funiculus, the intercalated nucleus, and the central autonomic nucleus, but none from the
intermediomedial cell column and the subpial nucleus (Cummings, 1969 and Petras and Cummings, 1972).

Then recently developed method of intracellular labeling with horseradish peroxidase (HRP), involving retrograde transport of this protein from the region of axon terminals back to the parent cell bodies, has been extensively used for the study of neural connections in the central nervous system (LaVail and LaVail, 1972; Nauta et al., 1974) and the peripheral nervous system (Kristensson et al., 1971), as well as in the peripheral autonomic nervous system (Ellison and Clark, 1975).

The present study was undertaken to determine the location of sympathetic preganglionic neurons within the spinal cord of the cat by utilizing the HRP method. The morphology of the labeled preganglionic neurons was examined with electronmicroscopy, with special attention paid to the synaptic inputs to the soma and proximal dendrites of these neurons. In addition, the location, distribution, and pathways of those neurons that contribute fibers to the stellate ganglia of the cat were studied experimentally with surgical intervention and quantitatively with light microscopy.
A. THE DISCOVERY OF THE AUTONOMIC NERVOUS SYSTEM

The earliest anatomical description of the autonomic nervous system probably dates back to Galen's account of "the nerve trunk running along the roots of the ribs, receiving fibers from the thoracic and lumbar portions of the spinal cord and being distributed to the viscera" (see p. 1081 Sheehan, 1936). A decisive account of the anatomical and physiological relationships of the autonomic nervous system did not appear until 1886, when Gaskell described the distribution of small myelinated fibers of less than 3.6 μ in diameter in the ventral roots of the thoracic cord and their connections to the white communicating rami. He noticed that the lateral horn of spinal gray matter formed a distinct column of cells in all parts of the cord, except in the cervical and lumbar enlargements, where it became indistinguishable. The close correspondence of the cell column to the segments from which the fine medullated fibers arose led Gaskell to postulate that the cells in the lateral horn were the origin of such fibers. He noted that the peripherally located motor ganglion cells were connected
with the central nervous system (CNS) by fine medullated nerve fibers from three separate outflows; the bulbar, the thoraco-lumbar and the sacral. Gaskell (1889) also traced fibers in the rami communicantes and roots of the spinal nerves, demonstrating the segmental nature of nerves to the periphery. From the above observations Gaskell concluded that the autonomic outflow consists of a two neuron pathway system, the white communicating rami containing the first part of the pathway and the gray rami communicantes containing the second part. In his later work, Gaskell (1916) used the term "involuntary nervous system" to designate those efferent neurons located outside the CNS which supply fibers to involuntary structures.

Langley and Dickinson (1890) used the ganglionic blocking action of nicotine to investigate the relationships of nerve fibers to peripheral ganglion cells. They demonstrated that nicotine injections into the vein stopped the sweating and skin pallor produced by electrical stimulation of white rami coming from the cord, but did not affect the stimulation of peripheral ramifications of the sympathetic chain, thereby substantiating Gaskell's concept of a two neuron system in the "involuntary nervous system". The results obtained by the use of this method
led Langley (1921) to call this the "autonomic nervous system".

This system as described by Langley consisted of the thoracolumbar outflow supplying fibers to all parts of the body and the cranial and sacral outflows supplying fibers only to specific parts of the body.

Furthermore, he noted that the functional effects induced by activation of thoracolumbar outflow, in general, were the opposite of those of the cranial and sacral outflows. Langley, therefore, regarded the thoracolumbar outflow as a system distinct from the rest of the autonomic nerves. He considered the thoracic outflow supplying the eyes as being part of the thoracolumbar system and, therefore, distinct from the cranial outflow. He noted that the cranial and sacral outflows constituted a system that innervated the alimentary canal and parts developmentally connected with it. At first, he divided the autonomic nervous system into tectal, bulbosacral and sympathetic systems. Following the discovery that effects produced by adrenalin were similar to those produced by stimulation of sympathetic nerves, and that certain other drugs produced effects similar to those produced by stimulation of tectal and bulbosacral nerves, he grouped
the tectal and bulbosacral autonomic nerves together as the "parasympathetic" system (1921). Langley's classification of autonomic nerves into sympathetic and parasympathetic, has been retained to the present day.

B. THE SYMPATHETIC TRUNK IN MAMMALS

The first description of the sympathetic trunk appeared with Galen's note (see p.1081 Sheehan, 1936) of a "nerve trunk running along the roots of the ribs, receiving fibers from the thoracic and lumbar portions of the spinal cord and being distributed to the viscera".

The sympathetic trunks are bilateral, paravertebral ganglionated cords which begin with the superior cervical ganglia near the first cervical vertebra and extend to the coccyx. In the cervical region each sympathetic trunk lies behind the homolateral carotid sheath and in front of the vertebral transverse processes; in the thorax behind the pleura and over the heads of the ribs; in the abdomen along the medial margin of the psoas major on the anterolateral surfaces of the lumbar vertebrae and discs; in the pelvis on the ventral aspect of the sacrum, medial to the anterior sacral foramina; and over
the coccyx the trunks of opposite sides unite in the ganglion impar (Mitchell, 1953).

Several investigators have focused their observations on the arrangement of the ganglia and their connections to the spinal nerves (Gaskell, 1886; Langley, 1892; Saccomanno, 1943; Sheehan and Pick, 1943; Pick and Sheehan, 1946; Pick, 1956). The ganglia of the sympathetic trunk vary in size, shape and position in different regions along the cord, in different animals, as well as in different individuals among the same species. Even though variability has been demonstrated by several investigators (Woollard and Norrish, 1933, Becker and Grunt, 1957), generally three ganglia are described in the cervical region: superior, middle, and inferior cervical ganglia. The sympathetic trunk begins with the superior cervical ganglion near the first cervical vertebra and extends caudally as the cervical chain. At the angle of the first rib, the sympathetic trunk divides to form the ansa subclavia. The anterior limb of the ansa subclavia passes in front of, and the posterior limb behind of, the subclavian artery to join the stellate ganglion. Just above the ansa subclavia is located the triangular middle cervical ganglion. The
inferior cervical ganglion fuses with the first thoracic ganglion, and sometimes with the second as well, to form the stellate ganglion (Saccomanno, 1943, and Langley, 1892 in the cat; Keng, 1893, and Gaskell, 1886 in the dog; Sheehan and Pick, 1943, in the monkey; Pick and Sheehan, 1946, and Woollard and Norrish, 1933 in man). Much variability occurs in the presence of an accessory cervical ganglion (Langley, 1894; Becker and Grunt, 1957), in the location of the middle cervical ganglion (between C4-C7), and/or the presence of an independent inferior cervical ganglion rather than fusion into a stellate ganglion (Woollard and Norrish, 1933).

From the second thoracic segment downward the sympathetic trunk ganglia are arranged in a fairly segmental manner, although there are usually some variations in the number of ganglia. The discrepancy may be due to the fusion or non-formation of certain ganglia (Mitchell, 1953). There is also a possibility that each primordial ganglion consists of cranial and caudal parts. These parts may remain together, may separate and reunite, or may persist as discrete ganglia (Pick and Sheehan, 1946) resulting in variations in the distribution of the rami communicantes. The exact number of a particular
ganglion (e.g., fourth thoracic ganglion) can be determined only by studying the attachments of its rami to the spinal nerves.

Since the work of Wrete (1935) the occasional presence of small ganglia on communicating rami (intermediate ganglia) has been well studied. These ganglia are most commonly found on gray rami in the cervical and lower lumbar regions, are less frequently found in the upper lumbar region and are seldom found in the thoracic and sacral regions (Pick and Sheehan, 1946; Wrete, 1951). In human fetal material, Wrete (1951) reported these intermediate ganglia are most commonly found in association with the third and fourth cervical nerves and the rami attached to them but are less frequent in the upper and lower cervical nerves and rami. The next peak of frequency occurs in the upper lumbar region. Pick and Sheehan's (1946) findings from a study of human adult subjects were substantially similar for the cervical region, but more intermediate ganglia were found in the lower lumbar region than anywhere else. The intermediate ganglia are not confined to the rami, but may also be located in the ventral roots, in the spinal nerves near the attachment of rami, and occasionally between the gray rami passing to
adjacent spinal nerves (Mitchell, 1953). Wrete and Pick and Sheehan all pointed out that most of these ganglia would escape removal by standard surgical trunk sympathectomy procedures. These ganglia represent groups of migrating cells from the neural tube which become arrested during development at intermediate stations between the neural tube and the primordia of the sympathetic ganglia (Mitchell, 1953).

Each ganglion is surrounded by a capsule of connective tissue, and connective tissue septa divide the ganglion cells into several groups. The capsule and septa are particularly thick in man (Pick, 1970) and represent about 30% of the wet weight in the superior cervical ganglion of the rat (Matthiew, 1970). In spite of the thickness of the connective tissue capsule, according to Eccles (1935) the potential changes recorded with an electrode on the surface of sympathetic ganglia are due to the action potentials of the ganglion cells themselves.

The total number of sympathetic ganglion cells has not been counted in any species, but neurons have been counted in individual ganglia, particularly the superior cervical ganglion. The estimated number of neurons in the superior cervical ganglion varies from 14,900 in the mouse
(Levi-Montalcini and Booker, 1960) to 911,000 in man (Ebbesson, 1968), with intermediate numbers for animals of intermediate size: 32,000 in the rat (Levi-Montalcini and Booker, 1960), 123,000 in the cat (Billingsley and Ranson, 1918), 220,000 in the monkey (Ebbesson, 1968).

Mammalian sympathetic ganglion cells are multipolar neurons (De Castro, 1932) showing great variability in the appearance of their cell processes. In silver-impregnated ganglia, the dendrites are usually numerous, relatively thick and profusely branched; with the Golgi impregnation method, many dendrites show short expansions similar to spines (De Castro, 1932). The dendrites can run a great distance from the cell body, or can branch locally into an intricate net. These dendrites abut on neighboring neurons and seem to form pericellular nests around these neurons. Sometimes several dendrites converge upon a single neuron. Occasionally, dendrites from two or more ganglion cells converge into a dendritic glomerulus to which preganglionic fibers also contribute (De Castro, 1932). Such glomeruli are common in man (Ranson and Billingsley, 1918; Gairns and Garven, 1953) but are rare or absent in smaller mammals (McLachlan, 1974). Other ganglion cell processes, the accessory or secondary dendrites or intracapsular
processes, are short and thin and are contained entirely within the satellite cell sheath. These short processes are very common in man and monkey (De Castro, 1932) but are rarely found in the dog and cat. By injecting a fluorescent dye (Procion Yellow) intracellularly into neurons of the superior cervical ganglion of the guinea pig, McLaclan (1974) has found 13 dendrites per cell on the average and has calculated the ratio of dendrites to soma surface area to be about 2.16 to 1.

Sympathetic ganglion cells have also been examined by electronmicroscopy in the superior cervical ganglion of the cat (Pick, 1970; Elfvin, 1963a). All ganglion cells and their processes are covered by a sheath or capsule of satellite cells. The nucleus is poor in chromatin and contains lattice-like inclusions, which are composed of microtubules and neurofilaments arranged in very regular patterns (Seite et al., 1971; Elfvin, 1963a). Nissl bodies are clumped throughout the cytoplasm and are more abundant in the deeper part of the cell. There are numerous mitochondria, Golgi profiles and dense bodies, and free ribosomes are present singly or in clusters (Elfvin, 1963a, 1963b; Matthews and Raisman, 1972). In the superficial part of the cells, clusters of small
granular vesicles (i.e., of the adrenergic type) are observed (Hökfelt, 1969), and these sometimes occupy a mushroom-shaped process evaginating from the cell surface (Van Orden et al., 1970). The granules of these vesicles are difficult to preserve and are rarely seen in preparations of sympathetic ganglia fixed with glutaraldehyde or osmium (Richardson, 1966; Grillo, 1966). However, they are consistently observed after permanganate fixation (Hökfelt, 1969; Taxi, 1974).

Preganglionic fibers provide the ganglionic neurons with a large number of synapses. The preganglionic fibers run parallel to or wind around the dendrites, forming trails of varicosities (en passant synapses) and ending as terminal boutons (Elfvin, 1963b; Matthews and Nelson, 1975). In mammalian sympathetic ganglia, synapses on the soma are far less numerous than on dendrites (Elfvin, 1963b; Pick, 1970; Matthews and Nelson, 1975). In the superior cervical ganglion of the rat only 14 percent of the synapses are axosomatic; 71 percent are on dendrites (Tamarind and Quilliam, 1971). In this ganglion the synapses on dendritic spines are 2.4 times more numerous than those on dendritic shafts; on the other hand, of those synapses on the soma, only 2 percent are on
spine-like projections (Matthews and Nelson, 1975). Pre-ganglionic nerve endings are about 0.3-1.5μ in diameter and contain small, clear-centered synaptic vesicles about 300-500Å in diameter, as well as some larger dense-cored vesicles of 600-1000Å in diameter and varying number of mitochondria (Elfvin, 1963b; Matthews and Nelson, 1975).

Sympathetic ganglia are known to contain catecholamines, predominantly noradrenaline (Euler, 1947). The majority of neurons in sympathetic ganglia display a formaldehyde-induced fluorescence throughout the cytoplasm but many cells also show intensely fluorescent granules near the outer cell membrane, revealing the presence of catecholamines (adrenergic neurons) (Eränko, 1972; Eränko and Härkönen, 1963).

Neurons devoid of specific fluorescence are present in the sympathetic ganglia of many species and are considered to be non-adrenergic. By treating adjacent cryostat sections of a cat lumbar sympathetic ganglion for visualization of noradrenalin and of actylcholine-esterease activity, Hamberger et al. (1965) showed that all the ganglion cells devoid of specific fluorescence had intense acetylcholinesterase activity and vice versa. Eränko (1966)
and Yamasuchi and Lever (1971) confirmed Hamberger et al.
in the superior cervical ganglion of the rat, sheep and pig.

Chromaffin-positive cells have been found in the
superior cervical ganglion of the cat (Bülbring, 1944).
With the Falck-Hillarp method, cells of similar description
have been found in most sympathetic ganglia; they are small,
intensely fluorescent (S.I.F.) cells and have a high
content of catecholamines (Norberg and Hamberger, 1964;
Eränko and Härkönen, 1965). These cells are identifiable
by electronmicroscopy (Tamarind and Quilliam, 1971) and are
described as granular or granule-containing cells. They
are ovoid in shape, 8 to 15μ in diameter, with a large,
non-fluorescent nucleus located somewhat eccentrically.
These cells often have intensely fluorescent varicose
processes of fairly large diameter which sometimes can be
followed for as long as 40μ in the superior cervical
ganglion of the rat (Norberg et al., 1966).

The great interest in chromaffin cells in sympa-
thetic ganglia is related to observations that catechol-
amines modify ganglionic transmission, as originally
observed by Bülbring (1944). Bülbring found that the
venous perfusate of the cat superior cervical ganglion
during preganglionic stimulation contained an adrenaline-like substance. She suggested the chromaffin cells as a possible source of this adrenaline-like substance. A variety of findings suggest that catecholamines have a physiologically inhibitory role in sympathetic ganglia (DeGroat and Saum, 1971). The action of dopamine and other catecholamines is primarily due to an inhibition of the acetylcholine output from the preganglionic nerve endings, and to a minor extent to a direct effect on ganglionic neurons (Christ and Nishi, 1971; Dun and Nishi, 1974).

C. SYMPATHETIC PREGANGLIONIC FIBERS

In 1886 Gaskell noted that white rami communicantes are formed by an outflow of myelinated nerves from anterior and posterior roots of the spinal nerves between the second thoracic and second lumbar levels, inclusive. These myelinated nerves pass not only into their metameric sympathetic lateral ganglia, but also form three main streams: upwards into the cervical ganglia, downwards into the lumbar and sacral ganglia, and outwards into the collateral ganglia. Furthermore, he suspected that the origin of these fine myelinated nerves was the intermedio-lateral cell column. Through many investigations (Gaskell,
1916; Bok, 1928; Sheehan, 1941; Mitchell, 1953), it became clear that preganglionic fibers reach the paravertebral ganglia through the ventral roots and the white rami communicantes, ending in the ganglia of the corresponding level, or ascending or descending along the sympathetic chain to more cranial or caudal ganglia.

From mainly physiological evidence, each preganglionic fiber has been shown to branch and supply several different ganglia. By studying the pilomotor responses to stimulation of different portions of the sympathetic chain, Langley (1899) observed that the fibers to single segmental ganglia often send branches to other ganglia. In the lower thoracic, lumbar, and sacral regions of the sympathetic chain in the cat, the great majority of the fibers send branches to three successive ganglia; a few send branches to four.

Obrador and Odoriz (1936) studied the transmission of nerve impulses through the fifth lumbar sympathetic ganglion of the cat, using electrophysiological techniques. They observed that some of the preganglionic fibers supplied branches which synapsed on fifth lumbar ganglion cells and then passed to more distally located ganglia.

More recently, Blackman and Purves (1969) showed
electrophysiologically that preganglionic fibers in the thoracic sympathetic chain of the guinea pig may either ascend or descend several segments, making synapses in more than one ganglion.

Preganglionic fibers represent a heterogeneous population of fibers in terms of size, presence of myelin sheath, and conduction velocity. Eccles (1935) classified four types of preganglionic fibers (S1, S2, S3 and S4) based on their excitability and conduction velocity. Based in order of stimulation threshold (i.e., decreasing diameter), Folkow, Johansson and Oberg (1958) differentiated three groups of preganglionic sympathetic fibers: (1) fibers to the neurons innervating the pupil, the nictitating membrane and the arterio-venous anastomoses of the skin; (2) fibers to neurons involved in vasoconstriction in the skin and skeletal muscles; (3) fibers to the neurons which supply vasodilator fibers to striated muscles.

Morphological evidence also supports the heterogeneity of preganglionic fibers. Foley and DuBois (1940) calculated the fiber composition in the cervical sympathetic trunk of the cat. They determined that of the myelinated fibers in the cervical sympathetic trunk 87 to 99% are preganglionic fibers. In addition, a variable number of
preganglionic fibers (5 to 60%, average 23%) are unmyelinated. Dunant (1967) also demonstrated three types of preganglionic fibers in the rat cervical sympathetic chain. Myelinated B1 and B2 fibers measured approximately 3μ and 1.5μ in diameter, respectively; and B2 fibers were the most numerous. The unmyelinated C preganglionic fibers were about 0.5μ in diameter and surrounded by Schwann cell cytoplasm.

Recent electron microscopic work has verified the presence of different sized fibers in the cat cervical sympathetic trunk. Williams et al. (1973) classified preganglionic fibers into 4 groups, based on electron microscopic observations. The preganglionic axons of greatest caliber were designated S1 fibers; S2 and S3 classes were more numerous and were distinctly smaller myelinated fibers with thinner myelin sheaths. The average number of myelin lamellae in the sheaths of these fibers were 52, 26 and 16 for classes S1, S2 and S3, respectively. The unmyelinated S4 preganglionic axons were about as numerous as the sum of the myelinated fibers and were interspersed between them.

Postganglionic neurons are far more numerous than preganglionic fibers. Billingsley and Ranson (1918)
counted the myelinated nerve fibers stained by osmic acid in the cervical sympathetic trunk and related their number to the number of cells in the superior cervical ganglion. The resulting ratio of preganglionic fibers to ganglionic cells was approximately one to thirty-two. Considering Foley and DuBois's (1940) observation that in some cats as many as 5 to 60% of preganglionic axons may be unmyelinated, Wolf (1941) calculated the ratio of preganglionic fibers to the ganglionic neurons to be between 1:11 and 1:17 in the superior cervical ganglion of the cat. This ratio varies in different species and even in individuals of the same species. Ebbesson (1963, 1968) estimated the ratio of preganglionic fibers to ganglionic neurons in the superior cervical ganglia of monkeys and humans to range from 1:28 in a squirrel monkey ganglion to 1:96 in a human ganglion. In man, the ratio even varied from one individual (1:68) to the next (1:96). Since all the counts showed that the number of ganglionic neurons was in excess of that of preganglionic cells, there is a clear indication of divergence of axons along the efferent pathway.

Several rami communicantes contribute fibers to the cervical sympathetic trunk. Langley (1892) furnished indirect evidence that the ventral roots of upper thoracic
spinal nerves project preganglionic fibers into the sympathetic trunk. By stimulation of thoracic roots, he obtained characteristic effects in the pupil, nictitating membrane and eyelids, as well as in submaxillary glands and blood vessels, and piloerection of the hair of the face and neck of the cat. Foley and DuBois (1940) could eliminate 87 to 90% of the myelinated fibers in the cervical sympathetic trunk of the cat by ventral root section between C8 and T5, indicating this to be the major region of supply. By selective destruction of thoracic roots of the cat, Foley and Schnitzlein (1957) quantitatively calculated the number of preganglionic axons contributed by each root from T1 to T8. Thus, the first thoracic segment contributes fewer preganglionic axons to the upper cervical sympathetic trunk than any other of the upper five thoracic levels. The second, third, fourth, and fifth thoracic roots contribute approximately equal numbers of preganglionic axons to the upper cervical trunk, whereas the contribution below the level of T5 is minimal.
D. THE SPINAL ORIGIN OF THE PREGANGLIONIC AUTONOMIC OUTFLOW

In 1851 Clarke described a "lateral horn" of the gray substance of the thoracic region of the spinal cord. Later (1859), he named this nucleus the "tractus intermediolateralis" and noted that it was not present in the cervical region but continued through the lumbar region, becoming smaller in size.

Gaskell (1886) theorized the association of the cells of the lateral horn with the thoracic outflow of visceral nerves. He pointed out that the lateral group of cells commences at the origin of the second thoracic nerve and continues along the thoracic part of the cord to about the origin of the second lumbar nerves. These cell groups corresponded to the origin of the fine myelinated fibers which he found particularly plentiful in the ventral spinal roots and rami communicantes of the thoracolumbar region.

Subsequently, several investigators attempted to verify Gaskell's hypothesis experimentally. Onuf and Collins (1898) examined chromatolytic cells with the Nissl method after removing various sympathetic ganglia. They concluded that the efferent sympathetic fibers arose from a "paracentral group" (a number of cells lying near the
central canal), from small cells in the lateral horn, from small cells in the "intermediate zone" lying between the bases of the anterior and posterior horns, and to a limited extent from cells in Clarke's column. Anderson (1902) and Herring (1903) also studied the cat spinal cord after section of the cervical sympathetic chain and found atrophy exclusively in the small cells of the lateral horn on the side of the lesion.

The Nissl method following sympathectomy was criticized by Bok (1928), claiming that preparations did not show pathological changes but merely variations occurring in normal ganglion cells. Bok (1928) and Poljak (1924) furnished direct proof of the origin of preganglionic fibers by demonstrating their continuity with intermedio-lateral horn cells (IML) in silver preparations of spinal cord of guinea pig and bat, respectively. They also described, as preganglionic neurons, an intermediomedial (IMM) nucleus, which was located laterodorsal to the central canal. Some of these IMM cells were interpreted as intercalated neurons (Bok, 1928) which connect afferent, as well as inter- and suprasegmental efferent fibers with the cells of the IML column; others give rise to nerve fibers which uninterruptedly emerge with the
ventral spinal roots, thus forming part of the autonomic outflow (Poljak, 1940). Poljak also proposed that some preganglionic neurons are located along a line from the IMM cell column to the subpial nucleus. Bok (1928) divided the IML cell column into four subgroups; 1) apical, 2) reticular, 3) central, and 4) lateral funicular groups.

Extensive histological studies of spinal cord with silver and Nissl preparations, especially in longitudinal section, provided similar results. Laruelle (1937) proposed the spinal autonomic region to include the whole intermediate gray area, which extends from the central canal to the lateral funiculus and between the bases of the anterior and posterior horns. The most obvious nuclear groups within the gray matter are the IML columns. These are continuous with each other across the midline by means of many fibrocellular bands which include the intercalated and intermediointernal (or paraependymal) cell groups, thus forming a ladder-shaped architecture along the axis of the spinal cord.

Descriptions of spinal autonomic neurons also appear in Rexed's cytoarchitectonic study of the cat spinal cord (1954). Intermediolateralis thoracolumbalis is described
as a nucleus in Rexed's lamina VII at the border of the gray substance directly lateral to the central canal. The nucleus intermediomedialis appears as a small group of cells that lie at the transition of lamina VII to the substantia grisea centralis (Rexed's lamina X) at the level of the central canal.

A more comprehensive search for autonomic neurons, especially among the cell groups of the intermediate zone of the spinal cord, was carried out recently by Cummings (1969) in the dog and Petras and Cummings (1972) in the monkey. A cytoarchitectonic study of cell groups in the zona intermedia of the thoracolumbar spinal cord led to the identification of the following nuclei in the monkey: nucleus intermediolateralis thoracolumbalis pars principalis et funicularis (ILp, ILf), nucleus intercalatus spinalis (IC); nucleus intermediomedialis (IM), and nucleus intercalatus pars paraependymalis (ICpe). Unilateral thoracic and/or abdominal sympathectomy performed in dogs (Cummings, 1964) and monkeys (Petras and Cummings, 1972) resulted in massive numbers of chromatolytic preganglionic neurons in IC, ILp and ILf. Dorsal rhizotomy following sympathectomy in monkeys demonstrated hardly any degenerating dorsal root terminals in the vicinity of the
somas or proximal dendritic stems of chromatolytic sympathetic motor neurons (IC, ILp and ILf). The degenerating terminal fibers were found spreading into many areas, such as the n. marginalis of Waldeyer, substantia gelatinosa of Rolando, n. proprius cornus dorsalis (Pd), Clarke's column, n. intermediomedialis and among axial and appendicular somatic motor neurons. Thus, from their anatomical data, these authors suggest that, within the spinal cord, visceral reflex pathways are polysynaptic.

Using the method of intracellular labeling with horseradish peroxidase (HRP), Schramm et al. (1975) demonstrated HRP labeled preganglionic neurons in the rat spinal cord following HRP injection into the adrenal gland. The adrenal preganglionic neurons were located in ILp, ILf, IC and the central autonomic nucleus (CA). They were distributed between spinal segments T1 and L1, with approximately 50% of the neurons being within segments T7 to T10.

The rostral and caudal limits of the intermediolateral column have been described by several investigators. In the rat (Navaratnan and Lewis, 1970), cat (Laruelle, 1937; Rexed, 1954; Henry and Calaresu, 1972), and man (Bok, 1928; Gagel, 1928) the rostral end of the
column is located at the limit between the cervical and thoracic spinal cord. The caudal end of the cellular column is located by most authors between the first and third lumbar levels (Gagel, 1928; Laruelle, 1937). Henry and Calaresu (1972) and Rexed (1954) found that the intermediolateral nucleus in the cat becomes less distinct at lumbar levels but can be followed as far down as the fourth lumbar segment. Bok (1928), however, reported that in man the intermediolateral column, although becoming thin in midlumbar segments, extends down to the sacral spinal level.

The numerical distribution of sympathetic preganglionic neurons in the spinal cord of human (Bruce, 1908) and cat (Henry and Calaresu, 1972) was studied. Bruce (1908) counted 88,500 cells in the intermediolateral (IML) cell column in one half of the spinal cord of one human; as many as 50 to 60 cells were found in each transverse section. In the cat (Henry and Calaresu, 1972) the total number of neurons in the intermediolateral cell column ranged from 32,790 to 53,340. The mean numbers of neurons on the left and right sides were not significantly different. A statistically significant (P<0.02) difference, however, was demonstrated between the mean number in four
female cats (35,543 ± 1,411) and those in four males (45,765 ± 2,556). The number of IML neurons was found to vary considerably from segment to segment, with the highest counts occurring in segments T1 to T2 and L3 to L4.

Information concerning the morphology of sympathetic preganglionic neurons is very limited to only a few studies. In Nissl and silver preparations, preganglionic neurons are small (9μ) to medium (25-35μ) sized cells, varying in shape from fusiform to round. Each contains a relatively large, vesicular nucleus and prominent Nissl granules (Laruelle, 1937; Rexed, 1954; Petras and Cummings, 1972). Recently, in a detailed morphological study, Réthelyi (1972) noted that the neurons of the intermediolateral column are elongated (25-35μ), and oriented longitudinally. Most of the dendrites originate from the poles of the cells and run in a longitudinal direction, giving the intermediolateral column, when stained with the Golgi method, a fence-like appearance in longitudinal sections. Each axon emerges with a conspicuous axon-hillock from the soma or, more often, from a dendrite. In electron microscopy three types of nerve endings are seen synapsing on these neurons: 1) small terminals containing clear vesicles of spherical shape and synapsing with small
spine-like dendrite projections; 2) relatively large terminals containing clear vesicles of flattened shape and synapsing on cell bodies or large dendrites; and 3) terminals containing mixed large osmiophilic and small clear spherical vesicles and synapsing on smooth parts of large and medium sized dendrites. Different types of nerve endings have also been observed in the intermediolateral nucleus of the rat, where they form synaptic glomeruli with the dendrites (Wong and Tan, 1974).

Reorientation of sympathetic preganglionic neurons during development was reported by Schramm et al. (1975, 1976), using the HRP labeling technique. They first observed (1975) that the neurons of the intermediate zone were mainly oriented transversely in 15-day-old rats. In a later experiment (1976) they demonstrated that transversely oriented neurons lost their orientation or became longitudinally oriented and that many neurons of indeterminate orientation at 22 and 32 days of age subsequently became longitudinally oriented. Réthelyi (1972) also noted that transverse dendrites in the intermediate zone were more readily observable in kittens than in adult cats.
E. THE USE OF HORSE RADISH PEROXIDASE AS AN INTRACELLULAR MARKER IN NEUROANATOMICAL INVESTIGATIONS

Until recent years, two basic approaches have been employed to map neural pathways. One approach is based on the special affinity of neurons for metallic stains (e.g., the Golgi method) or basic dyes, such as methylene blue. A novel variant of this approach is the Falck and Hillarp (1964) fluorescent method, which permits the specific identification of neuronal cell bodies and axons containing monoamine. The other major approach to studying neuronal projections is based on Wallerian degeneration and chromatolysis following axotomy. Such degenerating axons and cell bodies can be identified at the light microscopic level by employing a silver stain or Nissl's stain, respectively.

Although these traditional methods have been invaluable and have provided most of the available information concerning neural circuitry, a substantial number of connections within the nervous system have escaped precise description because of the limitations inherent in these methods.

One of the more recently developed methods is an intracellular labeling technique with the enzyme
horseradish peroxidase (HRP), based on the retrograde movement of proteins within neurons. Kristensson et al. (1971) injected exogenous protein (horseradish peroxidase) into the tongues of suckling and adult mice and rats, and noted the presence of the protein tracer as numerous brown or black cytoplasmic granules in hypoglossal neurons 16-24 hours after injection.

Subsequently, the usefulness of this method as a neuroanatomical tool for identifying the origin of neuronal connections within the central nervous system was tested by several investigators: LaVail et al. (1973) for the retinotectal system in the chick; Kuyper et al. (1974) in the thalamocortical system of young and adult rats; and Nauta et al. (1974) for caudoputamen-substantia nigra connections in the rat. Conclusions based on their observations were as follows:

1) Since the HRP remains rather localized around the site of the injection, the degree of localization at the injection site is compatible with approaching some problems of neuroanatomy.

2) Horseradish peroxidase is picked up and transported in significant amounts in the retrograde direction by axons which terminate near the site of injection rather
than by axons of passage. Thus, labeling due to axons in passage does not appear to be a problem.

3) Although the HRP marker is taken up by cell bodies at the site of injection, the anterograde transport of the marker protein, if it occurs, does not appear to confound the interpretations of retrogradely labeled cell bodies.

4) HRP product is distinctly visible after retrograde transport in the form of small brown granules, and there is little risk of confusing these granules with background staining of tissue.

5) The method appears applicable to adult and young animals of several species.

6) Many, though not all, afferent cell populations can be identified. Thus they concluded that this method might be adapted to identify specific neurons on the basis of their axonal projection to an injection site.

The uptake of exogenous peroxidase by neurons was studied by electron microscopy, based upon the improved ultrastructural cytochemical technique of Graham and Karnovsky (1965) for the ultrastructural demonstration of peroxidase activity. Eighteen to 24 hours after injection of horseradish peroxidase in the rat cerebrum, Becker et al.
(1968) localized the HRP reaction products in membrane bound, small, multivesicular bodies and simple vesicles, as well as in larger dense bodies in neurons and in proximal dendritic and axonal processes. Rarely, a coated pinocytotic vesicle, containing peroxidase, was seen fused with neuronal plasmalemma and in continuity with the tracer-filled intercellular space.

Uptake of exogenous peroxidase was also observed in peripheral nerve fibers. Krishnan and Singer (1973) applied surgical cotton soaked with horseradish peroxidase dissolved in amphibian Ringer solution on the brachial spinal nerves of adult newts. After six hours exposure to peroxidase, the HRP reaction product was localized in the axoplasm of the nodal regions. It was always concentrated within the vesicles and agranular endoplasmic reticulum. Pinocytotic vesicles containing peroxidase were seen in various stages of formation from the axolemma.

More recently LaVail and LaVail (1974) studied retrograde transport of horseradish peroxidase from the region of retinal ganglion cell axon terminals back to the cell bodies, by electron microscopy. After injection of HRP into the chick optic tectum, HRP was taken up by axon terminals and unmyelinated axons as well as by other
processes and cell bodies of the outer tectal layers. The uptake of extracellular HRP appears to occur by pinocytosis into membrane-bound vesicles of two distinct classes of size: a) small, 43-50 nm (presumably synaptic) vesicles within the axon terminals, and b) larger, 100-125 nm coated vesicles restricted to axons near the injection site, but rarely within the axon terminals. By 7.3 hours after a restricted tectal injection, HRP-positive cup-shaped organelles and multivesicular bodies, as well as large vesicles, are found in axons of the contralateral optic nerve, despite the apparent absence of extracellular HRP in the optic nerve. This observation indicates that the multivesicular bodies can move from the distal axon to the cell body.

The possible involvement of microtubules in the retrograde transport of HRP has been suggested. Hansson (1973) illustrated HRP-positive vesicles in retrograde transit near microtubules in a ganglion cell axon in the rat optic nerve. LaVail and LaVail (1975) also noticed the frequent observation of at least three microtubules arranged regularly around HRP-containing vesicles in optic nerve of chick.
These morphological observations have been supported by several functional findings based on the disruption of microtubule formation by colchicine and vinblastine sulfate. A relatively low concentration (5-10 μg/eye, or 1.8 x 10^{-1} mM) of colchicine was administered in the vitreous humor of chicks four days before HRP introduction into the optic tectum (LaVail and LaVail, 1974). After sufficient time was allowed for the normal HRP transport (24 hours) to the retina, peroxidase could not be identified in ganglion cells in the retina which had received intravitreal doses of at least 10 μg of colchicine. When vinblastine sulfate (VLB, 0.1 to 30 μg in 1 to 3 μl saline) was injected intravitreally into the eye of adult albino rats, Bunt and Lund (1974) observed the blockage of retrograde axonal transport of HRP from the superior colliculus to the retinal ganglion somas of the drug treated eye. Since vinblastine sulfate is known to bind specifically to tubulin in low concentrations, results suggest that the transport mechanism may involve free tubulin and/or intact microtubules within the axoplasm.

The rate of retrograde transport of HRP has been estimated. When horseradish peroxidase is injected into
the chick optic tectum, axons of ganglion cells transport it centripetally to their cell bodies in the retina at a rate of about 72 millimeters per day (LaVail and LaVail, 1972). Following intravitreal injections, the marker enzyme was found in neuronal cell bodies of the isthmo-optic nucleus four hours after injection, indicating a rate of retrograde transport of at least 84 mm per day in these neurons (LaVail and LaVail, 1974).

The marker that accumulates in cell bodies after retrograde transport by neuronal axons remains variously for 3-4 days (LaVail and LaVail, 1974; Turner and Harris, 1974) or 6-11 days (Kristensson and Olsson, 1973) after injection. Thereafter, the proteins disappear from the neurons, presumably as a result of lysosomal degradation. Repeated injections of HRP do not seem to induce any sign of nerve cell necrosis (Kristensson and Olsson, 1973), as seen by both light and electron microscopy. The lysosomal system therefore appears to protect neurons against toxic effects due to intracellular penetration of these substances.

The early negative findings concerning orthograde transport of HRP (LaVail et al., 1973) turned out to be misinformation due to the small size of HRP-containing
granules which are below the resolution of light microscopy. Later electron microscopic observation proved the presence of orthograde transport of HRP in several instances. Hansson (1973), LaVail and LaVail (1974) and Colman et al. (1976) studied orthograde transport of HRP in the visual system of rat, mouse and chick, and observed vesicles containing electron-opaque reaction product in axons of the optic nerve and in terminals in the superior colliculus and lateral geniculate body after intraocular injection of HRP. Reperant (1975) could also trace retinal ganglionic cell projections in the rat to primary optic centers after intravitreal injection of HRP, by confirming the presence of peroxidase only within axonal profiles, as far out as the axon terminals, but not in extracellular space. In studying the caudoputamen-substantia nigra connections in the rat, Nauta et al (1975) observed the presence of HRP in the neuropil as well as in cell bodies of the substantia nigra after HRP injection into the caudoputamen complex, thus confirming that there is a bidirectional connection between them, as well as bidirectional transport (retrograde and orthograde) of proteins. The HRP within boutons and preterminal axons was localized in tubular profiles and vesicles of varying
size, but the vesicles were always much larger (50 nm or more) than typical microtubules would be (25 nm). Since the tubular profiles frequently gave the impression of representing cross sections through a branched coiled network, the investigators suggested that agranular reticulum may be involved in orthoerade transport of HRP. The estimated rate of orthoerade transport of HRP varies between 9.2 mm/day (chick ganglion cell, LaVail and LaVail, 1974) and 216 mm/day (mouse ganglion cell, Colman et al., 1976).
MATERIALS AND METHODS

A. ANIMAL PREPARATION AND HORSE RADISH PEROXIDASE INJECTION

Experiments were performed on 19 adult cats (2-4Kg) of either sex. All surgical procedures were carried out under sodium pentobarbital (35-40 mg/Kg, ip) anesthesia. Thoracotomies were performed in the fourth intercostal space and the animals were then maintained on a positive pressure respirator (EnSCO RU-4M) with room air throughout the surgical procedures. Respiration rate and depth were adjusted to approximate the spontaneous breathing patterns. Stellate ganglia were identified and cleared of surrounding fat in order to see their connections to the sympathetic chain and spinal nerves.

For the localization of the sympathetic preganglionic cell bodies, which project their axons to the stellate ganglia, horseradish peroxidase was injected into either the right or left stellate ganglion. The spinal cords from these animals were studied for the distribution of preganglionic cell bodies, both in the transverse and longitudinal planes. The uninjected side of the cord in each instance was used as control.
For a comparison of the numerical distribution of the preganglionic neurons on right and left sides of the spinal cord, the stellate ganglia of both sides in one animal were injected with horseradish peroxidase. The population distribution of preganglionic neurons on one side of the spinal cord was compared with that of the other side.

For determination of the specific routes by which preganglionic axons travel from the spinal cord to the stellate ganglion, all the connections between a given stellate ganglion and the spinal cord were cut, except the particular rami communicantes associated with the single spinal nerve in question in each experiment (Fig. 1). Before closing the thorax, horseradish peroxidase (HRP) was injected into both right and left stellate ganglia. Since it is known that preganglionic neurons project their axons exclusively ipsilaterally, the unoperated/injected side was used as a control. For the operated/injected side, the population of HRP-labeled neurons was compared with that of the control side. This procedure was carried out individually for spinal nerves T1 and T2.

Horseradish peroxidase (HRP, type VI, Sigma Chemical Co.) was stored in a vacuum desiccator in the freezer prior
FIGURE 1

CUTS THROUGH CONNECTIONS BETWEEN

STELLATE GANGLION AND SPINAL NERVES
A schematic drawing of the cat stellate ganglion and its connections (represented by solid black) to the spinal nerves. A stellate ganglion is connected to the cervical sympathetic chain rostrally through the subclavian ansa and directly to the thoracic sympathetic chain caudally. It is connected to the C7, C8, T1 and T2 spinal nerves by rami communicantes. In the study of the efferent pathways of preganglionic fibers to the stellate ganglion, a specific cut was made for each individual experiment. For determination of the preganglionic projections through the T1 white ramus, the C7, C8 and T2 rami communicantes, the subclavian ansa and the thoracic sympathetic chain (just caudal to the stellate ganglion) were cut on one side of the animal just before the HRP injection into that ganglion. The specific cuts made for this experiment are shown by solid lines. Broken lines represent the cuts made for the study of the preganglionic projection to the stellate ganglion through the T2 white ramus.
to use. HRP solution was made to a final concentration of 5 to 10% with physiological saline (0.9% sodium chloride) just before injection.

HRP solution was injected into the stellate ganglion by means of a Hamilton syringe with a 27-gauge needle over a period of 2 to 3 minutes. About 5 to 10 μl of HRP solution was injected into each ganglion at 3 to 4 sites, filling almost the entire ganglion. Care was taken to prevent the spread of HRP into the surrounding tissue; this caution was aided by the fact that the stellate ganglion is surrounded by a thick capsule.

B. LIGHT MICROSCOPY

After 24 to 36 hours postoperative survival time, cats were reanesthetized intraperitoneally with sodium pentobarbital (40 mg/Kg). The cats were perfused intracardially with 500 ml of normal saline (0.9% NaCl) and followed immediately by perfusion with 2,000 ml of fixative consisting of 2% paraformaldehyde and 2.5% glutaraldehyde in a 0.1M phosphate buffer containing 4% sucrose. The final pH of this solution was between 7.2 and 7.4.

Immediately after perfusion a laminectomy was performed from C7 to L2. The spinal cord was taken out and
placed in the perfusing fixative and stored in the refrigerator (4°C) overnight. The length of each spinal cord segment was measured and recorded. The borders of adjacent segments were arbitrarily determined as being midway between the lowermost rootlet of the upper spinal cord segment and the uppermost rootlet of the lower spinal cord segment. Dissected segments were placed in 0.1M phosphate buffer solution containing 10% sucrose and stored in the refrigerator until the tissue sank to the bottom (12 to 24 hrs).

Sections were cut at 40 μ on the freezing microtome, and every fourth section (in serial sections) or randomly selected sections (in random sampling) were collected in phosphate buffer. In the case of random sampling, at least two thirds of the length of each spinal cord segment was cut at 40 μ, and one third of these sections were randomly selected for histochemical localization of HRP reaction products.

Sections were incubated for 30 minutes at room temperature in a medium containing 5 mg of 3,3'-Diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co.), 10.0 ml of 0.05M Tris-HCl buffer (pH 7.6) and 0.1 ml of 1% hydrogen peroxide. The sections were then briefly washed
with phosphate buffer, mounted on albuminized slides, dehydrated, cleared and mounted. Some sections were counter-stained lightly with toluidine blue. Sections were observed with light and darkfield microscopy. The HRP reaction product was visualized as brownish granules of various sizes under light field and as bright granules in the darkfield microscope.

To determine the extent of HRP spread at the injection site, HRP-injected ganglia were histologically processed using the above method.

The spinal cord sections were examined to determine: 1) the location of the HRP-labeled sympathetic preganglionic neurons in cross sections of the cord; 2) the longitudinal shape of the preganglionic cell column; and 3) the numerical distribution of preganglionic neurons along the long axis of the spinal cord.

C. ELECTRON MICROSCOPY

After 24 to 36 hours postoperative survival time, cats were reanesthetized with sodium pentobarbital (40 mg/Kg, ip). The cats were perfused with 500 ml of 0.1M phosphate buffer (pH 7.4) containing 4% sucrose. This was followed immediately by perfusion with 2,000 ml of fixative
consisting of 2.5% glutaraldehyde and 2% paraformaldehyde in a 0.1 M phosphate buffer with 4% sucrose. The final pH of this solution was between 7.2 and 7.4.

Immediately after perfusion of each animal, a laminectomy was performed all along the vertebrae from C8 to T9. The spinal cord was removed and placed in the perfusing fixative and stored in the refrigerator (4°C) overnight. The following morning, the spinal cord was cut in the transverse plane into blocks of about 1 mm thickness, which were then placed in the fixative for another hour. The tissues were subsequently rinsed twice in 4% sucrose-phosphate buffer over a period of one hour, then the tissues were incubated for one hour at room temperature in the medium of 5 mg diaminobenzidine (DAB) in 10 ml of 0.05 M Tris-HCl buffer (pH 7.6) without hydrogen peroxide, followed by a second incubation for one hour in the complete DAB substrate including hydrogen peroxide. The tissues were then washed in buffer and the small areas of intermediolateral cell columns were cut out. These small dissected pieces were post-fixed in 2% osmium tetroxide phosphate buffer for two hours. They were then rinsed in buffer, dehydrated in ethyl alcohol, and transferred through propylene oxide into Araldite (Fluka). After curing, the
blocks were thick-sectioned at 1μ on a Porter-Blum MI-1 ultramicrotome. The sections were collected on glass slides and stained with 1% toluidine blue. The block face was trimmed into a small trapezoid encompassing the intermediolateral column (IML), especially the neurons which showed HRP granulations. Ultrathin sections were then made on a Sorval MT-2 ultramicrotome. The sections were stained with uranyl acetate and lead citrate and examined and photographed with a Hitachi HU-11B-2EM electron microscope. In this study, a total of 30 blocks, representing the left or right intermediolateral cell column, were examined in 3 animals. Approximately 45 sections from the IML cell column were analyzed.

In the peroxidase reaction the DAB acts as an electron donor and is oxidized at the site of the enzyme activity to a highly insoluble, brown, polymeric compound. On postfixation with osmium tetroxide, the reaction product reacts strongly with the osmium, yielding a black, insoluble, non-crystalline deposit which is highly electron opaque (Karnovsky, 1968). This technique gives a fine, sharp localization at the ultrastructural level.

The profiles of labeled preganglionic neurons and the proximal parts of their dendrites were photographed,
and montages were constructed from the photographs. On each montage, the transition between the neuronal cell body and its dendrite, roughly defined as the inflexion point of the membrane, was demarcated. By using the inflexion point as a boundary between dendrite and soma, the diameter of each cell body was measured by taking the long axis of the cell body and the perpendicular axis, and the two measurements were averaged. The widths of dendrites were measured at a region 5 μ distal to the inflexion point. The length of the membrane of each profile was measured with a rolling map distance measurer. Each bouton found on the cell bodies and dendrites was characterized as to type. The length of the total synaptic contact per neuronal profile was determined.
RESULTS

LIGHT MICROSCOPY

A. DISTRIBUTION OF SYMPATHETIC PREGANGLIONIC CELL BODIES IN CROSS SECTIONS OF THE SPINAL CORD

The preganglionic neurons which had taken up horse-radish peroxidase retrogradely were distinctively labeled with granules (Fig. 2A). These granules were rather uniform in size and were distributed throughout the cytoplasm and large dendrites. Since the accumulation of HRP granules was limited to the cytoplasm, in darkfield microscopy, the nucleus appeared as a dark, round area approximately in the center of the cell body, while the cytoplasm was filled with light diffracting granules (Fig. 2B).

The HRP-labeled preganglionic neurons were found in the gray matter of the thoracic spinal cord, exclusively ipsilaterally to the side of the injection. The uninjected side did not show any trace of HRP granules in any neurons of the spinal cord (Fig. 2C).

These HRP-labeled preganglionic neurons were distributed throughout the zona intermedia (Rexed's laminae VII and X), which extends from the central canal to the lateral horn of the spinal gray matter. Some labeled
FIGURE 2
HRP-LABELED PREGANGLIONIC NEURONS
A: A light field micrograph of HRP-labeled neurons. Tissue sections were prepared just for HRP reaction without counterstain. Dark granules are clearly identified in the cytoplasm. 40 X oil immersion obj. 800 X. B: A dark field micrograph of the same neurons. C: A dark field micrograph of a spinal cord in cross section. HRP-labeled neurons are observed only on the experimental (exp) side of the cord. Cont. = control side; c.c. = central canal. 120X.
neurons were found in the lateral funiculus, in a fashion somewhat like radiating projections from the lateral horn. Based on their location, neurons were subgrouped into four cell-groups: 1) nucleus intermediolateralis (IML); 2) nucleus lateral funicularis (LF); 3) central autonomic nucleus (CA); 4) nucleus intercalatus (IC) (Fig. 3).

Despite several descriptions of the presence of sympathetic preganglionic neurons in the nucleus intermediomedialis (IMM) (Rexed, 1954; Petras and Cummings, 1972), the present experiment did not show any HRP-labeled neurons in the IMM, suggesting no direct projections from IMM neurons to the stellate ganglion.

The nucleus IML is located in the lateral gray matter at its junction with the lateral funiculus. Thus, this nucleus represents the lateral horn of classical anatomical descriptions based upon the customary transverse sections of the spinal cord. It also includes, often times, the cells dorsoventrally arranged along the lateral margin of the ventral horn (Fig. 3). This vertical band of cells begins near the lateral horn and may occupy one-third to one-half of the lateral margin of the ventral horn gray matter.
FIGURE 3

THE LOCATIONS OF PREGANGLIONIC CELL BODIES
FIGURE 3

A diagrammatic drawing of a cat spinal cord in cross section at the T2 level. Round dots represent sympathetic preganglionic neurons in four different locations. The number of dots does not represent the actual number of neurons. CA: central autonomic nucleus; IC: nucleus intercalatus; IML: nucleus intermediolateralis; LF: nucleus lateral funicularis.
The somal size of preganglionic neurons was measured on the light microscope at a magnification of 450X by using an ocular micrometer which was calibrated against a stage micrometer. The cell diameters were measured by taking the long axis of the cell body and the perpendicular axis, and the two measurements were averaged.

A graph of cell diameters from 211 preganglionic neurons has been plotted in Figure 4. The cell size ranged between 10µ and 28µ, and the cells can be seen to form one cell population with a mean size of 18µ ± 0.2 S.E.

The cells in the IML varied in shape from round to fusiform to distinctly multipolar, and they contained a relatively large, round nucleus and prominent Nissl substance. Fusiform cells were as small as 12 x 16µ, but mostly measured about 15-20 x 23-27µ. The round neurons of this nucleus ranged from 10µ to 19µ, or occasionally as much as 27µ in diameter. The distinctly multipolar cells were few in number and usually larger in size, ranging between 22µ to 28µ.

All three types of cell (round, fusiform and multipolar) were found intermingled with each other randomly in the IML region, without any specific subgrouping according to their cell types within the area. These preganglionic
FIGURE 4
SOMAL DIAMETERS OF PREGANGLIONIC NEURONS
FIGURE 4

A graph of the perikaryal lengths (average of long axis and perpendicular short axis of the soma) of a sample of 211 preganglionic neurons. Somal lengths appear to be dispersed along a continuum within a range of 10µ and 28µ. Mean somal size is 18µ ± 0.2 (S.E.). This suggests that the preganglionic neurons of the cat constitute, at least with regard to size, a unimodal population.
cell bodies were often quite crowded in a very small area, such that it was common to find two or three cells seemingly overlapping each other in histological sections.

The neurons of the intermediolateral nucleus merged laterally with the lateral funiculus. Laterally the group extended as far as about two-thirds of the width of the lateral funiculus from the lateral margin of the gray matter.

The nucleus lateral funicularis (LF) contained two types of cells, most commonly fusiform but occasionally round. The cell bodies of fusiform neurons ranged from 12 x 18 μ to 18 x 36 μ, and round neurons were in the range of 12-16 μ in diameter. It was quite common to observe that the fusiform cells of the LF projected their long dendrites toward the IML column. However, any actual contact between the two cell populations was beyond the resolution of this technique with the light microscope.

The nucleus intercalatus (IC) consisted of a series of transverse cellular bands or bridges within transverse fiber tracts which spanned the zona intermedia and mid-line of the cord, thus appearing to unite the clusters of the nucleus intermediolateralis of the right and left sides of the spinal cord. On their lateral aspect the bands of
IC cells merged with the neurons of the IML. Medially, the cells merged with the neurons of the central autonomic nucleus.

The nucleus intercalatus was chiefly composed of large fusiform neurons which lay with their long axes transversely oriented to the spinal cord. These neurons contained oval nuclei and cytoplasm filled with large and deeply stained Nissl bodies. Some triangular or round neurons were found along with the fusiform cells. The fusiform neurons predominated and measured from 12 x 23μ to 17 x 34μ. The triangular or round neurons varied from small (12μ) to medium (17μ) sizes.

The central autonomic nucleus (CA) was located lateral and dorsolateral to the central canal. The area of the nucleus intercalatus merged with it laterally. The shape of its neurons varied from round to fusiform, in small to medium sizes ranging from 10-15μ or 11-17 x 20-28μ in diameter, respectively. The long axis of the fusiform cells was oriented either mediolaterally or dorso-ventrally in transverse sections of the spinal cord.

The population distribution of the thoracic pre-ganglionic neurons in different subgroups was calculated from cell counts in 871 cross sections of spinal cord from
T1 to T6 levels inclusive, from three animals. Only the HRP-labeled neurons which showed definite nuclei were counted, at a magnification of 450X, using an AO microscope. A total of 4307 HRP-labeled neurons were counted. The numerical distributions of the observed HRP-labeled neurons in each subgroup are shown in Table 1. About 78.2 per cent of the total observed preganglionic cells were located in the nucleus intermediolateralis, while 18.5 per cent of them were found in the lateral funiculus. The remaining 3.3 per cent were found in the nucleus intercalatus and the central autonomic nucleus.
Table 1
THE HORIZONTAL DISTRIBUTION OF PREGANGLIONIC CELL BODIES WITHIN THE CORD

<table>
<thead>
<tr>
<th>Level of cord</th>
<th>No. of sections</th>
<th>No. of neurons (%) of total in each level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(IML)</td>
</tr>
<tr>
<td>T1</td>
<td>125</td>
<td>400(51.7)</td>
</tr>
<tr>
<td>T2</td>
<td>178</td>
<td>822(78.7)</td>
</tr>
<tr>
<td>T3</td>
<td>203</td>
<td>938(85.0)</td>
</tr>
<tr>
<td>T4</td>
<td>127</td>
<td>476(89.0)</td>
</tr>
<tr>
<td>T5</td>
<td>123</td>
<td>466(84.6)</td>
</tr>
<tr>
<td>T6</td>
<td>115</td>
<td>266(88.7)</td>
</tr>
<tr>
<td>TOTAL (T1-T6)</td>
<td>871</td>
<td>3368(78.2)</td>
</tr>
</tbody>
</table>
B. LONGITUDINAL DISTRIBUTION OF SYMPATHETIC PREGANGLIONIC NEURONS WITHIN THE SPINAL CORD

Following injection of HRP solution into the stellate ganglion of the cat, the HRP-labeled sympathetic preganglionic neurons were studied in horizontal longitudinal sections as well as in cross sections of the spinal cord. The extent and the shape of the sympathetic preganglionic cell column which innervates the stellate ganglion, as well as the numerical distribution of preganglionic cell bodies along the long axis of the spinal cord, were examined.

With HRP injection into the stellate ganglion, the level of the first appearance of labeled preganglionic neurons in the spinal cord varied from animal to animal. They started to appear between the caudal portion of the eighth cervical and the middle of the first thoracic levels. The caudal extent of the HRP-labeled neurons also varied, from the rostral portion of the seventh thoracic to the caudal portion of the eighth thoracic spinal cord levels.

At the rostral pole of the thoraco-lumbar sympathetic preganglionic cell column the distribution pattern of the neurons in cross section of the spinal cord was
different from the typical mid-thoracic pattern which is shown in Figure 3. Rostrally the majority of the HRP-labeled neurons were widely scattered in the lateral funiculus adjacent to the intermediate gray matter, forming a radiating web. A few cells were found at the base of the anterior horn at the junction of gray and white matter. Since the cervical enlargement in the cat extends down to the T1 level, the large lateral motor nuclear group persists in the ventral horn at this level. This lateral motor nuclear group is somewhat isolated from the rest of the ventral horn by fiber tracts which run medial to it (represented by broken lines in Figure 5). Scattered preganglionic neurons were found along the fiber tracts which surrounded this lateral motor nuclear group. Rarely neurons were also found at the ventral border of the anterior horn and along the ventral root pathways (indicated by arrows in Figure 5).

More caudally, the cells gradually merged into the lateral border of the gray matter. At the most caudal region of the first thoracic level, the lateral horn first started to take shape by accumulating more preganglionic neurons in the region, while losing most of the lateral funicular nuclear group. This distribution pattern only
FIGURE 5

THE ROSTRAL POLE OF THE THORACO-LUMBAR SYMPATHETIC PREGANGLIONIC CELL COLUMN
This three dimensional drawing schematically illustrates the change in distribution of the sympathetic neurons at the rostral pole of the preganglionic cell column. The figure represents the whole length of the T1 spinal cord segment, and the four transverse planes were drawn from sections taken at about equal distance (2.4 mm) from each other. At the most rostral end the majority of preganglionic neurons are located in the lateral funiculus. Cells are frequently observed along the ventro-lateral border of the ventral horn (indicated by arrows). Cells gradually gather at the lateral border of the intermediate gray, thus forming a more distinctive intermediolateral cell column at the caudal part of the T1 level.
appeared at the transition region within a very short length of the spinal cord. HRP-positive neurons were also found in the central autonomic area and the intercalated nucleus (Fig. 5). Due to this fact, the population distribution of neurons in cross sections of the T1 level was quite different from that of other spinal cord levels. The differentially very high population in LF at level T1 can be seen in Table 1.

The preganglionic cell columns were also examined in horizontal longitudinal sections of the cat spinal cord. From histological serial sections, each HRP-positive neuron was mapped on paper, using a Leitz microscope equipped with a camera lucida. One example of cumulative data is represented in Figure 6. This particular mapping was made from mid-T5 to mid-T6 levels of one side of the spinal cord of one animal; each dot represents one HRP-labeled neuron.

The nucleus intermediolateralis appeared to be a somewhat segmented cell column, with clusters of 6 to 30 neurons alternating with less populated patches. These alternations were repeated several times within one segmental length of spinal cord, with more or less obvious clumping. Any difference in population distribution corresponding to regions of rootlet input or outflow was
FIGURE 6
THE LONGITUDINAL DISTRIBUTION PATTERN OF
THE PREGANGLIONIC CELL COLUMN
FIGURE 6

The longitudinal distribution pattern of the sympathetic preganglionic cell column. The individual HRP-labeled neuron was mapped from serial horizontal (longitudinal) sections of the middle thoracic cord (T5 and T6) with the help of a camera lucida. All the labeled neurons from one side of the spinal cord were then accumulated into this two dimensional figure. One dot represents one labeled neuron.
not observed in this study. This clumping phenomenon of preganglionic neurons was also observed in the examination of spinal cross sections, where the number of HRP-labeled neurons varied from 0 to 20 from one section to the other.

After injection of HRP into the stellate ganglion, preganglionic neurons from a very extensive length of spinal cord segments accumulated HRP in their cytoplasm. Of the 15 animals in this study, eleven showed labeled neurons from T1 to T8; three from C8 to T8; and one from C8 to T7.

The numerical distribution of preganglionic neurons which contribute to the stellate ganglion was estimated along the long axis of the spinal cords in seven cats. The cats weighed between 2-3 Kg, and five were female and two male. In five cats HRP was injected into the left stellate ganglion and in two cats into the right. In three animals serial cross sections were cut, and every fourth section was collected for HRP reaction. In the rest of the animals, sample sections were collected randomly from each spinal cord segment. In the random sampling, at least two-thirds of the length of each spinal cord segment was cut in cross section, with about one-third of the sections being collected for histochemistry. In this way, between 30 to
60 sections (each 40μ thick) from each spinal cord level from C8 to T9 spinal cord segments were collected and run for histochemistry of HRP with DAB.

From every section, the HRP-positive neurons were counted by using an AO light microscope (X450 magnification), and the estimated total numbers of preganglionic neurons were calculated for each segmental level of the spinal cord in each experimental animal. Table 2 shows one example of the protocol used in the estimation of the total number of preganglionic neurons in each segmental level in one animal (#H10). In this instance, using level T2 as a sample, the calculation of the total number of preganglionic neurons used the following items of information:

1) the thickness of section: 40μ.
2) the level of the spinal cord: T2.
3) the number of sections observed: 36 (A).
4) the total number of HRP-positive neurons observed from 36 sections: 378 (B).
5) the estimated number of neurons in 1 mm length (25 sections) of spinal cord level T2: 378 x 25/36 = 263 (C).
6) the length of the spinal segment T2: 7.5 mm (D).
Table 2
THE METHOD OF ESTIMATION OF THE TOTAL NUMBER OF PREGANGLIONIC NEURONS CONTRIBUTING TO THE STELLATE GANGLION IN A SINGLE ANIMAL

#H10   2.3 Kg body weight. Male cat. 40μ thick sections.

<table>
<thead>
<tr>
<th>level of spinal cord</th>
<th>C8</th>
<th>T1</th>
<th>T2*</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
<th>T7</th>
<th>T8</th>
<th>T9</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. No. sections</td>
<td>30</td>
<td>30</td>
<td>36*</td>
<td>36</td>
<td>34</td>
<td>37</td>
<td>34</td>
<td>27</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>B. No. labeled neurons (N)</td>
<td>0</td>
<td>119</td>
<td>378*</td>
<td>283</td>
<td>145</td>
<td>156</td>
<td>85</td>
<td>39</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>C. No. N / mm</td>
<td>0</td>
<td>99</td>
<td>263*</td>
<td>197</td>
<td>107</td>
<td>105</td>
<td>63</td>
<td>36</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>D. length (mm)/seg.</td>
<td>7.0</td>
<td>7.2</td>
<td>7.5*</td>
<td>8.7</td>
<td>9.5</td>
<td>10.0</td>
<td>11.0</td>
<td>9.8</td>
<td>10.0</td>
<td>10.5</td>
</tr>
<tr>
<td>E. total No. N/seg.</td>
<td>0</td>
<td>713</td>
<td>1973*</td>
<td>1714</td>
<td>1017</td>
<td>1050</td>
<td>693</td>
<td>353</td>
<td>80</td>
<td>0</td>
</tr>
</tbody>
</table>

* shown as a sample in the text description.
Using this information, the total number of neurons in the whole length of segment T2 of the spinal cord was estimated \((E)\) to be 1973 (or \(263 \times 7.5\)). The above procedures were performed for each spinal cord level from C8 to T9 inclusive in seven animals. The number of observed (actually counted) HRP-positive neurons in each animal varied from 1050 to 1726. The estimated total number of neurons which contribute to the stellate ganglion varied from animal to animal. The highest value in this study was 9794 in a 2.9 Kg male and the lowest value was 6069 in a 2.1 Kg male. The average number was \(7,974 \pm 628\) S.E.

Histograms of the numerical distribution of estimated total numbers of sympathetic preganglionic neurons along the long axis of spinal cords are shown in Figures 7 and 8. In Figure 7, each histogram represents a different experimental animal. The abscissa represents the level of spinal cord (from 8th cervical to 8th thoracic) and the ordinate represents the number of cells (0 to 2,000). In three out of seven animals \((C,E,G)\), the cells are distributed from the lower C8 to the T8 levels of the spinal cord, but in the rest of the animals they extend from T1 to T8 levels. As shown in these histograms, the population distribution varied from animal to animal. In
FIGURE 7
THE PREGANGLIONIC PROJECTION TO THE STELLATE GANGLION
FIGURE 7

Histograms of the longitudinal distribution of preganglionic neurons projecting to the stellate ganglion. Each graph (A to G) represents a single experimental animal. Neurons are located within a spinal cord range of C8 to T8. The distribution pattern along the long axis of the spinal cord is different in each animal.
FIGURE 8
SUMMARY HISTOGRAM OF THE PREGANGLIONIC PROJECTION TO THE STELLATE GANGLION

![Histogram showing the number of preganglionic neurons at various levels of the spinal cord.]
FIGURE 8

A summary histogram of the longitudinal distribution of preganglionic neurons which projected to the stellate ganglion from the spinal cord. Data are expressed by the mean number of preganglionic neurons in each spinal cord level ± S.E. The highest population is located in spinal cord level T2 and gradually decreases caudally.
some animals, the stellate ganglion received a high number of axons from neurons located within a very narrow range of spinal cord segments, such as from T1 to T2 in cat D. But in some other animals neurons were rather evenly distributed throughout a wide range of spinal cord levels: in cat E, from T1 to T7 and in cat F, from T2 to T7. A definite drop-off was shown between T6 and T7 spinal levels in most of the animals. The 8th thoracic spinal cord level provided only minor portions (averaging less than 3 per cent) of the total preganglionic projections to the stellate ganglion. No definite difference was observed between sexes or between right and left sides of the animals.

The longitudinal distribution data of preganglionic neurons from all seven animals are summarized in Figure 8. Data are represented by the mean ± SE. These data show that the stellate ganglia studied here were innervated by neurons located from the 8th cervical to the 8th thoracic spinal cord segments. The major populations originated from levels T1 to T7; among them the T2 level contributed the highest number of axons to the stellate ganglia. The number of neurons projecting their axons to the stellate ganglia decreased gradually from the T5 level caudally, and T8 comprised a very minor portion of them. The presence of
some preganglionic neurons in the cervical spinal cord was shown in this study, but proportionally to the total they were minimal. No HRP-labeled preganglionic neurons were observed below the 8th thoracic level of the spinal cord.

C. COMPARISON OF THE DISTRIBUTION OF PREGANGLIONIC NEURONS ON THE RIGHT AND LEFT SIDES OF THE SPINAL CORD.

Reported above (section B) were the various longitudinal distribution patterns of the thoracic preganglionic neurons in individual animals. In this portion of the study, the distribution of the preganglionic neurons was compared on the right and left sides of individual spinal cords.

Two animals were prepared for this purpose by injecting HRP solution into both right and left stellate ganglia of each animal. The HRP-labeled neurons were counted from the histological sections, and the data were treated as in the previous experiment.

The histograms of the population distribution are shown in Figure 9. Each histogram represents an individual animal. The empty bar represents the right side and the solid bar the left side.
FIGURE 9
COMPARISON BETWEEN RIGHT AND LEFT PREGANGLIONIC PROJECTIONS

A

B

LEVEL OF SPINAL CORD

NUMBER OF NEURONS

RIGHT
LEFT
FIGURE 9

Histograms comparing the longitudinal distribution of HRP-labeled preganglionic neurons on the right and left sides of the spinal cord. A and B represent two experimental animals. The empty bar represents the right side and the solid bar represents the left side of the spinal cord.
Despite the variations in the pattern of distribution between the two animals, the pattern within a given animal showed a close correlation from one side of the spinal cord to the other. In cat A, the right side of the cord showed a somewhat greater population than the left side. The estimated number of neurons totalled 6,542 on the right side and 5,526 on the left, making a difference of about 16%. In cat B, the total population difference between right and left sides was less than 1%. Thus, it seems reasonable to conclude that the distribution pattern and the total population of the sympathetic preganglionic neurons on the right and left sides of the spinal cord in one animal are not markedly different from each other.

D. THE EFFERENT PATHWAYS OF PREGANGLIONIC NEURONS TO THE STELLATE GANGLION

Based on the data from the experiment described in B, it becomes obvious that neurons from a wide range of spinal cord segments (from C8 to T8) project their axons to the stellate ganglion exclusively ipsilaterally. In the present experiment, an attempt was made to find the actual route of the preganglionic axons from the spinal cord to
the stellate ganglion, especially through the different white rami communicantes. Furthermore, experiments were designed to find the locations of the preganglionic neurons which send their axons through a single, specific ramus communicans to the stellate ganglion.

Experiments were designed in such a way that all possible routes of preganglionic axons from the spinal cord to the stellate ganglion were cut, except one specific route on one side of the animal (either right or left), before HRP injection into the stellate ganglion. Specifically, T1 and T2 rami communicantes were chosen for this study, since the stellate ganglion usually represents a fusion of the metameric chain ganglia T1 and T2 as well as the inferior cervical ganglion in most cats (Langley, 1892; Saccomanno, 1943). Thus it could be conjectured that T1 and T2 white rami communicantes are the main routes for preganglionic projection to the stellate ganglion.

The specific cuts of the sympathetic chain and of the white and gray rami communicantes for these experiments are shown in Figure 1. Solid lines represent the cuts made when only the T1 ramus communicans connected the spinal cord with the stellate ganglion; the broken lines
represent the cuts when the connection was via the T2 ramus communicans. In each surgical procedure, the fat around the stellate ganglion was cleared as much as possible in order to see all the connections from the stellate ganglion to the spinal cord. T1 and T2 rami communicantes were identified, as well as C8 and T3 rami communicantes. After the specific cuts for each experiment were made, it was always confirmed that the stellate ganglion was completely isolated except for one link, either the T1 or T2 white ramus communicans, connecting it to the spinal cord. Injections of HRP were then made into both right and left stellate ganglia, and the injected operated side of the spinal cord was considered to be the experimental side in each animal. Based upon the similar population distributions of preganglionic neurons on the right and left sides of the spinal cord (see section C), the injected but unoperated side, where all the connections from the stellate ganglion to the spinal cord were intact, served as a control in each experimental animal. Histological sections of spinal cord (from C8 to T8) were prepared as in previous experiments.
HRP-positive neurons were counted on both the experimental and control sides of the spinal cord. The estimation of the total number of preganglionic cells was carried out on both sides following the method described in section B.

Histograms of the longitudinal distribution of HRP-positive neurons throughout the spinal cord on both sides are shown in Figures 10 and 11. Figure 10 shows the longitudinal distribution of the sympathetic preganglionic neurons when the T1 white ramus communicans was the only available path to the stellate ganglion of one side. Experiments were carried out in five cats, and each histogram represents a single experimental animal. The empty bar represents the total estimated number of neurons on the control side, and the cross-hatched bar represents that of the experimental side. Neurons from a wide range of spinal cord levels (T1 to T8) project via the T1 ramus. In fact, among the five animals studied here, between 40-70 per cent of the total preganglionic projections to the stellate ganglion took their path through this ramus.

The longitudinal distribution of labeled preganglionic neurons found when only the T2 white ramus communicans connected the stellate ganglion to the spinal cord is shown...
FIGURE 10
PREGANGLIONIC PROJECTIONS THROUGH T1 WHITE RAMUS

LEVEL OF SPINAL CORD

CONTROL

EXPERIMENT
The longitudinal distribution of preganglionic neurons which project to the stellate ganglion through the T1 white ramus (the cross-hatched bars). Data were collected from five animals and each histogram represents a single experimental animal (A to E). Empty bars represent the total population of the preganglionic neurons which terminate in each normal stellate ganglion (control side).
FIGURE 11

PREGANGLIONIC PROJECTIONS THROUGH T2 WHITE RAMUS

LEVEL OF SPINAL CORD

A

B

C

D

E

NUMBER OF NEURONS

CONTROL

EXPERIMENT
FIGURE 11

The longitudinal distribution of preganglionic neurons which project to the stellate ganglion through the T2 white ramus (cross-hatched bars). The bars represent the total preganglionic projection to each control stellate ganglion. Data were collected from five animals and each histogram represents a single experimental animal.
in Figure 11. Five adult cats were used for this experiment, and each histogram represents a single experimental animal. The empty bars represent the total estimated number of neurons on the control side and the cross-hatched bars represent that of the experimental side. Among the five animals studied, between 12-68 per cent of the total preganglionic projections to the stellate ganglion took their route through the T2 white ramus communicans.

Figure 12 summarizes the longitudinal distribution of those spinal preganglionic neurons from which axons projected to the stellate ganglion via T1 and T2 white rami. Data were collected from five animals in each experiment. The data are expressed as the percentage (+ SE) of total neurons which are labeled in each spinal segmental level. Figure 12A shows the neuronal distribution through the T1 white ramus, and Figure 12B through the T2 white ramus.

As shown in Figure 12A, the T1 white ramus carried axons whose cell bodies were located all along the spinal cord from C8 to T8 levels. The highest population was located at the T3 level, and the neurons within cord levels
FIGURE 12

SUMMARY HISTOGRAMS OF PREGANGLIONIC DISTRIBUTION

THROUGH THE T1 AND T2 WHITE RAMI

A  T1 RAMUS

B  T2 RAMUS

MEAN % OF TOTAL NUMBER OF NEURONS

LEVEL OF SPINAL CORD
Summary of the longitudinal distribution of preganglionic neurons from which axons project to the stellate ganglion via T1 and T2 white rami. The data were collected from five animals in each experiment. The data are expressed as the percentage (± S.E.) of labeled neurons in each segmental level against the total labeled neurons in each animal. Figure A shows the neuronal distribution through the T1 white ramus and Figure B through the T2 white ramus.
T3-T5 comprised about 57% of the total population projecting through the T1 ramus. The neuronal population gradually decreased rostrally and caudally from the peak point at T3. The eighth cervical and 8th thoracic spinal cord together comprised only 2 per cent of the total outflow population.

The T2 white ramus carried preganglionic axons whose cell bodies were located in the upper eight thoracic spinal levels, as shown in Figure 12B. The majority of the neurons were located in the upper three thoracic levels (about 75% of the total) and the cell population decreased abruptly from the fourth thoracic level caudally. The neurons originating from T4-T8 levels comprised 25 per cent of the total. Compared to the greater neuronal outflow at the T3-T5 levels through the T1 ramus, the T2 ramus carried axons whose cell bodies were located in a more rostral portion of the spinal cord (mostly from T1 to T2).

The relative proportion of preganglionic neurons which took the route of either the T1 or T2 white ramus was compared in each spinal cord level. For this purpose, the total number of preganglionic neurons at each spinal level that terminated in the stellate ganglion was used as
a control. Then the proportion of the neuronal population in each spinal cord level which was taking the route of either the T1 or T2 white ramus was computed against the control side. These calculations were carried out for each spinal level from C8 to T8 in five animals, and results are summarized in Figure 13. The data are expressed as mean per cent number of labeled neurons (± SE) on the experimental side as compared to the total number of labeled neurons on the control side. The empty bar represents the percentage of neurons using the T1 white ramus, and the cross-hatched bar represents that using the T2 white ramus. For example, among the total preganglionic population which originated from the T2 spinal level and innervated the stellate ganglion, an average of 47 ± 9% of them projected their axons through the T1 white ramus while an average of 59 ± 5% of them were carried through the T2 white ramus. Black dots above the bars indicate data in which the T1 ramus was significantly different from that of the T2 ramus.

As shown in Figure 13, within spinal cord levels T1 to T3 the neuronal population which projected their axons through the T1 white ramus was not significantly different from that through the T2 white ramus. On the
FIGURE 13

COMPARISON BETWEEN USE OF T1 AND T2 WHITE RAMI AS PATHWAYS TO STELLATE GANGLION

% OF CONTROL (NUMBER OF NEURONS)

LEVEL OF SPINAL CORD

T1 RAMUS
T2 RAMUS
FIGURE 13

The relative proportion of preganglionic neurons which took the route of either the T1 or T2 white ramus is compared in each spinal cord level. To obtain the percentage of the ordinate, the number of neurons using the T1 or T2 white ramus was computed against the total neuronal population that terminate in the stellate ganglion in each spinal level from C8 to T8 in five animals. The summarized results are shown in this figure. The data are expressed as mean percent numbers of labeled neurons (+ S.E.) on the experimental side as compared to the total number of labeled neurons on the control side. The empty bars represent projections through the T1 ramus, and the cross-hatched bars represent the T2 ramus. Black dots above the bars indicate data for which the T1 ramus was significantly different from the T2 ramus.
other hand, in spinal cord levels T4 to T7, a majority of the neuronal population (between 53% and 82%) projected their axons through the T1 white ramus, while a smaller population was projecting through the T2 white ramus (between 14-27%).

Based on these data, it became clear that in the lower thoracic spinal cord (T4-T7), a greater population of neurons were projecting their axons through the T1 white ramus than through the T2 ramus. This suggested that a large population of preganglionic axons travel rostrally within the spinal cord before they exit through T1 ventral roots, while a large population of preganglionic neurons from the T1 spinal level send their axons caudally within the cord to exit through T2 ventral roots.

Using these two sets of experimental data, the longitudinal distribution of preganglionic cell bodies and their specific routes to the stellate ganglia are schematically represented in Figure 14. A large population of neurons from the mid-thoracic spinal cord travel rostrally within the cord, exit through the T1 ventral root, and project to the stellate ganglion via the T1 white ramus communicans. On the other hand, another large population of preganglionic axons, from upper thoracic levels (T1-T3),
Figure 14

The longitudinal distribution of preganglionic neurons using T1 and T2 routes to the stellate ganglion.
FIGURE 14

A schematic illustration of the longitudinal distribution of preganglionic neurons and their specific routes to the stellate ganglion. A large population of neurons from the mid-thoracic (T3-T5) spinal cord project axons rostrally within the cord and thence to the stellate ganglion via the T1 white ramus, while the T2 white ramus carries axons which originate mainly from the upper thoracic cord (T1-T3).
travel either caudally or rostrally within the cord before they exit by the T2 ventral root and join the T2 white ramus communicans, to terminate in the stellate ganglion. That suggests that many preganglionic axons pass each other within the T1 and T2 spinal cord levels on their way to the ganglion. It is of interest that the T1 white ramus carries more axons than the T2 ramus does from neurons located in rather caudal portions of the thoracic spinal cord.

In summary, application of the HRP-labeling technique to the study of the thoraco-lumbar sympathetic preganglionic system successfully revealed the following points. First, the localization of the sympathetic preganglionic neurons in the spinal cord was clarified and identified as four subgroups: IML, LF, IC and CA. The majority of neurons are located in IML, with LF providing the next greatest population, and CA and IC containing only a minor number of cells. Second, the stellate ganglion is innervated by the axons of preganglionic neurons located in a wide range of spinal cord segments (from C8 to T8), with the majority coming from T3 to T5 levels. Third, the distribution pattern of preganglionic neurons within a given animal closely correlates
from one side of the spinal cord to the other, despite the variations in the pattern of distribution among animals. Fourth, more preganglionic neurons in spinal cord levels T3 to T5 project their axons through the T1 white ramus communicans to the stellate ganglion, while the T2 white ramus carries more axons originating from cord levels T1 to T3.

**ELECTRON MICROSCOPY**

**A. MORPHOLOGY OF PREGANGLIONIC NEURONS**

In the light microscope or in the electronmicroscope at low magnification, the cell bodies of the sympathetic preganglionic neurons are recognized by the presence of dark brown or electron dense granules in the cytoplasm, respectively (Fig. 15). Small to medium sized neurons have round to fusiform perikarya, ranging in length from 12-24μ, which give off 1 or 2 dendrites. A typical small neuron can be seen in Figure 15A, a light micrograph taken from a toluidine blue stained, plastic section. Large neurons have perikarya ranging in length from 25-35μ and give off 2 or 3 large primary dendrites (Fig. 16). Large neurons are distinctly multipolar in
FIGURE 15
SMALL PREGANGLIONIC NEURON
FIGURE 15

A. HRP-labeled, small, oval preganglionic neuron in cat spinal cord. One micron, toluidine blue stained, plastic section. HRP-reaction products are shown as dark granules (indicated by arrows) in cytoplasm. 40 X oil immersion obj. X 2,500. B. A low power electronmicrograph of a small preganglionic neuron. X 8,000. N: nucleus; M: mitochondria; EDG: electron dense granules.
FIGURE 16

LARGE PREGANGLIONIC NEURON
A. HRP-labeled, large preganglionic neuron in cat spinal cord. One micron, toluidine blue stained, plastic section. HRP-reaction products are shown as dark granules (indicated by arrows) in the cytoplasm. 40X oil immersion obj. X 1,500. B. An electron micrograph of a large multipolar preganglionic neuron in cat spinal cord. X 4,500. N: nucleus; RER: rough endoplasmic reticulum; EDG: electron dense granules.
shape and are found randomly mixed among the smaller pre-ganglionic neurons.

At the ultrastructural level, neurons possess round, oval, or polygonal perikarya and generally round nuclei containing homogenous karyoplasm and prominent nucleoli. The cytoplasm contains rough endoplasmic reticulum (RER) which is aggregated as distinct clumps of Nissl substance (Fig. 16B). Free ribosomes are also scattered throughout the cytoplasm. Although it is not prominent in this figure, several Golgi complexes are usually found lying near the nucleus. Each complex consists of stacks of irregular cisternae and rounded vesicles. Round gray lipid droplets are present throughout the cytoplasm. The presence of electron dense material (EDG) in the lysosomes is typical of HRP-labeled neurons.

Small to medium cell profiles are seen to give off primary dendrites ranging in width from 0.6 to 5.9μ with a mean value of 3.85μ and SE of 1.30, based on measurements of 48 dendrites. The proximal dendrites of these cells take a curving course through the neuropil and can seldom be traced more than 10μ in any one section. They contain long tubular mitochondria, free ribosomes, clumps of ER,
and lysosomes. Neurotubules and neurofilaments are seen dispersed among the other cytoplasmic elements (Fig. 15B).

A photograph of a large neuron from a toluidine blue-stained plastic section is shown in Figure 16A. Such neurons possess polygonal perikarya ranging in length from 25-35\(\mu\). They contain dense clumps of Nissl substance, and an oval nucleus with a very prominent nucleolus. Large cell profiles are often seen to give off 2 to 3 massive primary dendrites.

In lower power electronmicrographs, large cells (Fig. 16B) possess polygonal perikarya with a comparatively vast cytoplasmic area. They have a large oval or round nucleus containing homogeneous karyoplasm and a large, round nucleolus. Their cytoplasm contains an abundance of various organelles, such as Golgi apparatus and lysosomes, which are scattered throughout the cytoplasm. The RER consists of dense stacks of cisternae studded with ribosomes; free ribosomes are also scattered throughout the cytoplasm in small clusters. Large cells also contain moderate amounts of lipofuscin. Occasionally small, rounded spines are seen to protrude from the somas of these neurons.
Large cells give off massive primary dendrites 6-13.5 μm wide, with a mean value of 8.18 and SE of ± 0.33, based on measurements of 36 primary dendrites. They contain dense clumps of RER, as well as lysosomes, neurotubules, lipid droplets, and long, rod-like mitochondria.

B. CLASSIFICATION OF TERMINALS

The somas and dendrites of the thoracic sympathetic preganglionic neurons of the cat are contacted by three types of boutons, classified according to vesicle shape (round or pleomorphic) and the presence or absence of a postsynaptic density associated with the postsynaptic membrane (symmetric or asymmetric):

1) **Round asymmetric terminals (RA)** exhibit a variety of sizes between 0.5-4.0 μm and contain numerous round or oval vesicles 350-500 Å in diameter (Fig. 17A). These boutons generally exhibit a clear ground substance and occasionally contain large dense core vesicles. The synaptic cleft is about 150 Å wide, and the postsynaptic membrane is underlain by a prominent postsynaptic density. A variant or subtype of RA terminal possesses a more abundant vesicle population and a 200-300 Å thick layer of postsynaptic dense material, below which postsynaptic
FIGURE 17

ROUND ASYMMETRIC TERMINALS
FIGURE 17

A. A round asymmetric bouton (RA) synapsing on a dendrite of a preganglionic neuron. X 77,000.
B. A variant of RA bouton in which postsynaptic dense bodies (Taxi bodies, indicated by arrows) are associated with the postsynaptic membrane.
dense bodies are situated; 3 to 7 of these bodies can ordinarily be seen in one synapse (Fig. 17B).

2) **Pleomorphic symmetric terminals (PS)** contain flattened synaptic vesicles, 150-300 x 400-600 Å in diameter. The vesicles are less uniform in shape than those of the RA type of terminal, and some rounded vesicles are commonly found among the population of flattened vesicles. These terminals nearly always contain a clear ground substance, associated with a rather narrow (90-130 Å) synaptic cleft, and make symmetrical synaptic contacts in which both pre- and postsynaptic membranes are of similar density (Fig. 18).

3) **Round symmetric terminals (RS)** contain round to oval vesicles of 5000 Å mean diameter and are associated with a 250 Å wide synaptic cleft. The postsynaptic membrane is similar in density to the presynaptic membrane and is not bordered by a postsynaptic density (Fig. 19).

C. RELATIVE DISTRIBUTION OF SYNAPTIC TYPES

To obtain an estimate of the approximate frequency of occurrence of the various synaptic types contacting the cell membranes of preganglionic neurons, high power electronmicrographs of boutons in transverse sections of
FIGURE 18

PLEOMORPHIC SYMMETRIC TERMINAL

.5 \mu
FIGURE 18

A pleomorphic symmetric (PS) bouton synapsing on the soma of a preganglionic neuron. X 58,000.
FIGURE 19

ROUND SYMMETRIC TERMINAL
A round symmetric (RS) bouton synapsing on a dendrite of a preganglionic neuron. X 67,000.
the intermediolateral cell column were used to make random counts of over 940 synaptic knobs. The boutons were pooled according to their type as well as to their location on the cell surface. The relative number of boutons on either the soma or dendrites was expressed as a percentage of the total number of bouton profiles photographed on that particular area of the cell. The relative percentages of given populations are expressed as histograms in Figure 20.

These histograms reveal that on preganglionic cell somas PS terminals comprised 67% of the total bouton population, RA terminals comprised 30% and RS terminals the remaining 3%, based on a total count (N) of 310 boutons. On the other hand, axo-dendritic terminals, specifically on proximal dendrites, were composed of 44% PS terminals, 49% RA terminals and 7% RS terminals.

The percentage of the total bouton population comprised of a given bouton type varied when comparing the somatic distribution with that on the dendrites. Thus, the per cent population of terminals containing round vesicles is greater on the dendrites than on the soma.
FIGURE 20

COMPARATIVE POPULATIONS OF BOUTONS

PERCENT OF BOUTONS

N = 310

N = 636

SOMA  DENDRITE
FIGURE 20

A histogram showing the relative percentages of various types of boutons on the somas and proximal dendrites of preganglionic neurons of the intermedio-lateral cell column. PS: pleomorphic, symmetric boutons; RA: round, asymmetric boutons; RS: round, symmetric boutons; N: number of boutons in sample.
D. DENSITY OF SYNAPTIC COVERING

There are obvious limitations to any attempt to calculate synaptic density on an actual three-dimensional neuron when measurements are made on two-dimensional profiles. However, other workers have deemed it a worthwhile undertaking (Lemkey-Johnston and Larramendi, 1968; Conradi, 1969). The method used here to calculate the total synaptic density on a given profile is similar to that of Conradi (1969).

A rolling map-distance measurer was used for measuring the total membrane length of a given profile. The same instrument was then used to measure the total bouton surface length contacting the given profile. The mean ratio of total bouton length to profile membrane length was taken as a two-dimensional expression of the percentage of the somatic or dendritic surface covered by boutons. Seventy-seven somas and 96 dendrites were used in this analysis. Comparison of synaptic covering was made between soma and dendrites of the preganglionic neurons.

Somal profiles had a mean bouton coverage of 12.3% of the profile surface, with SE of ± 1.86, based on N = 77 profiles. The dendritic profiles had a mean bouton
coverage of 39.6% of the profile surface, with SE of $\pm$ 2.48, based on $N = 96$ profiles. These data show that the percentage of the dendritic profile surface covered by boutons is greater than the percentage of the somatic surface covered by boutons. The non-paired Student's T-test revealed that the increment in synaptic density observed when comparing dendrites with soma is statistically significant ($P < 0.001$).

The question arose as to whether the observed increase in bouton coverage as a function of spatial location could be explained by an increase in bouton size, or whether it was due to an increase in the number of boutons contacting a given profile. To ascertain the former possibility, high magnification photos of all the boutons observed on a given part of the cell were measured with a calibrated scale and the results pooled. The longest diameter through the terminal was taken as its width.

Based on a total sample of 135 boutons contacting the soma, the width of the bouton ranged from 0.5-3.7$\mu$, with a mean value of $1.54\mu \pm 0.057$. The mean value of the bouton width on dendrites was $1.44\mu \pm 0.054$, from 135 samples, and they ranged from 0.5 to 4.5$\mu$ (Fig. 21A). The paired Student's T-test revealed that the difference in
FIGURE 21
MEAN BOUTON WIDTH AND MEAN BOUTON NUMBER

A

WIDTH OF BOUTON (μ)

N=135  N=135

SOMA  DENDRITE

B

NUMBER OF BOUTONS

N=69  N=91

SOMA  DENDRITE
FIGURE 21

A. A histogram showing the range in widths of boutons synapsing on the somas and proximal dendrites of preganglionic neurons. B. A histogram showing the mean number of boutons per profile of the somas and dendrites of preganglionic neurons. N: the number of profiles analyzed.
synaptic bouton width on soma and dendrites is not significantly different (P>0.1). Thus, the increase in bouton coverage as a function of surface location could not be explained on the basis of any increment in bouton size. It, therefore, seemed that the increase in bouton coverage might be due to an increase in mean number of boutons per profile.

This possibility was examined by counting boutons on each profile of soma and dendrites. Micrographs of ninety-one dendrites and 69 somas were studied. The number of boutons on each dendrite varied from 0 to 24, with a mean value of 7.24 ± 0.49 (SE). On the other hand, the average number of boutons on each soma was 2.79 ± 0.37 (SE), with a range of 0-17 (Fig. 20B). The Student's T-test revealed that the increased number of boutons on profiles of dendrites is statistically significant (P<0.001).

The present data show the following points concerning the morphology of sympathetic preganglionic neurons in the cat spinal cord. Most of the cells are round to fusiform in shape and measure 12-24μ in length. Some larger neurons are distinctly multipolar and 25-35μ in length. They all contain a large homogenous nucleus with a prominent nucleolus. The cytoplasm is filled with
organelles: distinct clumps of Nissl substance, free ribosomes, Golgi apparatus, lipid droplets, mitochondria, lysosomes, neurofilaments and neurotubules. Two predominant bouton types (RA and PS), as well as a few of the RS type, terminate on preganglionic somas and dendrites. The PS type of bouton outnumbers the RA type on the soma, but the proportion of the two types is almost equal on dendrites. A very small portion (2.8-6.7%) of the total bouton population is composed of RS type boutons. The surface area covered by synapses is greater on dendrites than on the soma, due to the increase in the number of boutons contacting dendritic profiles.
DISCUSSION

A. AN EVALUATION OF THE RETROGRADE TRANSPORT METHOD OF HRP

Although the horseradish peroxidase technique has been widely utilized for identifying neuronal pathways, several questions should be considered in interpreting the results: 1) the possible uptake of the marker by axons of passage, and, in particular, by fibers injured during the injection procedure, 2) the diffusion of the marker from the injection site, 3) the possibility that the transport phenomenon might be a feature of a restricted class of neurons, and 4) the presence of possible sources of error of identification, such as endogenous peroxidase and melanin pigment.

Concerning the first question, it has been shown in light microscopy that the accumulation of HRP-positive granules is not found in cell bodies of neurons whose axons pass through an injection site (LaVail et al., 1973; Nauta et al., 1974). On the other hand, Holtzman (1971) demonstrated that surfaces of many cells are capable of pinocytosis of extracellular material, and DeVito et al. (1974) found that when the cut ends of axons of the vagal motor neurons are dipped into HRP solution, the marker
accumulates in their cell bodies. Concerning pinocytosis, axonal uptake of HRP by this means has been demonstrated along the length of peripheral nerves, particularly at nodal regions (Krishnan and Singer, 1973), in electron microscopy, although such instances were very rare compared to the pinocytotic activity at terminals and preterminals (LaVail and LaVail, 1974). It seems unlikely that such a rare form of axonal pinocytosis could accumulate enough HRP to interfere with the interpretation of results, particularly at the light microscopic level. But the possibility of HRP uptake from injured axons during the injection procedure can not be completely eliminated. Thus, extreme care was taken during injection by using very small injection needles and making the same number of injections into each ganglion.

From studies involving injections of HRP into the mammalian central nervous system, adequate localization of the HRP at the injection site is seen to be an important factor in making an interpretation of the origin of the transported HRP protein. HRP is originally picked up from an area smaller than that which is seen stained a day or more after the injection (Jones and Leavitt, 1975). However, the effective area of uptake is not limited in all
cases to the tissue surrounding the needle tip (LaVail and LaVail, 1974). Several factors are likely to be important determinants of the effective area of uptake, including the concentration of the injected marker (Kim and Strik, 1976), the local cytoarchitecture at the injection site, the pressure of injection and the survival time (Nauta et al., 1974).

Because the stellate ganglion is encapsulated, diffusion from the injection site is limited. Furthermore, in these experiments the aim was to expose all of the axon terminals in the stellate ganglion to the injected HRP. Histological slides of the injected ganglia, taken 24 hours after injection, demonstrated that the entire ganglion was completely filled with dark brown granules. This suggested that all of the terminals were exposed to the exogenous HRP. To minimize variability, the concentration of HRP was the same in each experiment.

The question of the possible transport of HRP by a restricted class of neurons still remains. In experiments using animals of several species, HRP transport has been demonstrated in a variety of systems such as retinal-tectal, cerebellar-hippocampal and septal afferent fibers. However, Nauta et al. (1974) found that neurons of the neocortex did
not transport HRP as expected after its injection into the caudaputamen, and Stökel et al. (1974) also found lack of transport of HRP in sympathetic axons. These results suggest a specificity of retrograde transport, involving the uptake, actual transport, and possible inactivation within the axon and/or cell body. Specificity of transport was demonstrated by the lack of transport of HRP isoenzyme A and periodateborohydride-treated isoenzyme C in the rat visual system, while demonstrating the transport of isoenzyme B and C in the same system (Bunt et al., 1976). Such specificity may depend on different species, different neuronal systems, the age of animals, or even on the physiological state of the animal. For the present, the reasons for differential transport are unknown but must be kept in mind in using and interpreting the HRP technique.

In the present study it is assumed that discriminative transport of HRP does not occur. In other words, all the preganglionic neurons whose axons were exposed to HRP were assumed to accumulate the HRP in their cytoplasm. The present results demonstrated that the total number of preganglionic projections to the stellate ganglion varies between 6,069 and 9,794 from one animal to the next, with
a mean value of 7,974 \pm 628; this range of variation supports the above assumption.

The final possible source of errors in interpreting results of retrograde transport of HRP is the presence of endogenous peroxidase activity in the central nervous system. The demonstration of the presence of HRP depends on the ability of this enzyme to oxidize diaminobenzidine (DAB) in the presence of hydrogen peroxide (H₂O₂) to form an electron-dense and light-dense precipitate. It has been shown that neurons within certain regions of the brain, such as the dorsal motor nucleus of the vagus and the nucleus tractus solitarius (Keefer and Christ, 1976), and extrapyramidal motor systems and certain cranial nerve nuclei (Wong-Riley, 1976), consistently stain in media of DAB and H₂O₂, even in control animals not injected with HRP. Also, melanin granules can be easily mistaken for retrograde HRP product (Mensah and Finger, 1975).

In the present work this problem was overcome by running control experiments without HRP injection and also by controlling the pH and H₂O₂ concentration of the reaction medium. None of the control animals in this study demonstrated any trace of brown granulations in the spinal cord. Thus, all the brown granulations in neuronal cell
bodies of experimental animals were interpreted as resulting from retrograde transport of exogenous HRP.

The most commonly used protocol for HRP reaction is the method of Graham and Karnovsky (1966). Several experiments have demonstrated that a high concentration of paraformaldehyde in fixative (4% or higher) reduces HRP activity (Straus, 1964; Kim and Strick, 1976; Adams, 1977). Very little effect has been shown by varying the presoak in DAB from 10 to 30 minutes or changing the reaction time in DAB and $\text{H}_2\text{O}_2$, or varying the amount of $\text{H}_2\text{O}_2$ (0.15-0.6%) or DAB (10-20 mg/10 ml) (Kim and Strick, 1976). It is interesting to note that the average number of HRP positive granules found in the cell body and proximal dendrites of individual neurons is consistently the same, regardless of the HRP concentration used in the injections, suggesting that the amount of protein transported per cell reaches saturation levels at a low concentration of HRP (Kim and Strick, 1976).

The velocity of retrograde transport of HRP has been estimated to be at least 84 mm/day in the chick visual system (LaVail and LaVail, 1974) and, at most, 96 mm/day in the rat sympathetic system (Schramm et al., 1975). It has also been shown that HRP accumulated in cell bodies
after retrograde transport disappears within 3 to 4 days (Turner and Harris, 1974) in cortical neurons of rabbit, cat and monkey, and within 6 to 11 days in hypoglossal neurons in mice (Kristensson and Olsson, 1973). Thus, selecting the optimal survival time after injection in the neuronal system in question is also important. After running test experiments with HRP injection into the stellate ganglion, the optimum survival time selected in this study was between 24-36 hours, when the intensity of HRP accumulation and the population of labeled neurons were the greatest.

B. THE DISTRIBUTION OF SYMPATHETIC PREGANGLIONIC NEURONS

The preganglionic neurons of the thoracolumbar spinal cord have been the subject of past investigations, yet no unanimity exists in regard to their location. Onuf and Collins (1898), on the basis of retrograde degeneration studies in the cat, reported that preganglionic fibers arose from paracentral neurons, neurons in the lateral horn, and probably small neurons in the intermediate zone. Poljak (1924), with Golgi preparations, found that the sympathetic motor center of the bat consisted of several cell groups: cells of the lateral horn, ganglion cells of
the central region, ganglion cells in the lateral funiculus, and the cells in the stratum subpiale.

Bok (1928) felt that the retrograde degeneration method was inadequate for unequivocal identification of preganglionic neurons. Using reduced-silver preparations in guinea pig embryos, he identified preganglionic neurons in the lateral horn. Although Bok described the nucleus intermediomedialis just laterodorsal to the central canal and differentiated paracentral and postcentral cell groups within this nucleus, he was not certain that these represented collections of preganglionic neurons. Gagel (1928) was also critical of investigations in which retrograde degeneration had been used to detect preganglionic neurons. He considered the cells of the lateral horn and the intermediate zone as "vegetative" neurons. Among the neurons of the moelle grise vegetative, Laruelle (1937) included the cells of the intermediolateral cell columns, the cellules intercalatus which were inserted in transverse fiber bands that spanned the zona intermedia, and the cells of the paraependymal or intermediointernal column. In a recent investigation with both silver staining and chromatolysis, Petras and Cummings (1972) identified the thoracolumbar
sympathetic preganglionic neurons in the nucleus intermediolateralis thoracolumbalis pars principalis and funicularis (ILp, ILf), the nucleus intercalatus spinalis (IC), the nucleus intercalatus pars paraependymalis (ICpe) and the nucleus intermediomedialis (IM).

The conflict between investigators concerning the locations of sympathetic neurons has resulted partly from the inherent limitations of the techniques which they have applied, involving dependence upon pure speculation in the case of direct histology and difficulty in identification in chromatolysis. In the present study, the HRP-labeled sympathetic preganglionic neurons were clearly identified, contrasted with the unstained neurons on the control side without any uncertainty. Thus, the HRP technique permitted more detailed and complete information about preganglionic neurons in the cat spinal cord than has previously been obtained.

The present investigation confirmed many of the observations of Onuf and Collins (1898), Poljak (1924), Bok (1928), Laruelle (1937) and Petras and Cummings (1972). HRP injection into the stellate ganglia labeled sympathetic preganglionic neurons which project their axons to these ganglia. The majority of HRP-positive neurons (78.2%) were located in the nucleus intermediolateralis. Lesser numbers
were found among the nucleus lateral funicularis (18.5%), while the nucleus intercalatus and the central autonomic nucleus contained only a minor portion (3.3%) of the total population.

The central autonomic neurons identified in this investigation appeared to correspond topographically to the intermediomedial nucleus of Bok (1928), cells of the paraependymal (or intermediointernal) column of Laruelle (1937) and the central autonomic neurons of Cummings (1969). However, it is questionable that these same neurons correspond to the nucleus intermediomedialis as described by other authors such as Rexed (1954), Szentágothai (1966) and Petras and Cummings (1972). The location and composition of the nucleus intermediomedialis (Rexed, 1954; Szentágothai, 1966; Petras and Cummings, 1972) takes on added significance when viewed in conjunction with the results of studies on the terminal distribution of dorsal root fibers. Using Nauta staining, Petras (1966) and Petras and Cummings (1972) reported that following dorsal rhizotomy there was massive fiber degeneration around clusters of small neurons which might correspond to the nucleus intermediomedialis. Similarly, Szentágothai (1966) noted a center of heavy terminal
degeneration corresponding to the nucleus intermedio-medialis. Smith et al. (1968) also demonstrated that the major termination site of the hypothalamo-spinal tract was the IMM.

Furthermore, there is some information in the literature which suggests that intermediomedial neurons may participate in autonomic functions of the spinal cord. Bok (1928) described a tractus intermediolateralis which he believed originated in the nucleus intermediomedialis and then projected laterally to divide near the IML by emitting cranial and caudal branches which ran parallel to the IML. Bok believed that this tract was capable of furnishing a source of afferent fibers to the IML. It is conceivable that IMM axons project through, or are neighbors of, the nucleus intercalatus. Poljak's account (1924) of the zona intermedia would be in accord with this view.

The present anatomical data, along with all the previous results, suggest the following notions. First, the majority of sympathetic preganglionic neurons are located in the nucleus intermediolateralis. A lesser number lie in the nucleus lateral funicularis. The nucleus intercalatus and the central autonomic nucleus provide only a minor portion of the population. Second,
the cells of the nucleus intermediomedialis do not project axons directly to the sympathetic ganglia, but may relay afferent impulses to visceral motor neurons.

The rostral pole of the sympathetic preganglionic cell column in the 15 specimens studied here was observed to be between the caudal portion of C8 and the middle of T1. This finding is in general agreement with the results of previous workers. Laruelle (1937) in the cat and Bok (1928), Gagel (1928) and Laruelle (1937) in man, located the rostral pole in the eighth cervical segment; Rexed (1954) in the cat and Navarathnan and Lewis (1970) in the rat located it in the first thoracic segment. Based on present observations, the exact location of the rostral pole of the sympathetic preganglionic cell column varies with individual animals, even in the same species. The disagreement between groups of authors may be due not only to species differences but also to the lack of samplings within a given species, differences in the definition of the limits of C8 and T1, or difficulties in observing small numbers of neurons scattered throughout the finger-like projections of gray matter in the lower cervical area.

The variability in the number of HRP-positive preganglionic neurons from one cross section of spinal cord to
another was a consistent finding throughout the length of the IML. This uneven longitudinal distribution has been referred to by Laruelle (1937), Rexed (1954) and Cummings (1969) as "beading" or a segmented cell column. The present observation of longitudinal sections confirmed this phenomenon, revealing segmenting of the IML cell column along the long axis of the spinal cord. The neurons are more clustered together in some areas than in others, and this alternation of heavily and lightly clustered cells is repeated several times in any one segmental length of spinal cord. No correlation could be detected between degree of cellular density and location of rootlet outflow from the cord.

Information concerning the segmental distribution of neurons in the sympathetic preganglionic system is useful for experiments in which intracellular or extracellular single unit recordings from preganglionic neurons are attempted, because it gives an indication of the relative probability of encountering preganglionic neurons at the different segmental levels. The present quantitative study shows that each animal has its own distinctive distribution pattern of neurons along the long axis of the spinal cord. The greatest contribution of neurons from a
single spinal cord segment occurs at the second thoracic level (20% of total population), and the least contribution at either the 8th cervical (0.2% of total) or the 8th thoracic (3% of total) level. The majority of the population originates from spinal cord segments T1 to T6. The caudal limit of the preganglionic neurons lies at level T8 in the majority of animals.

The wide range of distribution of preganglionic neurons which innervate specific organs or comprise specific nerves has been documented in earlier works. Onuf and Collins (1898) and Herring (1903) observed chromatolytic neurons in the spinal cord from T1 to T9 and from C8 to T6 following either removal of the stellate ganglia or lesioning of the cervical sympathetic chain of the cat, respectively. Cummings (1969), after ablation of adrenal medulla of mice, traced either chromatolytic or HRP-labeled preganglionic neurons in the ipsilateral spinal cord from levels T4-L1 or T1-L1, respectively. The present observations agree well with the previous work, indicating that the innervation of a single organ by neurons originating from a wide range of spinal cord levels is the general pattern of distribution of preganglionic neurons.
A remarkable agreement between the counts on the two sides of the cord was demonstrated in the present study. Henry and Calaresu (1972) also showed this marked closeness in their counts of total presumptive sympathetic preganglionic neurons in the cat spinal cord. These data demonstrate a symmetrical spinal representation of the sympathetic outflow, suggesting a symmetrical distribution of sympathetic fibers to the visceral organs on both sides. Also, this allows one to use one side of the spinal cord as a control while using the other side as an experiment.

Although Henry and Calaresu (1972) demonstrated a significantly higher number of preganglionic neurons in the male than in the female, no population difference according to sex was observed in the present experiment. Henry and Calaresu claimed that the difference in population may have been due to differences in weight between the two sexes. Even though there is no way of knowing the exact ages of the cats we have used, all the cats were at least older than eight weeks. Considering the early development of the central nervous system and its constancy thereafter, it seems unlikely that the present data were biased by differences in age of the animals. Discrepancies in the literature may result from technical limitations, such as
the purely speculative identification of certain neurons in normal histological sections as being neurons projecting only to the stellate ganglion.

C. ULTRASTRUCTURE OF PREGANGLIONIC NEURONS

Compared to the quantity of work that has been reported on the ultrastructure of spinal motor neurons in mammals, the information specially concerning sympathetic preganglionic neurons is very limited. This is, in part, due to the difficulty of differentiating preganglionic neurons from other neurons.

The only ultrastructural study of preganglionic neurons in mammals was done on neurons in the intermedio-lateral (IML) cell column of the cat spinal cord by Réthelyi (1972). He described IML neurons as elongated and oriented longitudinally, with a somal length of 25–35 μ. He identified preganglionic neurons on the basis of their location, cell arrangement and the path of their axons, which he could trace ventrally along the lateral border of the ventral gray matter and the ventral funiculus. However, such a method of identification was necessarily speculative in part.
The application of the HRP-labeling method has greatly aided in the ultrastructural study of these neurons. HRP-positive neurons are clearly distinguished by the presence of electron-dense material in their cytoplasm; furthermore, the labeled neurons are healthy and normal.

In the present study, the general features of the preganglionic neurons appear to be similar to those described in earlier works (Rethelyi, 1972). Cells have a small to medium sized soma (12-35 μ in diameter), and the cytoplasm contains an abundance of various organelles: Nissl substance, Golgi apparatus, mitochondria, lysosomes, free ribosomes, lipofuscin granules, glycogen particles, neurotubules and neurofilaments. The nucleus has a homogeneous appearance, with one or two prominent nucleoli.

There appear to be two prominent bouton populations terminating on the sympathetic preganglionic neurons: one with round or spherical vesicles, a wide cleft, and a prominent post-synaptic density, and another with pleomorphic vesicles, a narrow cleft, and little or no post-synaptic density. These boutons were termed round-asymmetric (RA) and pleomorphic-symmetric (PS), respectively, after the terminology of Colonnier (1968). He
demonstrated that boutons with round vesicles were almost invariably associated with a wide cleft and an asymmetric membrane thickening, whereas boutons with pleomorphic vesicles were usually associated with a narrow cleft and a symmetrical thickening. The same correlation between vesicle shape and cleft width has been verified by numerous workers, in various areas of the CNS and in a variety of species: in cat spinal cord (Bodian, 1966), and in mouse and cat cerebellum (Lemkey-Johnston & Larramendi, 1968; Uchizono, 1970).

Recently Pfenninger (1973) has confirmed with freeze-etch studies the concept that there are at least two distinct populations of boutons in the CNS of vertebrates. One type of bouton is associated with a rather wide synaptic cleft, a prominent post-synaptic density, and spherical vesicles averaging 480 Å in diameter. The other type of bouton has a narrower synaptic cleft, little or no post-synaptic density and spherical vesicles averaging 390 Å in diameter. However, a question arose as to why terminals with symmetric synaptic clefts contained pleomorphic vesicles in aldehyde-fixed material, but spherical ones in freeze-etched material. The enigma was solved by demonstrating the induction of vesicle
flattening by the high osmotic pressure generated by the aldehyde fixative commonly used to perfuse nervous tissue (Valdivia, 1971). Valdivia found that when aldehyde fixatives with a lower osmotic pressure were used, boutons with symmetric clefts contained predominantly small round vesicles similar to those seen in freeze-etched material. It is thus concluded that the flattened vesicles seen in PS terminals in this material are in reality a by-product of the osmotic pressure produced by the glutaraldehyde fixative, and in reality are spherical vesicles which are smaller than those seen in RA boutons. Similarly, Conradi (1969) and McLaughlin (1972) have seen RA terminals (S-type) which contained round vesicles ranging in diameter from 300-700 Å, as well as PS terminals (F-type) possessing pleomorphic vesicles measuring either 200 x 600 Å (Conradi) or 350 x 650 Å (McLaughlin) associated with a synaptic cleft width of 90-130 Å. A variant of the RA terminal associated with post-junctional, or Taxi, bodies (see Taxi, 1961) was considered by Conradi and McLaughlin to belong to a distinct and separate group (T-type).

Several workers have attempted to correlate the morphology of RA and PS boutons with their physiological functions. In the mammalian cerebellum, the mossy
fiber-granule cell contact and climbing fiber-Purkinje cell contact have been proven to be excitatory, while the basket cell-Purkinje cell synapses and the Golgi cell-granular cell contact were inhibitory (Eccles et al., 1967). Uchizono (1967) demonstrated that excitatory systems are associated with axon terminals possessing round vesicles and an asymmetric cleft, while inhibitory systems possess axon terminals having pleomorphic vesicles and symmetric clefts. Furthermore, Bodian (1966) demonstrated that in the developing neuropil of the monkey spinal cord, synaptic bulbs with round vesicles appeared initially, when the first reflexes could be elicited, and presynaptic terminals with elongate vesicles occurred later, when inhibitory inputs developed. A similar correlation has been shown in the mammalian hippocampus (Gottlieb & Cowan, 1972).

Recent autoradiographical studies have shown that axon terminals with round vesicles concentrate acetylcholine, a putative excitatory neurotransmitter (Marchbanks, 1969), whereas terminals with flattened vesicles concentrate glycine (Matus & Dennison, 1971) or GABA (Hodfelt & Lundahl, 1972), both of which are putative inhibitory transmitters.
Since examples demonstrating the converse situation have not yet been documented, one can speculate tentatively that RA terminals are generally associated with excitation, whereas PS terminals are usually involved in inhibition. One might use this line of evidence, although circumstantial at best, to suggest that in spinal sympathetic pre-ganglionic systems RA terminals are also involved in excitation, whereas PS terminals are mediating inhibition.

RS boutons comprise a small population of boutons on preganglionic neurons. While such a type has not been reported in the majority of classical papers (Bodian, 1966; Colonnier, 1968; Conradi, 1969), Ebner and Colonnier (1975) feel that they exist, and Palay and Chan-Palay (1974) speculate that they are probably mediators of inhibition in the cerebellar cortex.

The present study of terminal distribution on preganglionic neurons demonstrated that PS terminals outnumbered RA terminals on the soma, while the two types were represented almost equally on dendrites. Furthermore, the total coverage was found to be greater on proximal dendrites (39.6%) than on perikarya (12.3%). These findings are consistent with the evidence shown by Conradi (1969) and McLaughlin (1972) on cat spinal motor
neurons. Conradi demonstrated that F-type (PS) boutons comprised 58 per cent of the bouton population on motor neuron somas and 49 percent of the population on dendrites. McLaughlin observed similar patterns of PS distribution (47% on somas and 39% on dendrites) on motor neurons in cat spinal cord. Conradi also showed that 47 percent of the somal surface of cat spinal motor neurons was covered by boutons, and 72 percent of the surface of the dendrites.

If we hold to the argument that boutons with pleomorphic vesicles and symmetric clefts are inhibitory, it seems clear that inhibitory synapses are more concentrated on the cell soma, whereas excitatory synapses are relatively more numerous on dendrites.

These anatomical findings are supported by physiological evidence which has demonstrated that inhibitory synapses become increasingly more effective in decreasing the size of excitatory postsynaptic potentials recorded at the soma than the dendrite of cat motor neurons (Rall, 1967). Burke et al. (1971) have shown that the main inhibitory input to spinal motoneurons (Renshaw cells and Group Ia inhibitory interneurons) synapse solely on the soma and proximal dendrites of these cells.
This anatomical study of the preganglionic components of the sympathetic reflex pathway help to open a new stage in the neurophysiological study of the autonomic nervous system. Clarifying the exact locations of neurons with the HRP technique gives a more confident base for applying physiological methods such as intracellular recording of nerve activity. The differential distribution of preganglionic neurons along different segmental levels of the spinal cord may suggest a differential effect of each spinal segment on effector organs. Demonstration of three different types of boutons and different proportions of these terminals synapsing on the preganglionic neurons also suggests the possibility of at least three different sources of afferent input upon these neurons, as well as differential inputs on specific regions of individual neurons. An understanding of the origin of afferent inputs upon preganglionic neurons awaits further study.
CONCLUSIONS

The data presented in this study have led to the following conclusions regarding the anatomy of spinal sympathetic structures in the cat:

1) The sympathetic preganglionic cell bodies are located in four groups of nuclei in the spinal cord: (a) nucleus intermediolateralis (IML), (b) nucleus lateral funicularis (LF), (c) nucleus intercalatus (IC) and (d) central autonomic nucleus (CA).

2) The majority of neurons projecting to the stellate ganglion (78%) are located in the IML, with the LF providing the next greatest population (19%), and the IC and CA containing only a minor number of cells (3%).

3) The stellate ganglion is innervated by the axons of preganglionic neurons located in a wide range of spinal cord segments (from C8 to T8), with the majority coming from T2 to T5 levels.

4) The longitudinal distribution pattern of preganglionic neurons varies from one animal to the other.

5) The patterns of longitudinal distribution of preganglionic neurons on the left and right sides of a given spinal cord are very similar.
6) A large population of neurons from the mid-thoracic spinal cord project rostrally within the cord, exit through the T1 ventral root, and project to the stellate ganglion via the T1 white ramus. On the other hand, another large population of preganglionic axons, from upper thoracic levels (T1-T3), project either caudally or rostrally within the cord before they exit by the T2 ventral root and join the T2 white ramus, to terminate in the stellate ganglion.

7) The majority of preganglionic neurons are round to fusiform in shape and measure 12-24 μ in length. Some large neurons are distinctly multipolar and 25-35 μ in length.

8) The preganglionic neuron typically contains a large homogeneous nucleus with a prominent nucleolus. The cytoplasm is filled with cytoplasmic organelles: distinct clumps of Nissl substance, free ribosomes, Golgi apparatus, mitochondria, lysosomes, neurotubules and neurofilaments.

9) The somas and dendrites of the preganglionic neurons are contacted chiefly by two bouton types: round asymmetric (RA) boutons and pleomorphic symmetric (PS) boutons, as well as a few of the round symmetric (RS) type.
10) The PS type terminal outnumbers (75%) the RA type (22%) on the soma, but the proportion of the two types of boutons is almost equal on dendrites. The RS terminals comprise a very small portion of the total bouton population: 3% on somas and 7% on dendrites.

11) The surface area covered by synapses is greater on dendrites (39.6%) than on the somas (12.3%) because of the greater number of boutons contacting each dendritic profile.


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