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An immunocytochemical and ultrastructural investigation of canine cardiac ganglia

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AN IMMUNOCYTOCHEMICAL AND ULTRASTRUCTURAL INVESTIGATION OF CANINE CARDIAC GANGLIA

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DEDICATION

To those who loved me the most:

Mom and Dad

and

To those whom I love the most:

Ghada, Hatem, Hamza and Huda

and

To those who educated me the most:

Drs. Faith LaVelle and Robert Wurster

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LIST OF ABBREVIATIONS

Ach	Acetylcholine
AchE	Acetylcholine Esterase
ANOVA	Analysis of Variance
AVN	Atrioventricular Node
BOMB	Bombesin
CNS	Central Nervous System
ChAT	Choline Acetyl Transferase
CGRP	Calcitonin-Gene-Related-Peptide
DBH	Dopamine Beta Hydroxylase
DMN	Dorsal Motor Nucleus
E	Epinephrine
ENK	Enkephaline
HRP	Horse Radish Peroxidase
5-HT	Serotonin
IVC-ILA	Inferior Vena Cava-Inferior Left Atrium
NA	Nucleus Ambiguus
NE	Norepinephrine
NPY	Neuropeptide Y
PAP	Peroxidase-Anti-Peroxidase
PBS	Phosphate Buffer Saline
PNS	Peripheral Nervous System
PVFP	Pulmonary Vein Fat Pad

SAN	Sinoatrial Node
SIF	Small Intensely Fluorescent
SP	Substance P
TBS	Tris Buffer Saline
TH	Tyrosine Hydroxylase
VIP	Vasoactive Intestinal Peptide

CHAPTER I

INTRODUCTION

The heartbeat is considered an electromechanical process that is initiated by the sinoatrial node and regulated by both the sympathetic and parasympathetic limbs of the autonomic nervous system. Recent investigations have revealed that parasympathetic control of the sinoatrial node of the dog heart is mediated through distinct vagal branches that synapse on a group of cardiac neurons located in the pulmonary vein fat pad (PVFP) (Randall and Ardell, 1985). Cardiac ganglia were originally considered as simple parasympathetic relay stations, but this notion has been challenged by recent morphological and physiological investigations that show the cardiac ganglia contain three different, distinct populations of postganglionic parasympathetic neurons as well as sympathetic and afferent neurons (Moravec and Moravec, 1984, 1987, Xi et al., 1991, Yuan et al., 1991). The existence of parasympathetic, sympathetic, and afferent neurons within the intrinsic nerve plexus of the mammalian heart suggests that some regulation of cardiac functions can be achieved without intact connections with the central nervous system.

This dissertation will investigate several questions

regarding the morphology, immunocytochemistry, and ultrastructural organization of the canine cardiac neurons that regulate the sinoatrial node. The dog is used as a model because of the extensive body of anatomical and physiological data regarding the neural control of its cardiac functions. Specific denervation can be achieved because both the pre- and postganglionic pathways to the canine cardiac ganglia have been identified and can be interrupted surgically (Randall et al., 1991) using intrapericardial methods of cardiac denervation described by Randall et al. (1980). Using such well documented surgical procedures will permit accurate total and selective denervation of the canine cardiac neurons that innervate the sinoatrial node. My general hypothesis is that canine cardiac neurons are integrating centers that receive parasympathetic, sympathetic, afferent, and intraganglionic inputs and that these interneuronal connections can modulate cardiac functions even after cardiac denervation. To test this hypothesis several questions must be addressed:

Do cardiac ganglionic neurons receive their preganglionic innervation from both sympathetic and parasympathetic limbs of the autonomic nervous system?

Following total denervation, is there any ultrastructural evidence they are partly innervated by intraganglionic neurons?

Does transganglionic degeneration take place after 10 days

of cardiac denervation?

Are ganglionic neurons purely cholinergic or are some adrenergic and peptidergic?

CHAPTER II

REVIEW OF RELATED LITERATURE

A. Neural regulation of cardiac functions: an overview

Cardiac innervation and autonomic nervous system have been investigated by scientists for several centuries (French 1971; McC.Brooks and Seller 1981). As early as the seventeenth century, Thomas Willis and Benigne Winslow were able to study the gross anatomical features of the autonomic system and mention its involuntary "sympathetic actions". Winslow considered the autonomic ganglia as "little brains." In the eighteenth century, Jacob Johnson studied the autonomic ganglia and called them "reservoirs of nervous powers" (reviewed by McC.Brooks and Seller 1981). Since then, although the autonomic nervous system has been studied in many species, the detailed anatomy and physiology of vagal preganglionic and postganglionic neurons that regulate different cardiac functions still needs further investigation to answer many questions regarding the morphological and physiological nature of these neurons.

Neural control of the heart allows for adjustments of cardiac function to changing physiological conditions. The classic understanding of this control is that it is mediated

through long loop reflexes involving the central nervous system. The ascending part of the loop is formed by afferent neurons conveying information from mechanoreceptors and chemoreceptors located in the heart and in the major blood vessels (review by Armour, 1977). Integration of the incoming afferent information was originally thought to occur exclusively in the "medullary vasomotor center" (Alexander, 1947). More recently, the "center" concept has been replaced by the concept that cardiac functions are controlled by "circuits" involving several interconnected brain structures such as the spinal cord, brain stem, hypothalamus, limbic system, and other forebrain nuclei (Wurster, 1984). These central nervous system circuits exert their control of the heart via the rhythmic activity of both the sympathetic and parasympathetic efferent limbs of the autonomic nervous system. Such direct neural regulation exercises more control than either hormonal effects or intrinsic length-tension relationships (Randall 1974).

1. Sympathetic regulation of the heart:

The preganglionic neurons of the sympathetic innervation to the heart are located in the intermedial spinal gray regions of the upper thoracic segments of the spinal cord with their axons leaving the spinal cord by the second, third, and fourth (and occasionally first and fifth)

thoracic anterior roots (Randall et al., 1957, Wurster, 1977). Via nicotinic cholinergic receptors, these preganglionic axons synapse on the sympathetic ganglion neurons located mainly in the middle cervical ganglia and the cranial medial regions of the stellate ganglia (Armour and Hopkins, 1984).

The sympathetic postganglionic fibers leave these ganglia to course to the heart via different cardiac nerves associated with the vagal and stellate cardiac nerves. These adrenergic fibers most densely innervate the sinoatrial node, atrioventricular node, and atria, with somewhat less dense innervation to the ventricular muscle and coronary blood vessels (Dahlström et al., 1965, Shindler, et al., 1968). Electrical stimulation of sympathetic cardiac efferent fibers induces accelerated heart rate (positive chronotrophy), augmentation of myocardial contractile force (positive inotrophy), and faster atrioventricular and ventricular conduction times (positive dromotrophy). In addition to increased cardiac output and blood pressure, intense sympathetic activity may induce cardiac changes that may result in atrial and ventricular tachycardias and premature ventricular systole (Ueda et al., 1963, Randall, 1974)).

Norepinephrine released from these sympathetic nerves of the heart induces the above functional changes by its interaction with alpha and beta adrenergic receptors.

Several adrenergic receptor types have been identified in the mammalian heart both at the pre- and postsynaptic sites where different receptor subtypes mediate different physiological responses. For example, presynaptic alpha receptors are important for the feed back inhibition of the norepinephrine release from sympathetic terminals, $beta_1$ receptors are predominantly related to the positive inotropic response, while both $beta_1$ and $beta_2$ receptors are related to sympathetic chronotropic responses (review by Wikberg and Lefkowitz, 1984). In dog, the positive chronotropic and dromotropic responses to selective stimulation of sympathetic nerves to sinoatrial node or atrioventricular node can be blocked using selective $beta_1$ and $beta_2$ adrenoceptor blockers (atenolol and ICI 118,551 respectively), indicating that sympathetic regulation of heart rate and conduction is mediated through activation of both $beta_1$ and $beta_2$ receptors located in the sinoatrial node and atrioventricular node (Takei et al., 1992).

In addition to sympathetic norepinephrine release, during the last two decades many neuropeptides, such as neuropeptide Y and galanin, have been reported to co-localize and co-release with the classical transmitters in the sympathetic efferent pathway. These neuropeptides potentially increase the diversity of the neural control of the cardiac functions. These peptides will be discussed later in this review.

As indicated earlier, the postganglionic sympathetic innervation of the heart is considered to be of extracardiac origin, i.e., the cell bodies of the sympathetic postganglionic cardiac fibers are located outside the heart. Recently, this view has been challenged by physiological investigations that demonstrate that in atropinized dogs activation of some of the cardiac ganglion cells with nicotine resulted in a sympathetic-like response, including acceleration of heart rate and increase in ventricular contraction (Butler et al., 1990, Yuan et al., 1991, 1993). These physiological studies suggest that many of the cardiac ganglia neurons may be sympathetic ganglion cells. However, no anatomical data supporting this hypothesis are available; this issue is addressed in this dissertation utilizing immunocytochemistry.

Neural innervation of the coronary blood vessels may modulate coronary blood flow. In particular, sympathetic mediated coronary vasospasm may play an important role in angina that is not caused by excessive metabolic demands (Thomas, et al., 1984). Extrinsic cardiac denervation was used to treat angina caused by refractory coronary spasm (Klark et al., 1977). The occasional failure of extrinsic cardiac denervation to relieve coronary vasospasm and the recurrence of the coronary spasm after denervation were considered signs of sympathetic reinnervation or incomplete denervation. However, intrinsic cardiac innervation by

cardiac ganglionic sympathetic neurons may provide some explanation for the persistence of the coronary spasms after denervation.

2. Parasympathetic regulation of the heart:

In general, preganglionic parasympathetic neurons controlling the heart are mainly located in the medullary dorsal motor nucleus (DMN) and nucleus ambiguus (NA). The preganglionic fibers form nicotinic cholinergic synapses with postganglionic cardiac neurons located in the cardiac ganglia. These ganglia are in close association with the outer surface of the heart, especially on the posterior aspect of the atria and interatrial septum (anatomical details will be discussed later). Activation of the vagus nerve causes heart rate slowing or a negative chronotropic effect. This response has a very short latent period (100-300 ms) and rapidly reaches a steady-state level. Upon cessation of vagal activity, heart rate rapidly increase (Warner and Cox 1962). Parasympathetic activation causes a decreased conduction or a negative dromotropic effect with prolongation of atrioventricular conduction time and the development of various degrees of heart block (Hageman et al., 1975). Vagal activation also causes decreased contractile force which has highly specific regional distributions. Atrial contractility is more sensitive than ventricular contractility to vagal stimulation. These

atrial responses have a more rapid onset and termination compared to the ventricular responses (Randall et al., 1974). In addition, vagal activity suppresses the contractile force at the base of the ventricle more than that of the apex of the ventricle (Randall et al., 1968). These regional differences in response to vagal stimulation may reflect the variability of density of the postganglionic innervation to different parts of the heart. Whether it may also reflect a distinct variability of transmitters and peptides released by different populations of cardiac neurons is not known.

Acetylcholine released from the postganglionic parasympathetic nerve terminals activates the muscarinic receptors located postjunctionally on the myocardial cell membrane, e.g., on the sinoatrial node, atrioventricular node, and atrial myocytes. This activation of muscarinic receptors causes augmentation of an outward K^+ current which results in earlier repolarization of the membrane potential, shortening of action potentials, and reduction of contractility (Watanabe, 1984).

As noted above, the sympathetic and parasympathetic limbs of the autonomic nervous system have antagonistic effects on cardiac functions which may result in complex interactions between these effects. For example, the inotropic effects of sympathetic stimulation on the atrial contractility are determined by the degree of

parasympathetic activity, so that, as the vagal activity increases, the inotropic effects become less (Stuesse et al., 1979). Much of this interaction also occurs at the presynaptic level. For example, in the guinea pig atria, released acetylcholine can inhibit the release of norepinephrine from the sympathetic terminals (Manabe et al., 1991).

B. **Parasympathetic innervation of the heart - a detailed review:**

1. The preganglionic parasympathetic neurons:

The preganglionic neurons that contribute efferent fibers to the vagus nerve are located in several nuclei in the brain stem, including the dorsal motor nucleus (DMN), nucleus ambiguus (NA), nucleus retroambigualis, nucleus dorsomedialis, spinal nucleus of the accessory nerve, and reticular formation. Those that supply the heart are located mainly in DMN and NA (Nosaka et al., 1979, Sugimoto et al., 1979, and Kalia 1981). Geis and co-workers (1981) demonstrated that in cat and dog the preganglionic soma are located in DMN, NA, and the intermediate zone between the two nuclei (IZ). They also found that NA contained 72% of the total preganglionic cardiac neurons, while the DMN and IZ contained 19% and 9%, respectively (Geis et al., 1981). Interestingly, they also reported a different functional role for each nucleus, with the preganglionic neurons in the

DMN controlling ventricular contractility and the preganglionic neurons in the NA regulating heart rate.

Plecha et al. (1988) investigated the anatomical origin of both the right and left vagi using horseradish peroxidase injections into two different cardiac ganglionic locations in the canine heart, the PVFP (pulmonary vein fat pad) and IVC-ILA (inferior vena cava-inferior left atrium). More than 95% of the preganglionic somata that supply the cardiac neurons in the PVFP and IVC-ILA were located in the ventrolateral aspect of the nucleus ambiguus, while the rest were located in the dorsal motor nucleus and intermediate zone. Although cardiac ganglia within PVFP and IVC-ILA control different targets, with PVFP supplying the sinoatrial node and IVC-ILA supplying atrioventricular node, the labelled cells that supply PVFP or IVC-ILA do not fall in two different, distinct location in the brain stem (Plecha et al., 1988). However, whether the some preganglionic neurons supply the cardiac neurons in both of PVFP and IVC-ILA is not known.

The central regulation of the preganglionic cardiac neurons may be achieved by the afferent projections from other central nuclei to the DMN (e.g., from nucleus tractus solitarius, central nucleus of the amygdala, parabrachial nucleus, infralimbic cortex and insular cortex) or to the NA (e.g., A5 catecholamine cell group, central nucleus of amygdala, paraventricular hypothalamic nucleus), although no

specific projection has been directly seen on the cardiac preganglionic neurons (Loewy and McKellar 1980, Loewy, 1981, Hurley et al., 1991). In addition, the preganglionic neurons can be regulated by ascending afferent neurons located bilaterally in the dorsolateral sulcus of the spinal cord (Geis et al., 1981).

2. The anatomical course of the vagal cardiac branches:

The anatomical pathways taken by the vagal cardiac branches from cervical and thoracic vagi to reach their cardiac target vary considerably between different mammals (Armour and Hopkins 1984, Phillips et al. 1986). The extrapericardial anatomical pathways in dog were described for both right vagal cardiac fibers (Mizeres 1955) and left vagal branches (Mizeres 1957). However, the intracardiac pathways of both sympathetic and parasympathetic nerves to the canine heart were first documented by Geis and co-workers (Geis et al., 1973). Using a protocol of anatomical and physiological identification of the individual nerves along with several surgical interruptions of different pathways, Geis and his co-workers (1973) were able to demonstrate the exact pathways of both sympathetic and parasympathetic innervation to the atrial myocardium, sinoatrial node (SAnode) and atrioventricular node (AVnode) regions. The vagal fibers that control the SA nodal region reach the heart along the surface of the superior posterior

right atrium and along the adventitia of the superior vena cava, while vagal branches to the AV node reach their target by running at the junction of inferior vena cava and inferior left atrium (Geis et al. 1973, Randall et al. 1984, Randall and Ardell 1985). The possibility that specific separate vagal branches innervate the SA node or AV node was suggested because selective parasympathectomy of the AV node affected AV conduction without preventing parasympathetic regulation of the SA node (Randall and Ardell 1985 and O'Toole et al., 1986). Similar specific vagal innervation of cardiac ganglia, SA node, and AV node was also reported in a non-human primate (Billman et al., 1989) and in rat (Burkholder et al., 1992). A recent report by Carlson et al. (1992) indicated that a similar selective vagal innervation of cardiac ganglia localized in a fat pad near the sinoatrial node exists in the human too. Stimulation of the vagal fibers situated in that fat pad caused increased atrial cycle length without affecting the conduction of the atrioventricular node (Carlson et al., 1992).

3. Postganglionic neurons:

Vagal control of the heart is mediated through synapses with different groups of atrial and ventricular postganglionic neurons known collectively as the cardiac ganglia. In mammals the cardiac ganglia are mainly seen along the posterior surface of the right atrium, the

interatrial septum and groove, and around the openings of the pulmonary veins, the superior vena cava and the inferior vena cava, (King and Coakley 1952, Shavele and Sosunov 1985, Randall et al., 1986, Wong et al., 1987, Pardini et al., 1987, Billman et al., 1989). The atrial cardiac ganglia are usually located on the epicardium, but in some animals, especially dog, cardiac ganglia situated within the myocardium of the upper part of the right atrium are reported to be quite numerous (King and Coakley 1952). In addition, a few ventricular cardiac ganglia also exist in the mammalian heart; these neurons are multipolar and rarely unipolar and are found scattered within the ventricular subepicardial nerve network and along the coronary plexus (Davies et al., 1952, Smith 1970, Armour and Hopkins 1990).

The assumption that the cardiac ganglia send their axons to the closest cardiac tissue has been challenged by Randall and co-workers (1986, 1987, 1988), who demonstrated the exact postganglionic cardiac ganglia that mediate the parasympathetic effects on the SAN and AVN regions in dog. Using surgical excision or chemical ablation of either the cardiac ganglia located at the pulmonary vein-left atrial junction (PVFP) or the cardiac ganglia located at the junction of inferior vena cava and inferior left atrium (IVC-ILA), they reported that destruction of PVFP cardiac ganglia interrupted both right and left vagal chronotropic effects on SAN only, while destruction of the IVC-ILA

cardiac ganglia prevented the vagal control of the AV nodal region but left vagal control of the SA node intact.

4. The anatomical course of the axons of postganglionic neurons:

Mapping of the parasympathetic postganglionic neuronal pathways has shown that neurons in the PVFP send axons that run adjacent to the sinoatrial node artery along the sulcus terminalis and that there may also be a few more small ganglia scattered along the pathway and embedded in the connective tissue of the sulcus terminalis (Bluemel et al. 1990). An additional pathway for the PVFP cardiac neurons is via axons coursing in a dorsolateral direction to reach the interatrial septum and from there to the SAN (Randall et al. 1991).

Throughout these anatomical and physiological studies, the fundamental conclusion drawn by the investigators was that selective pathways and functions were evident for different groups of cardiac ganglia. However, the existence of selective pathways does not answer another pertinent question: To what extent may the cardiac ganglia influence their targets without vagal activation? A logical approach to investigate this question is to study the morphological characteristics of cardiac neurons with and without the preganglionic innervation to determine how dependent are these neurons on their vagal input.

C. Morphology of the cardiac ganglia:

It is generally accepted that knowing the morphological characteristics of a particular type neuron such as its size, number of dendrites, number of synaptic contacts, and location of synaptic contacts will help to explain the known physiological characteristics of this type of neuron and will help predict how this neuron will behave under certain physiological or pathological conditions. The following pages will summarize what is currently known about the morphological features of principal cardiac neurons and SIF cells.

1. Morphological feature under light microscopy:

Cardiac ganglia vary in shape and size, some consisting of only two or three neurons while others have several hundred cells. They commonly are located on the course of a nerve trunk or as isolated ganglia connected with several small nerve fibers to a large nerve trunk (King and Coakley 1952, Davies et al 1952, Xi et al. 1991, Pardini et al. 1987, Mick et al 1992, Burkholder et al 1992). King and Coakley (1952) noted that most cardiac ganglia in all species examined contained different sizes of neurons, with the majority being multipolar and only a few unipolar. They also noticed that most cardiac ganglia contained both myelinated and unmyelinated fibers and some of the ganglionic neurons had myelinated axons. In agreement, Xi et al. (1991) have shown

that the canine cardiac ganglia contain neurons of different sizes and different dendritic complexities that may possess two to twelve primary dendrites.

2. Morphological features under electron microscopy:

a. Ultrastructure of the principal neurons:

The ultrastructure of the innervation of the normal mammalian heart has been the subject of many investigations (Yamauchi, 1973; Kaye, 1984; Moravec and Moravec, 1987). Other animal groups investigated include frog (Sargent and Pang, 1989), portunid crab (Mirolli et al., 1987), amphibian (Kumar, 1974), chick (Kirby et al., 1980) and turtle (Yamauchi, et al. 1975). However, most of the ultrastructural investigations of cardiac ganglia have been of pure descriptive nature, and no quantitative studies have been devoted to the synaptic organization of these neurons. In general, the principal ganglionic neurons vary in size, but most fall within the range of 30-45 μm in diameter, with one nucleus and one or two nucleoli. Ultrastructurally, the cytoplasm of the ganglionic neurons is typical of an active neuron, with unevenly distributed granular endoplasmic reticulum and occasional Nissl bodies. Mitochondria with both circular and elongated profiles are seen in the soma and in the terminals. The nucleus is located either centrally or peripherally and contains one or two prominent nucleoli. Satellite cells have a dark nucleus and little

cytoplasm and usually surround the ganglionic neurons.

b. Ultrastructure of intraganglionic contacts:

Ganglionic neurons have both synaptic and non-synaptic contacts. The presynaptic elements of the autonomic ganglia arise mainly from extraganglionic sources. Two types of synaptic boutons make contacts with the somas or dendrites of the principal ganglionic neurons: boutons with clear vesicles and boutons with both clear vesicles and large, dense-cored vesicles (Shvalev and Sosunov, 1985, Papka, 1974, 1976, Wong et al., 1987). Small, dense-cored vesicles, which are characteristic of sympathetic innervation, were reported by Shvalev and Sosunov (1985) in rat, rabbit and guinea pig. The ultrastructure of canine cardiac ganglia has not been previously investigated.

Intraganglionic contacts were first seen in superior cervical sympathetic ganglia in cat (Elfvin et al. 1963), but Kondo and co-workers were the first to suggest the existence of dendro-dendritic and dendro-somatic synaptic contacts between the principal ganglionic neurons of mammalian sympathetic ganglia (Kondo et al. 1980). Non-synaptic attachments have been seen between different neuronal profiles, especially dendrites, spines, terminals and rarely somas (Matthews 1983). The prevalence of such intraganglionic contacts was further investigated by using ultrastructural preparations of principal sympathetic

postganglionic neurons injected with HRP; about 87% of the labelled neurons had dendro-dendritic contacts, but none formed classical synaptic contacts. Rather, they formed simple apposition or dense attachment plaques with dense symmetrical densities on the intracellular sides of the involved membranes (Kiraly et al. 1989). Because of the high incidence of these contacts, Kiraly et al. (1989) suggested that such contacts may be involved in interactions between neurons related to slow potentials or to influencing the metabolic activity of the cells. The attachment plaques were considered to be important for the mechanical support of the neuronal connections, especially since they are commonly seen in close relation to the synaptic contacts (Matthews, 1983).

In addition to the intraganglionic contacts between the principal neurons, Elfvin and Forsman (1978) reported the existence of gap junctions and tight junctions between the satellite cells in the sympathetic ganglia of guinea pig and rabbit.

The source of the ganglionic boutons and synapses could be elucidated by cutting preganglionic input to the ganglia and seeing whether the boutons would persist. The preganglionic denervation of the superior cervical ganglia in cat leads to loss of all synapses in the ganglia (Raisman et al 1974). However, there are also a few myelinated sensory fibers crossing through the ganglia, and usually

running with the preganglionic fibers. These were cut during the preganglionic denervation, making it difficult to determine whether they had collaterals and terminals within the ganglia (Matthews 1983).

c. The small, intensely fluorescent (SIF) cells in the cardiac ganglia:

Collections of chromaffin cells are commonly found within nerves and ganglia of the autonomic nervous system. Different names have been given to these cells: chromaffin cells, interneurons, and small intensely fluorescent cells (reviewed by Taxi et al., 1983). Grillo et al. (1972) used the term "small granule-containing cells" because of the dense-cored vesicles which characterize these cells. Matthews and Raisman (1969) studied the ultrastructure of SIF cells in superior cervical ganglion of rat. They reported that these cells tend to be arranged in clusters and have a high ratio of nucleus to cytoplasmic volume. In the heart, SIF cells have been seen within the atrial ganglion of rat, cat, guinea pig and mouse (Jacobowitz, 1967), as well as in the interatrial septum near the AV node in guinea-pig (Ellison and Hibbs, 1976) and rabbit (Papka, 1974, 1976). The number of SIF cells varies according to the species and type of ganglia. For example, there are more SIF cells in the abdominal ganglia than the stellate ganglia of the rat, and very few SIF cells in the stellate

ganglia of the dog. When different species were compared, the highest number of cells was seen in the rat, where there were 323 cells per 1 mg of wet ganglia, as compared with only five cells per mg in the cow, four per mg in the cat and two per mg in the monkey (Williams et al., 1976).

Morphologically, two types of SIF cells can be distinguished: Type I cells, which tend to be in clusters, show no branches, and mainly associate with the intraganglionic blood vessels; and Type II cells, which are usually scattered between the principle ganglionic neurons and typically have 1 or 2 processes of up to 100 μm in length (Jacobowitz 1967, Ellison and Hibbs 1976). Both types contain the characteristic dense-cored vesicles which range in size from 100-250 nm in diameter and usually lie peripherally beneath the plasma membrane of the cell. The dense core vesicles are variable in shape, position and density. The functional role of SIF cells is thought to be either interneuronal or endocrine. As interneurons, they may have an inhibitory feed-forward action on the principal neurons following preganglionic stimulation (Libet,1970, Yamauchi 1975, Papka 1976). As endocrine cells, Type II cells can affect other neurons by diffusion or by influencing the fenestrated blood vessels lying next to them (Matthews and Raisman 1969, Mekhail et al., 1990).

D. Nature of the parasympathetic ganglia and their preganglionic innervation:

Originally, the cardiac ganglia were assumed to be wholly parasympathetic due to their cholinergic nature and their location in the parasympathetic pathways. The cholinergic nature of the cardiac ganglia begins at different embryonic stages in different mammals (Navaratnam 1965). In monkey all cardiac neurons stain positive for acetylcholinesterase, and no adrenergic terminals are seen within the ganglia, even though many positive adrenergic neurons were seen in the myocardium (Osborne and Silva 1970). Similar observations regarding the absence of fluorescent adrenergic principal neurons or adrenergic terminals within the cardiac ganglia of rabbit, guinea pig, and dog were consistently reported for many years (Angelakos et al,1963; Dahlstrom et al 1965; Ehinger et al 1968 and Malor et al 1974). For example, Hirsch et al. (1963) described a large group of ganglionic neurons in the interauricular portion of the septum in dog and man, assumed that they were purely parasympathetic, and referred to all nerves associated with the ganglion as being vagal fibers. In addition, Kent et al. (1974) agreed with the notion of the purely parasympathetic nature of cardiac ganglionic neurons of the human and dog, and Navartnam et al. (1968) studied the effect of vagotomy on the cholinesterase content of cardiac ganglia of the rat and referred to all cells as

vagal postganglionic neurons. In agreement, Saito et al. (1988) called the cardiac ganglia of the rat "cardiac vagus ganglia." In a recent review of the morphology of synapses in the autonomic nervous system, Smolen (1988) considers all preganglionic terminals to parasympathetic ganglia of mammals to be of the cholinergic type and all postganglionic neurons to be of one cholinergic type, with no known interneurons.

However, Jacobwitz (1967) reported that very fine adrenergic fibers were seen in close contact with cholinergic neurons in the cardiac ganglia of rats, cats, guinea pigs and mice, and that some of the principal cardiac neurons of rat and guinea pig showed some monoamine fluorescence after pretreatment with both the monoamine oxidase inhibitor tranylcypromine and a precursor of catecholamines (3,4-dihydroxy-phenylalanine). In addition, recent studies by Moravec and Moravec (1984, 1987, 1989, 1990) have shown that some of the cardiac ganglionic neurons of the rat, especially those located at the atrioventricular junction, are adrenergic in nature. Moravec and Moravec (1987) demonstrated that this same group of adrenergic neurons stain positive for neuropeptide tyrosine (NPY) and tyrosine hydroxylase (TH) and project peripherally to the atrioventricular node where they form sensory capsule-like bodies. They proposed that some of the intracardiac neurons may have a sensory function and that the adrenergic neurons

may be involved in a proprioceptive feedback loop in the heart similar to that in the gut.

At the ultrastructural level, Papka (1976) reported that cholinergic terminals make axosomatic synapses while adrenergic terminals make axodendritic synapses on the principal ganglionic neurons of the cardiac ganglia of the rabbit (Papka 1976). In addition, cardiac ganglia of rat, rabbit, and guinea-pig receive cholinergic, adrenergic, and afferent synaptic contacts, with the adrenergic synapses seen only on the dendrites (Shvalev and Sosunov 1985).

Physiological evidence from Yuan et al. (1991, 1993) has suggested that the pulmonary vein fat pad contains not only parasympathetic postganglionic neurons but also some sympathetic and some afferent neurons as well. Xi et al. (1991), using intracellular injection of HRP in cardiac ganglia of dog, reported that almost half of the injected neurons did not have distinguishable axons and suggested that they may be intraganglionically active interneurons. They also showed that the ganglia contained three types of neurons with different morphological and physiological characteristics.

E. Morphological and functional subpopulations in autonomic ganglia:

Electrophysiological studies on cardiac neurons in dog

(Xi et al., 1991) and rat (Selyanko, 1992) have indicated that there are several distinct types of neurons.

Xi et al. (1991) reported that canine cardiac neurons consist of heterogeneous types of multipolar neurons, divided electrophysiologically and morphologically into three functional groups: (S) single firing, (R) repetitive firing, and (N) non-firing neurons. These investigators also suggested that there are a large number of intraganglionic interneurons in addition to the classical, small, intensely fluorescent cells. Because of the small number of cells in these electrophysiological studies, they were unable to conclude whether the canine cardiac neurons are morphologically one population with large variations in somal area and dendritic complexity or whether these are really three distinct morphological and physiological subpopulations.

Other autonomic ganglia, such as superior cervical ganglia, have been shown to have three morphologically distinct neurons with different dendritic arborization patterns (Kiraly et al., 1989).

F. Transneuronal degeneration:

The dendritic complexity of neurons has been shown to be related to both preganglionic and postganglionic innervation. For example, the dendritic configuration and number of dendrites of submandibular parasympathetic

ganglionic neurons are correlated with the size of the target (Snider, 1987). Also, Purves and Hume (1981) reported that dendritic complexity correlates with, and determines, the number of axonal inputs to the nerve cells in the rabbit ciliary ganglion. This relationship between the number of dendrites of parasympathetic neurons and number of axons innervating them persists after preganglionic nerve regeneration (Hadley, 1990). The role of preganglionic fibers in determining the overall morphological characteristics of canine cardiac neurons has not been tested.

Transneuronal degeneration due to denervation is more conspicuous in the central nervous system than the peripheral nervous system. Among the signs of such trans-synaptic degeneration are shrinkage of the nucleus, increased electron density of cytoplasm, swollen mitochondria, dilated rough and smooth endoplasmic reticulum, darkened dendrites, and an increased amount of free ribosomes (Knyhár-Cisillik et al.,1989). The extent of these changes depends on several factors such as age, species, size of the neuron and extent of denervation (Matthews et al.,1960, Wiesel and Hubel, 1963, Knyhár-Cisillik et al.,1989).

One-eye enucleation of adult monkeys caused shrinkage of the neurons of the lateral geniculate bodies in two stages. The early phase occurred in the first week, and the

later phase started in the second month and extended to the end of the fourth month. However, the shrinkage of the neurons was not accompanied by cell loss (Matthews et al.,1960).

Even sensory deprivation without direct damage to presynaptic fibers has been reported to cause transneuronal changes. In kittens visual deprivation for a 3 month period by unilateral eyelid suturing caused a 40% shrinkage of neuronal cytoplasm in the dorsal layers and a 25% shrinkage in neurons of the ventral layers of the lateral geniculate body supplied by that eye. These histological changes were less apparent in older kittens and were not seen in adult cats (Wiesel and Hubel, 1963). In rabbit visual cortex only the denervated part of the single neuron will lose its dendritic spines (Globus and Scheibel, 1966).

Similar transneuronal degeneration of soma and dendrites was seen in the neurons of olfactory bulb after removal of nasal mucosa (Pinching and Powell, 1971).

In spinal cord severing of the sciatic nerve causes second order transneuronal degeneration in 10% of neurons in the substantia gelatinosa after 30 to 90 days (Knyihár-Cisillik et al., 1989).

The effect of preganglionic denervation on autonomic ganglia has not had the same degree of study, and the reports are not conclusive. For example, the human parasympathetic ganglionic neurons in the heart and lungs

survived after preganglionic denervation with no apparent morphological changes (Rowan and Billingham, 1989, Springall et al., 1990). However, significant neurochemical changes in phenotype from typical cholinergic parasympathetic neurons to tyrosine hydroxylase and neuropeptide Y positive neurons have been seen in respiratory tract ganglia after heart-lung denervation (Springall et al.,1990). The studies of cardiac ganglia of humans were done at the light microscopy level, and it is possible that some changes were missed. In addition, ultrastructural studies on denervated cardiac ganglia of non-human primates showed early widespread glycogen accumulation in ganglionic neurons followed by intense darkening of the dendrites in 10% of the principal ganglionic neurons, indicating the occurrence of some transneuronal degeneration in cardiac ganglia (Tay et al., 1984; Wong et al., 1987).

The mechanisms for transneuronal degeneration are not clear. However, several causes have been suggested, such as depression of nucleic acid metabolism (Cook et al., 1951), dependence of these neurons on incoming trophic factors, indirect cellular reactions to degenerating terminals (Tay et al., 1984), and voltage dependent synaptic activity (Benshalom, 1989).

The possibility of transneuronal degeneration in canine cardiac ganglia and the rate of survival of these neurons without CNS connections has not been investigated.

Also, it is not known whether such cardiac neurons will maintain their normal phenotypic properties or can function in a way that can still affect the cardiac functions of denervated heart.

G. **The immunocytochemistry of cardiac ganglia:**

Recent studies have shown that cardiac ganglia may contain sympathetic and afferent neurons in addition to the classic parasympathetic neurons (GU et al., 1984; Moravec and Moravec, 1984, 1987, 1989, 1990) and that they receive sympathetic, afferent, and parasympathetic innervation (Parsons and Neel, 1987, Parsons et al., 1987). For example, in the rat some neurons appeared adrenergic because they stained positively for tyrosine hydroxylase (TH), dopamine beta hydroxylase (DBH), and neuropeptide Y (NPY). Other cardiac neurons appeared to be sensory neurons because they formed sensory, capsule-like bodies in the atrioventricular node (Gu et al., 1984; Moravec and Moravec, 1984, 1987, 1989, 1990). A similar pattern was found in the mudpuppy where the cardiac ganglia receive preganglionic innervation from both parasympathetic and sympathetic fibers (Neel and Parsons, 1986), as well as presumably sensory fibers with substance P-like (SP) immunoreactivity (Parsons et al., 1987). Likewise, cardiac ganglia in the guinea pig contain fibers immunoreactive to both SP and vasoactive intestinal peptide (VIP); in addition some ganglionic somas

stain positive for VIP (Della et al., 1983; Papka and Urban, 1987). In human heart the cardiac ganglionic neurons that stain positive for acetylcholine esterase ((AchE) do not stain positive for NPY, calcitonin-gene-related-peptide (CGRP), SP, or TH, although positively stained fibers for the latter peptides are regularly seen around the somas (Wharton et al., 1990).

The immunocytochemical characteristics of the canine cardiac ganglia have not been extensively investigated, and the few reports about their cytochemistry conflict with physiological results. For example, recent work by Anderson et al. (1992) shows that, although VIP positive fibers exist in the heart after denervation, there is a 98% decrease in the NE levels in the canine heart, indicating that these neurons are not adrenergic in nature. In agreement, physiological studies have shown that cardiac ganglia that survive cardiac denervation function in a way similar to parasympathetic neurons, i.e., they decrease AV nodal conduction, heart rate and atrial contractility (Priola D.V. 1980). Also, intracellular electrophysiological studies have shown that all EPSPs from orthodromic stimulation could be blocked by hexamethonium, a nicotinic ganglionic blocker, which suggests that canine cardiac neurons are parasympathetic in nature (Xi et al 1991; Xi-Moy et al.1993). On the other hand, there is physiological evidence that suggests that canine cardiac neurons contain

not only parasympathetic neurons but also sympathetic and some afferent neurons as well (Yuan et al.,1991, 1993).

The question of the adrenergic and cholinergic nature of the parasympathetic ganglionic neurons is further complicated because the presence of both TH and DBH enzymes does not prove that these neurons are adrenergic in nature. To prove the adrenergic nature of neurons of autonomic ganglia more studies are required to demonstrate their ability to synthesize, store, and takeup catecholamine. The dual cholinergic and adrenergic staining has been seen in the ciliary ganglia of rat, mouse and guinea-pig where the postganglionic neurons stain positive for ChAt, TH, and DBH but lack the ability to synthesize and store catecholamines, as shown by the absence of catecholamine fluorescence and the absence of any of the characteristic sympathetic small dense-core vesicles in their terminals (Landis et al.,1987). Similar results have been seen in rat submandibular ganglia, where large amounts of DBH enzyme were seen but no detectable amount of NE and E could be detected, and none of the cells showed catecholamine fluorescence when glyoxylic acid was used (Grzanna and Coyle 1978).

In addition, peripheral autonomic neurons are able to change their phenotype in culture. Depending on the type of growth media, they become cholinergic, adrenergic or express both characters (Landis S. 1976, 1980 ,Potter et al., 1986). In vivo, the adrenergic neurons of superior cervical

ganglion (SCG) undergo change from adrenergic to cholinergic phenotype at the age of 21 days, but they retain the ability for uptake and storage of catecholamine (Landis and Keefe 1983).

In conclusion, this review has demonstrated that there exists a paucity of information concerning the canine cardiac ganglion and that no clear conclusion can be drawn from the existing literature regarding the nature of preganglionic innervation and the nature of its principal ganglionic neurons. Thus, certain questions should be addressed concerning canine cardaic ganglia:

1. Are these neurons sympathetic or parasympathetic?
2. Will these neurons degenerate after preganglionic denervation?
3. What is the synaptic organization of these neurons?

CHAPTER III

IMMUNOCYTOCHEMISTRY OF NORMAL CANINE CARDIAC GANGLIA

ABSTRACT

This study was designed to test the hypothesis that canine cardiac ganglia located in the PVFP contain heterogenous populations of neurons that express different neurotransmitters and neuropeptides. The results indicated that canine cardiac ganglia contain neurons express the cholinergic synthesizing enzyme ChAT. In addition, after colchicine pretreatment the principal neurons were found to also express adrenergic (TH, DBH) enzymes as well as a variety of different neuropeptides, including NPY, SP, VIP, and galanin in different intensities. Galanin and TH were the most intensely expressed. SIF cells were sparse and stained positively for TH, VIP, 5-HT and galanin. In addition, a few fibers stained positively for TH, galanin, and NPY, and some terminal-like structures positively stained for SP were seen in the ganglia. Colchicine was necessary for the detection of all the mentioned enzymes and peptides except ChAT, which did not require any pretreatment, and 5-HT, which was only detected in SIF cells even after colchicine pretreatment.

Introduction

Vagal control of the heart is mediated through synapses with different groups of atrial and ventricular postganglionic neurons known collectively as the cardiac ganglia. In mammals these ganglia are mainly found along the posterior surface of the right atrium, the interatrial septum and groove, and around the openings of pulmonary veins, superior vena cava and inferior vena cava, (King and Coakley 1952, Shavele and Sosunov 1985, Randall et al., 1986, Wong et al. 1987, Pardini et al. 1987 and Billman et al. 1989). Although the cardiac ganglia were long known to exist, it was only during the last decade that Randall and co-workers (1986) identified in the dog two different postganglionic cardiac ganglia that mediate the parasympathetic effects on the sinoatrial node (SAN) and atrioventricular node (AVN) regions.

The anatomical and physiological investigations of cardiac ganglia of frog have shown that the ganglionic neurons are unipolar in shape and that each neuron receives innervation from one vagal axon and that no integration is done at the ganglionic neurons (Dennis et al. 1971). So, the cardiac ganglia have been assumed to be simple parasympathetic relays. However, in recent years autonomic ganglia have also been shown to express anatomically and respond physiologically to many neuropeptides in addition to

the classical adrenergic or cholinergic neurotransmitters (reviewed by Schultzberg and Lindh, 1988). Recent studies have suggested that some of the cardiac ganglionic neurons of the rat might be adrenergic while others might be sensory neurons (Gu et al., 1984; Moravec and Moravec, 1984, 1987, 1989, 1990). A similar organization in which the cardiac ganglia receive preganglionic innervation from both parasympathetic and sympathetic fibers is found in the mudpuppy (Parsons and Neel, 1986) and in the guinea pig (Della et al., 1983; Papka and Urban, 1987). In the human heart the cardiac ganglionic neurons that stain positive for acetylcholine esterase (AchE) do not stain positive for neuropeptide Y (NPY), calcitonin-gene-related-peptide (CGRP), substance P (SP) or tyrosine hydroxylase (TH), although positively stained fibers for these peptides are regularly seen around the somas (Wharton et al., 1990).

In the dog the cardiac ganglia located in the pulmonary vein fat pad (PVFP) play an important role in the parasympathetic control of heart rate because they are the primary relay for vagal control of the SAN (Randall and Ardell 1985 and O'Toole et al. 1986). However, some physiological evidence suggests that PVFP contains not only parasympathetic postganglionic neurons but also sympathetic and some afferent neurons as well (Yuan et al., 1991, 1993). In addition, canine cardiac ganglia have been proposed to be modulated by sympathetic, parasympathetic and afferent

terminals (Gagliardi et al.1988; Armour and Hopkins 1990; Ardell et al. 1991). Three functional types of PVFP ganglionic relay neurons have been described in canine cardiac ganglia: single-firing, repetitive- firing, and non-firing cells; some intraganglionic neurons have also been suggested to exist (Xi et al., 1991).

The purpose of this study is to test the hypothesis that canine cardiac ganglia located in the PVFP contain heterogenous populations of neurons that express different neurotransmitters and neuropeptides.

Materials and Methods

The neurotransmitters and peptides studied included the following: choline acetyl transferase (ChAT), tyrosine hydroxylase (TH), dopamine-ß-hydroxylase (DBH), neuropeptide Y (NPY), substance P (SP), vasoactive intestinal peptide (VIP), serotonin (5-HT) and galanin.

Methods for localizing ChAT-positive somata and dendrites:

Four dogs of either sex were anesthetized with sodium pentobarbital (30 mg/kg i.v.) and placed on positive pressure ventilation. Following neuromuscular blockade (succinylcholine 1 mg i.v.), right thoracotomy in the fifth intercostal space was performed. The pericardium was opened, and the PVFP at the entry of the right pulmonary vein into the atrium was rapidly excised. Immediately, 2-3

cc of fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3) were injected slowly into the fat pad to ensure prompt fixation of the ganglia. The PVFP was immersion fixed for another 18 hours at 4°C, and then the ganglia were dissected out of the fat pad and placed in 30% sucrose in phosphate buffer for 24 hours at 4°C for cryoprotection. These ganglia were rapidly frozen and cut at 20 μm thickness using a Leitz 1720 Cryostat at -20°C. Sections were collected on polylysine coated slides and left overnight to air dry.

Sections were incubated with mouse anti-ChAT monocholonal antibody (Chemicon International) in a concentration of 1:250 for 2 hours at room temperature followed by 18 hours at 4°C. Sections were washed in cold Tris buffer saline (TBS) and then incubated with secondary antibody rabbit anti-mouse IgG (Chemicon International) for 2 hours at a concentration of 1:100. After another rinse in cold TBS, the sections were incubated with 50 μg/ml mouse peroxidase-anti-peroxidase (PAP) complex (Chemicon International) for 1 hour at room temperature. Sections were placed in 0.06% 3.3-diaminobenzidine-4 HCL (from HACH), diluted in Tris buffer, and reacted with a few drops of 0.012% H_2O_2. Sections were rinsed in Phosphate buffer saline (PBS), counterstained with 1% Pyronin Y for better visualization, and coverslipped with Depex (BDH Chemicals).

Methods for TH, DBH, 5HT, SP, VIP, NPY and Galanin:

For these antisera, under sterile conditions 500 μl of colchicine (Sigma, dilution of 20 mg/ml) was injected directly in the PVFP in 4 anesthetized dogs. Following a right thoracotomy (fifth intercostal space) and opening of the pericardium, the colchicine injection was performed slowly over a 3 minute period. After surgical closure of the thorax, the animals were allowed to survive for 24 hours. Then the dogs were reanesthetized, and PVFP were excised. The PVFP were processed as in ChAT methods, and 20 μm sections were collected on polylysine coated slides. After colchicine treatment of the dogs, the following primary antisera were incubated for 24 hours: TH (Pel-Freeze Biologicals), DBH, SP, VIP (INCSTAR), 5HT (Biogenex), Galanin, and NPY (Peninsula Inc.) The antisera were diluted 1:250 (SP, VIP, DBH), 1:500 (5HT, NPY), 1:100 (TH) and 1:1000 (Galanin) in a solution formed of PBS, 0.2% Triton X-100 and 1% normal donkey serum. Sections were first incubated with the primary antisera for 18-24 hours. Then the sections were washed in PBS for 30 minutes, followed by incubation for one hour with the secondary, biotinylated donkey anti-rabbit antibody (Immuno Research) diluted 1:500 in the same carrier as the primary antisera. After another rinse in PBS for 30 minutes, sections were incubated with streptavidin-horseradish peroxidase conjugate (Gibco Brl) for one hour and then rinsed in 0.1 M phosphate

buffer (pH 7.4) for one hour. Sections were conditioned in 0.1 M acetate buffer (pH 6.0) for 10 minutes before a nickel intensified chromagen reaction was started. In this chromagen reaction, sections were incubated in 3.3-diaminobenzidine-HCL (from HACH) 0.05% W/V in acetate buffer containing 2.5% nickel ammonium sulfate. Hydrogen peroxide (0.003% V/V) was slowly added while observing the color of the sections. When the color appeared slightly brown, the reaction was stopped by rinsing the slides in acetate buffer. Then the sections were counterstained with 1% pyronin Y, dehydrated and coverslipped.

Immunocytochemistry controls:

To control for antisera specificity, in some experiments sections were preincubated for 24 hours in 1 ml of the diluted antisera (NPY, VIP, SP) with 50 μg of the respective neuropeptide (Peninsula NYC). The control sections were processed at the same time as the experimental slides to test whether the stain would be eliminated or not. In other experiments antisera specificity was tested by omitting the primary antibodies. As both positive and negative controls for the ChAT, sections of the stellate ganglia of the dogs were used and were processed along with the cardiac ganglia. All experimental procedures were conducted in strict accordance with the standards established by the American Physiological Society for the care and use of laboratory animals.

Data Analysis:

A total of 8 dogs were used in this study. Four dogs were treated with colchicine and samples from 2 animals were used for each antibodies. The 4 non-colchicine treated dogs were used for ChAT antibodies. Sections were microscopically examined and the labelling was quantified in the following manner: for each antiserum, the total number of cell body profiles in every section were counted on one slide per animal, each slide contained 7-13 ± 3 non-consecutive sections and the total sum of all profiles represented the total number of cells; the sum of the totals for all animals for that stain was entered as (total) in Table 1. Any profile that had some staining was considered a positive cell, while negative cells were the profiles which were devoid of any trace of nickel staining. Percentages of positively stained profiles were calculated for each animal, and the means of percentages for 4 dogs (ChAT) or 2 dogs (all other staining) were calculated and entered as % positive in Table 1.

Results

The present study confirmed that canine cardiac ganglia contain neurons express the cholinergic synthesizing enzyme ChAT. In addition, after colchicine pretreatment the principal neurons were found to also express adrenergic (TH, DBH) enzymes as well as a variety of different neuropeptides, including NPY, SP, VIP, and galanin in different intensities. Galanin and TH were the most intensely expressed. The number of smaller neurons that resembled SIF cells was sparse, but these cells stained positively for TH, VIP, 5-HT and galanin. In addition, a few fibers stained positively for TH, galanin, and NPY, and some terminal-like structures positively stained for SP were seen in the ganglia. Colchicine was necessary for the detection of all the mentioned enzymes and peptides except ChAT, which did not require any pretreatment, and 5-HT, which was only detected in SIF cells even after colchicine pretreatment.

Cholinergic enzymes (ChAT)

Virtually all principal ganglionic neurons (99.3%) showed positive immunoreactivity for ChAT (Fig. 1 A). On rare occasions (less than 1%) cells were seen that lacked any detectable amount of enzyme (Table 1). In general, cells were evenly stained, although some of the peripherally located somas were stained more heavily. No positively

stained fibers or terminals were seen because the method used was specific for ChAT-positive somas and dendrites only.

Monoaminergic enzymes

A) TH

All cardiac ganglia presented TH-positive fiber staining located either in between the cell bodies or in the nerves associated with the ganglia (Fig. 1C). The immunoreactivity of the somas ranged from intense reactions to no staining at all (Fig. 1B). In one dog almost all the cells (96%) were positive, with occasional varicosities of nerve axons seen in between the neurons. The other dog, however, did not show that intense a reaction, and only a small number of cells (20.5%) were lightly stained. However, a few very small, fusiform cells that were scattered in between the principal neurons in a manner characteristic of type II SIF cells were seen with intense immunoreactivity in both dogs (Fig. 1C)

B) DBH

Although 91.5% of the somas stained positively, the immunoreactivity was very light. No positively labeled fibers were seen within the ganglia (Fig.1D).

C) 5-HT

The principal ganglionic cells did not stain for 5-HT

except on very rare occasions (Table 1). Among the few positive cells wee some which had the characteristics of type I SIF cells in that they were found in clusters of small cells that were arranged in groups, usually close to blood vessels. Also, small cells that resembled type II SIF cells were immunoreactive (Fig. 3A,B). No positive 5HT fibers were seen.

Peptides

A) SP

About 90% of the observed cells showed light immunoreactivity (Table 1); a few fine, immunoreactive fibers and terminal-like structures were seen in the ganglia (Fig. 2C).

B) VIP

All somatic profiles showed light immunoreactivity (Fig. 2D) comparable to that seen in SP staining . Some type I SIF cells, which are usually located in the periphery of the ganglia close to or within a nerve trunk, stained positive for VIP (Fig. 3C).

C) Galanin

Some ganglia contained intensely stained somas and fibers (Fig. 2A), while other ganglia in the same dog had only positive fibers or only a few positive cells. The overall counts showed that about 66% of the cells were

either lightly or densely stained (Table 1). Clusters of small cells that were located within the nerve trunk as it enters the ganglion stained intensely; these were considered type I SIF cells (Fig. 3D). Within the center of the ganglia, some other small cells, which might be type II SIF cells, were densely stained, although their exact nature cannot be determined since these ganglia also contained some very small neurons that might be mistaken for SIF cells.

D) NPY

About 47% of the cell somas were immunoreactive for NPY (Table 1, Fig. 2B). They often were evenly distributed within the ganglia, but occasionally formed clusters containing different numbers of somatic profiles or occurred as single cell bodies lying within or adjacent to a large nerve trunk. Immunoreactive fibers were seen in the ganglia (Fig. 2B), and some of them seemed to be closely associated with the somas, possibly terminating on them. Examples of non-staining following treatment with control procedures are shown in Figure 4A,B.

Discussion

This study has shown that canine cardiac ganglia contain large numbers of neuronal perikarya that stain positively for both cholinergic (ChAT) and adrenergic

(TH,DBH) synthesizing enzymes. The ganglia also contain fibers that stain positively for TH, SP, galanin and NPY. Positive staining for the TH and DBH enzymes as well as the NPY, VIP, SP, and galanin peptides could be detected in the perikarya of canine ganglionic neurons only after colchicine pretreatment, suggesting they are normally produced at such low levels that they can not easily be detected.

However, more investigations are needed to prove that these neurons do have all the anatomical and biochemical sympathetic characteristics before one can definitely ascribe a dual sympathetic-parasympathetic nature to them. For example, the neurons of the ciliary ganglia of rat, mouse and guinea pig stain positive for ChAt, TH and DBH, but they lack the ability to synthesize and store catecholamines as shown by the absence of catecholamine fluorescence and the absence of any of the characteristic sympathetic small, dense-core vesicles in their terminals (Landis et al.,1987). Also, the neurons of rat submandibular ganglia contain large amounts of DBH enzymes, but no detectable norepinephrine (NE) and epinephrine (E) could be seen and none of the cells showed catecholamine fluorescence when glyoxylic acid was used (Grzanna and Coyle 1978). However, in both the ciliary and submandibular parasympathetic ganglia there is no physiological evidence that suggests that these ganglia contain sympathetic, parasympathetic, and afferent neurons similar to that

suggested to be present in the cardiac ganglia of dog (Yuan et al., 1991).

In addition, there are limitation and technical consideration for the use of colchicine to study the anatomical distribution of neurotransmitters and different peptides throughout the nervous system. Colchicine has injurious effects on cells, and neurons have a differential susceptibility to colchicine's toxic effects (Goldschmidt and Steward 1982, Peterson and McGinty 1988, Morelli et al., 1980). The axonal retrograde transport interrupted by colchicine may carry some regulatory factors controlling peptide and protein synthesis. In addition, colchicine can cause deafferentation due to blockade of anterograde transport. This deafferentation interferes with delivery of factors to the postsynaptic site which may regulate the postsynaptic cell's phenotype (Cortès et al., 1990, Ceccatelli et al., 1991).

Principal neurons: a dual nature ?

The existence of both adrenergic and cholinergic enzymes in canine cardiac ganglia is similar to findings reported in other parasympathetic ganglia such as ciliary ganglia of rat, mouse and guinea pig. In these animals the parasympathetic postganglionic neurons stain positively for ChAt, TH and DBH (Landis et al.,1987). Similar results have also been seen in rat submandibular ganglia where large amounts of DBH enzymes were seen in the neurons (Grzanna and

Coyle 1978). Some cardiac neurons and tracheal parasympathetic neurons in guinea pig have been reported to express catecholaminergic characteristics (Baluk and Gabella 1989, 1990). This dual adrenergic / cholinergic characteristics has been reported in sympathetic ganglia (Wolinski et al. 1985, Wolinski and Patterson 1983, Potter et al. 1986).

The results of this study are also in agreement with other anatomical studies that suggest an even more complex organization for cardiac ganglia. For example, recent studies have shown that some of the cardiac ganglionic neurons of the rat might be adrenergic since some neurons stain positively for TH, DBH and NPY, while other cardiac neurons appear to be sensory neurons (Gu et al., 1984; Moravec and Moravec, 1984, 1987, 1989, 1990). A similar pattern was found in the mudpuppy where the cardiac ganglia receive preganglionic innervation from both parasympathetic and sympathetic fibers (Parsons and Neel, 1986), as well as presumably sensory fibers with SP-like immunoreactivity (Parsons et al., 1987). Cardiac ganglia in the guinea pig also contain fibers immunoreactive to both SP and VIP, and some ganglionic somas stain positive for VIP (Della et al., 1983; Papka and Urban, 1987). In human heart the cardiac ganglionic neurons that stain positive for acetylcholine esterase (AchE), do not stain positive for NPY, calcitonin-gene-related-peptide (CGRP), SP or TH, although

positive stained fibers for the latter peptides are regularly seen around the somas (Wharton et al., 1990).

Collectively, the data suggest that neurons of the PVFP canine cardiac ganglia have anatomical parasympathetic, sympathetic, and other peptidergic characteristics. Such diverse characteristics may, in part, explain the different physiological responses reported in these neurons by extracellular recording. For example, cardiac ganglia that survive chronic cardiac denervation function similarly to parasympathetic neurons,i.e., they decrease AV nodal conduction, heart rate, and atrial contractility (Priola D.V. 1980). More recent work suggests that the PVFP contains not only parasympathetic postganglionic neurons but may also contain sympathetic and some afferent neurons as well (Yuan et al., 1991, 1993). The immunocytochemical results of the present study suggest that almost all of these neurons function primarily as parasympathetic neurons as shown by Priola (1980), and some of these neurons may also function as sympathetic and afferent neurons, as suggested by Yuan (1991).

In addition, the positively stained fibers for TH, SP, galanin and NPY (although their intracardiac or extracardiac origin is not clear) may explain why stimulation of sympathetic nerves, parasympathetic nerves, or limited regions of the heart will activate the principal ganglionic neurons, indicating that canine intracardiac ganglia can be

modulated by sympathetic, parasympathetic, and afferent terminals (Gagliardi et al.1988; Armour and Hopkins 1990; Ardell et al. 1991).

Co-localization:

That most of the neurons stained positively for several peptides indicates the possible coexistence of these enzymes and peptides within the same neuron. The coexistence of neuropeptides and classical transmitters has been reported in many areas of the nervous system. For example, in the central nervous system, NPY and SP have been shown to coexist with norepinephrine and epinephrine, while SP,VIP, galanin coexist with acetylcholine (review by Hökfelt et al. 1992). Also, in autonomic ganglia co-localization of many of the peptides and transmitters has been reported, e.g., DBH, dynorphin, NPY and VIP in some neurons of the paracervical ganglia (Morris and Gibbins 1987). Also, galanin co-localizes with TH and NPY in celiac ganglia (Ahrèn et al. 1990) and co-localizes with VIP and SP in pancreatic ganglia (Salakj et al. 1992). Other parasympathetic ganglia, such as the sphenopalatine and otic ganglia, show high levels of co-localization between VIP, NPY, SP and DBH (Gibbins 1990). The functional significance of such coexistence of multiple peptides and transmitters in the PVFP cardiac ganglia is not clear, but it suggests the possible complex functional capabilities of these neurons on the sinoatrial node and other cardiac cells if all or some

of these peptides are really co-released in different amounts and combinations.

The adrenergic / cholinergic dual function of autonomic neurons has been reported in tissue culture. For example, 45-66% of cultured principal neurons derived from the superior cervical ganglion of newborn rats secreted both norepinephrine and ACh at the same time (Potter et al., 1986). When co-cultured with myocytes, stimulation of these neurons, using high-frequency train of impulses, caused slow cholinergic hyperpolarization of the myocytes followed by intense adrenergic excitation (Furshpan et al., 1986). Ultrastructurally, the terminals of these neurons were found to contain a mixture of adrenergic small dense core vesicles and cholinergic clear vesicles (Potter et al., 1986).

SIF cells in the ganglia:

Cardiac ganglia are known to contain different numbers of SIF cells, and many neurotransmitters and peptides have been detected in SIF cells including dopamine, NE, E, 5-HT, enkephalin (ENK), bombesin (BOMB), and SP (reviewed by Williams and Jew, 1983). In this study we detected the presence of 5-HT, galanin, TH and VIP in SIF cells of canine cardiac ganglia. The presence of these peptides in the SIF cells adds to the complexity of ganglionic structure and physiology. For example, SIF cells can be in close proximity to fenestrated blood vessels, as seen in the

ultrastructural paper of this dissertation, and also can be in direct contact with the principal ganglionic neurons.

If SIF cells release 5-HT in the microcirculation of the ganglia or in the direct vicinity of the neurons, they can influence the activity of the principal ganglionic neurons, as has been shown in other parasympathetic ganglia. In ciliary ganglia, 5-HT can increase neuronal membrane permeability to sodium and potassium and can cause depression of the ACh release from presynaptic terminals (Tatsumi and Katayama 1987). In contrast, in vesical pelvic parasympathetic ganglia, 5-HT facilitates the release of ACh from the presynaptic terminals by acting on presynaptic 5-HT_1 receptors (Nishimura and Akasu 1989). The possible functional importance of SIF cell content of VIP or galanin will be discussed later.

Fibers in the ganglia:

The presence of NPY and TH positive fibers in canine cardiac ganglia suggests the possibility of sympathetic innervation to these ganglia because NPY and norepinephrine have been shown to coexist in the sympathetic cardiovascular terminals in several species (review by Wharton and Gulbenkian 1989). However, the sympathetic origin of these TH and NPY positive fibers is questionable because NPY has been seen in cultured cardiac neurons of guinea pig (Hassall and Burnstock 1984). The co-localization of NPY and DBH or TH has also been seen in somas of cardiac ganglionic neurons

in rat and mouse heart (Allen et al. 1986), suggesting that the TH and NPY positive fibers seen in this study could be axonal profiles of the ganglionic neurons as they are traveling through the cardiac ganglion. In addition, no small dense-core vesicles characteristic of sympathetic, adrenergic terminals were found in the ultrastructural study of this dissertation, further suggesting an intracardiac origin of these TH and NPY positive fibers.

The functional significance of NPY in the cardiac ganglia may be important. NPY can influence cardiac functions through its vasoconstrictor effect or through modulating norepinephrine release at the prejunctional sites (Lundberg 1984). NPY also inhibits ACh release from parasympathetic terminals and attenuates the slowing effect of vagal stimulation (Potter 1985, 1987, 1988, Warner and Levy 1989).

The presence of VIP labelling in cardiac ganglion principal cells supports the finding of Anderson et al.(1992), who showed that VIP positive fibers continue to exist in the sinoatrial node after chronic total canine cardiac denervation. Dense VIP positive fibers have been shown to exist in the mammalian sinoatrial node in addition to some VIP positive cardiac neurons, and these were assumed to be part of an intrinsic cardiac regulatory system (Weihe and Reineche 1981, Weihe et al. 1984). VIP has positive chronotropic and mild inotropic effects on the heart of dog

and cat (Said et al.1972, Unverferth et al.1985). However, the functional significance of VIP in canine cardiac ganglia and its interactions with other peptides remains to be investigated.

Galanin immunoreactivity has been shown in several autonomic ganglia (Ahrèn et al. 1990, Salakj et al.1992) and also in the cardiac neurons and SIF cells of the mudpuppy (McKeon and Parsons 1990). Galanin can attenuate the vagal effect on heart rate in cat (Ulman et al. 1992), but its role in the canine heart is yet to be established.

In conclusion, the cardiac neurons of the PVFP may be playing an important and complex modulatory effect on cardiac function. The complexity of these functions is reflected in the diversity of the neurotransmitters that could be co-released and their possible dual sympathetic and parasympathetic nature.

Figure 1.

Light photomicrographs of canine cardiac neurons (arrows) stained positive for ChAT (A),TH (B,C) and DBH (D). Some ganglia have many TH intensely stained cells as seen in B, while others were slightly stained (arrows in C). A few TH positive fibers (arrowheads) and small fusiform SIF-like cells(open arrow) can be seen (C). Most principal neurons were immunoreactive for DBH (D).

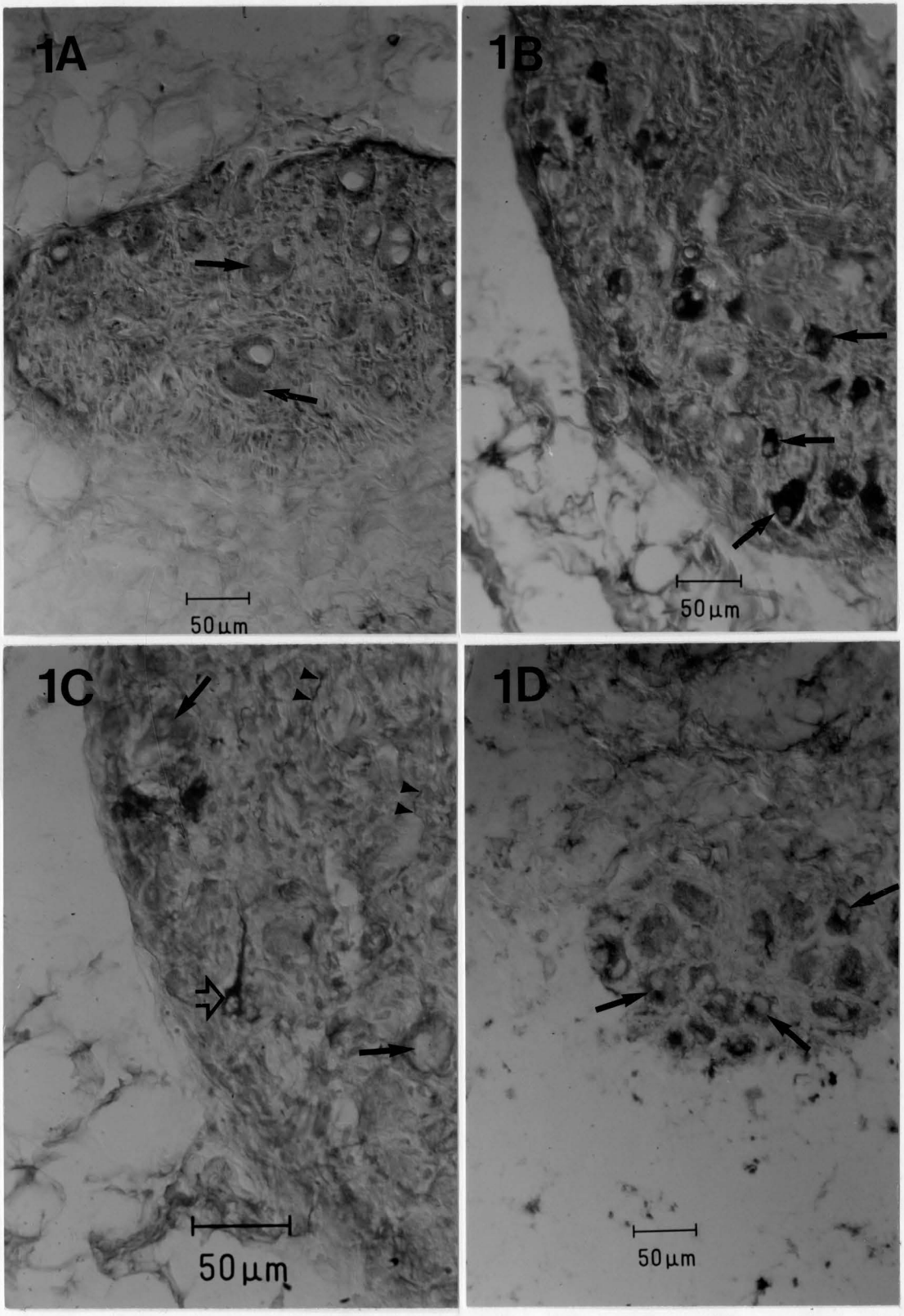
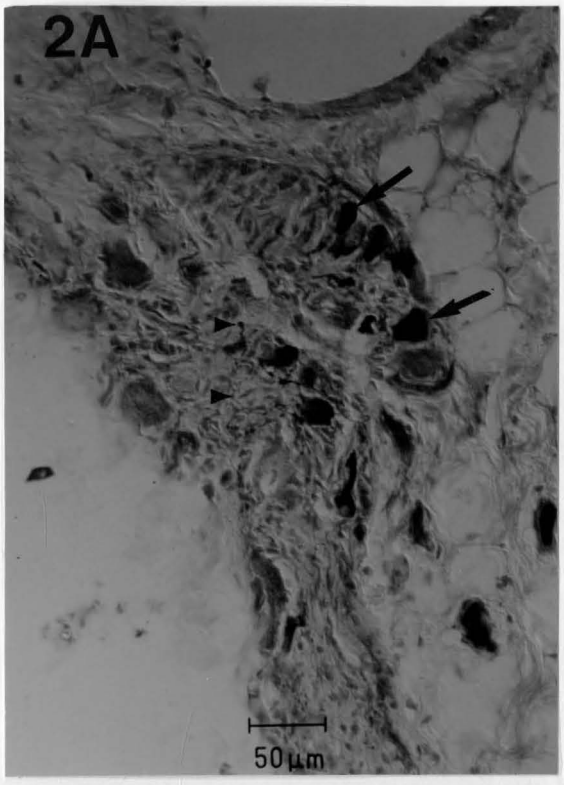

1A
50µm
1B
50µm
1C
50µm
1D
50µm

Figure 2.

Light photomicrographs showing the principal neurons (arrows) having dense immunoreactivity for galanin (A) and light staining for NPY (B), SP (C), and VIP (D). A few intraganglionic positive fibers (arrow heads) were seen with galanin (A), NPY(B) and SP(C) immunostaining. Notice the terminal-like structures positively stained with SP (short arrows in C).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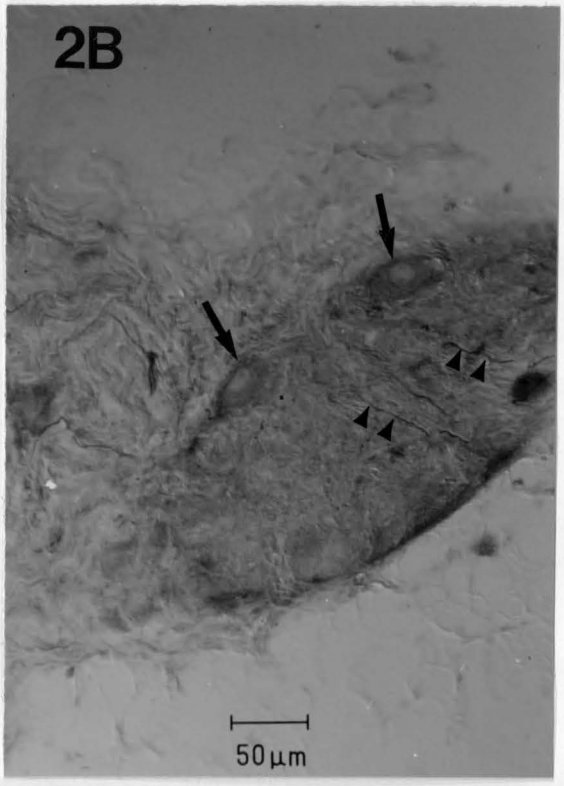
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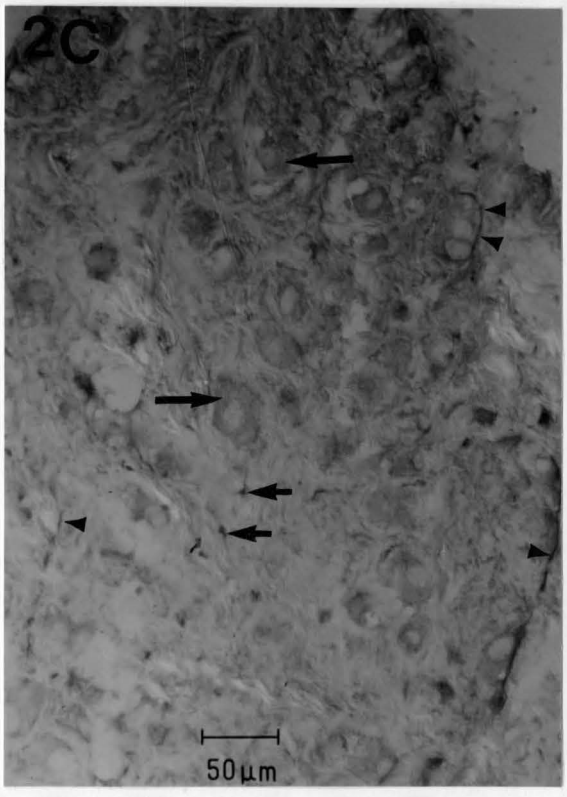
2C
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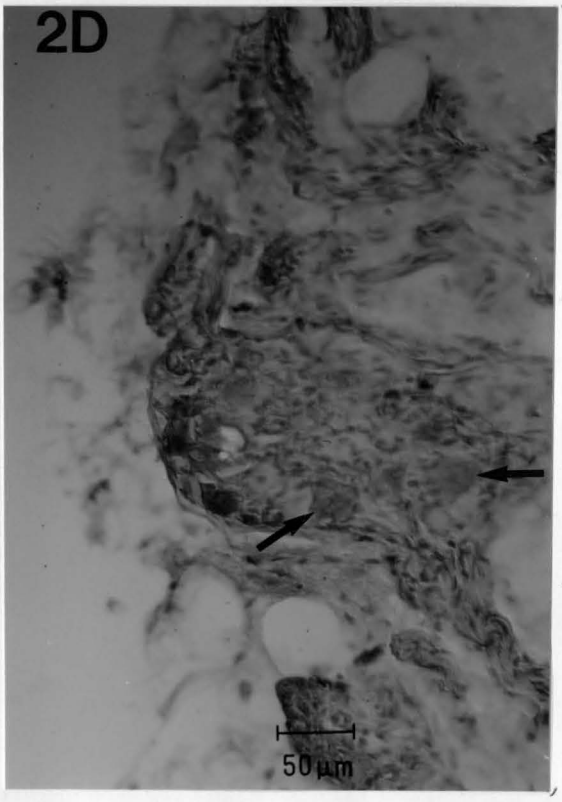

2D
50µm

Figure 3.

Both type II (A) and type I (B) SIF cells were positive for 5-HT, while only type I cells were positive for VIP (C) and galanin (D). Type I SIF cells were mainly seen in the course of nerve trunks associated with the ganglia as seen in C and D. In C some VIP positively stained principal neurons can be seen (small arrows), though they are out of the plane of focus. Scale bars = 50 μm in A,B,C; 20 μm in D.

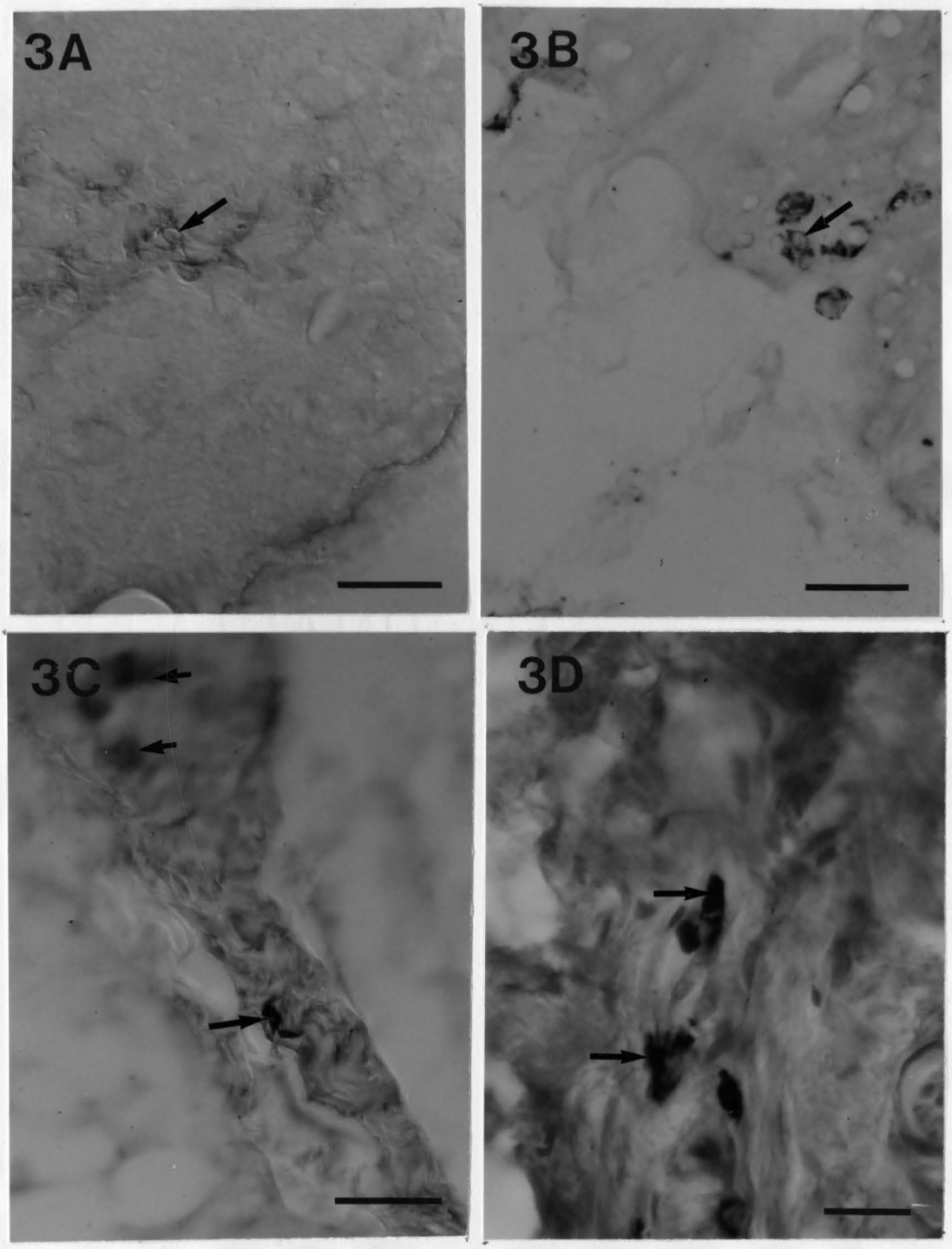
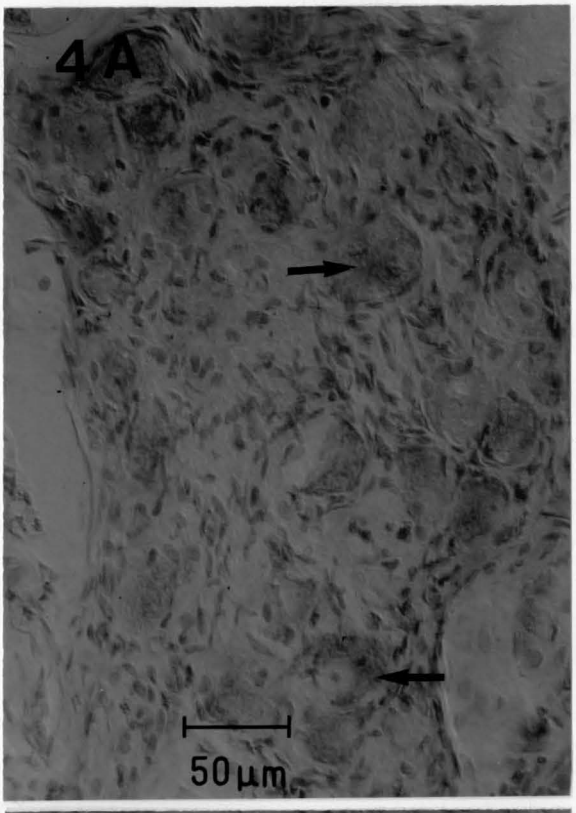

3A
3B
3C
3D

Figure 4.

Light photomicrographs of control samples: after using preincubated NPY antisera, staining of both fibers and somas was totally eliminated (A). Picture B shows another control done by omitting the galanin primary antibody, resulting in similar absence of positive staining.


4A
50µm

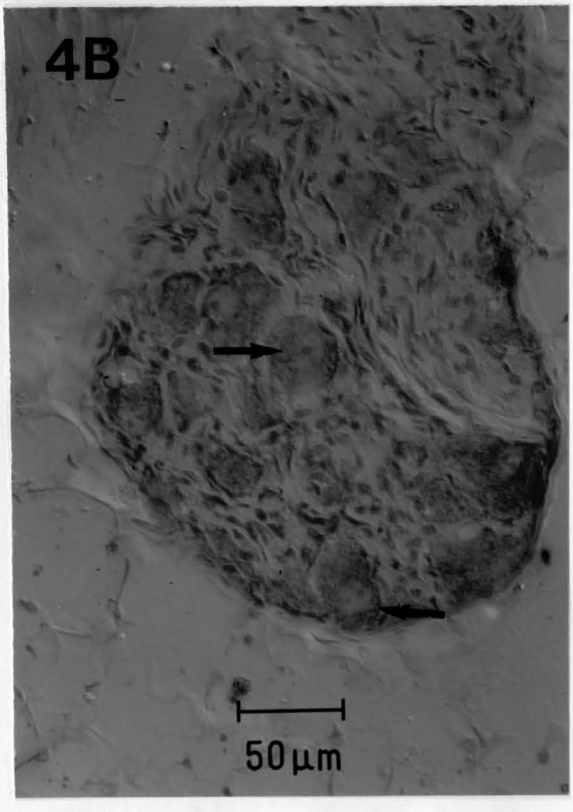

4B
50µm

Table 1.

Total number of neurons and the percentages of immunoreactive cells in the ganglia for each antibody studied. Total = number of examined neuronal profiles. Percentages = mean percentages of positively stained neurons. # Dogs = number of dogs used for that antibody.

Table 1

Immunoreactive cells in cardiac ganglia

Antibodies	Total Number	Percentages	# Dogs
ChAT	792	99.3	4
TH	736	57.4	2
DBH	531	91.5	2
NPY	410	46.8	2
5HT	349	0.8	2
VIP	242	100	2
SP	363	90	2
Galanin	563	66.4	2

CHAPTER IV

LIGHT MICROSCOPIC EXAMINATION OF THE EFFECT OF PREGANGLIONIC DENERVATION ON THE MORPHOLOGY OF CARDIAC NEURONS

ABSTRACT

This study is designed to test the hypothesis that neurons of canine cardiac ganglion consists of more than one population and that the neurons maintain their morphological characteristics independently from preganglionic innervation.

The results demonstrate that canine cardiac neurons are morphologically one population with large variation in somal area and dendritic complexity. These results also show that, in general, the ganglionic neurons do not depend on preganglionic innervation for maintenance of their morphological characteristics. A few neurons were severely affected by the denervation, but they did not change the overall population characteristics. What did appear to change was the amount of variability between animals. This variability was significantly reduced in the denervated groups.

Introduction

Transneuronal degeneration has been reported in both the central and the peripheral nervous system. After brain or nerve damage, transneuronal degeneration occurs in the CNS in the lateral geniculate bodies, visual cortex and substantia gelatinosa of spinal cord among other regions, (Matthews et al.,1960; Knyihár-Cisillik et al., 1989). In the peripheral nervous system (PNS) transneuronal degeneration occurs in the superior cervical ganglia after preganglionic denervation (Hamlyn 1954; Chang et al. 1976) and in cardiac ganglia after vagotomy (Tay et al.1984; Wong et al.1987). The role of neurons of cardiac ganglia after heart transplantation is not clear. Investigation of the transplanted heart and lung in human revealed that parasympathetic ganglionic neurons in the heart and lungs survived after preganglionic denervation with no apparent morphological changes (Rowan and Billingham, 1989, Springall et al., 1990). However, no systematic quantitative studies were done on denervated ganglia. The present study addresses the effect of selective and total cardiac denervation on different populations of neurons in canine cardiac ganglia to determine if these neurons are susceptible to deafferentation-induced transneuronal degeneration.

Materials and Methods

Three groups of mongrel dogs were used in this study: control, total cardiac denervation and selective parasympathectomy. Denervation was done using the intrapericardial method described by Randall et al. (1980).

Methods for cardiac denervation:

A. Total cardiac denervation

Seven dogs of either sex were anesthetized with sodium pentobarbital (30 mg/kg i.v.), supplemental doses of same anesthetic were administered as required. After intubation respiration was maintained with a positive-pressure respirator. Under sterile conditions the right fourth intercostal space was opened, and the pericardium exposed by retracting the lung medially and caudally. The left thoracic vagus, right thoracic vagus and left and right stellate ganglia including the two ansae branches (anterior and posterior), were identified and freed from surrounding tissue. Electrical stimulation was done to ensure the identification of the structures. A bipolar electrode was used to stimulate the isolated nerves (10 Hz, 5 msec, 5V for sympathetic stimulation and 20 Hz, 5 msec, 5 V for parasympathetic stimulation) while recording heart rate and electrocardiographic responses to stimulation using lead II electrocardiogram. The pericardium was opened, and all nerves were cut along their intrapericardial pathways around

the adventitia of the great blood vessels. The first stage of denervation started by cutting the ventrolateral cardiac nerve, then stripping all the adventitia at the bifurcation of the pulmonary artery and its branches. In the second stage, all nerves between the aorta and the main pulmonary artery were cut. Finally, all adventitia around the superior vena cava were dissected and cut, and the azygos vein was tied and cut. After denervation was complete, the stimulation procedure was repeated to ensure total denervation of the heart. The pericardium was closed by sutures, the lungs inflated, and the chest closed in layers. The chest was aspirated to create negative pressure, and the endotracheal tube was removed when the dog started to breathe. An analgesic therapy and antibiotics were administered post-operatively for 3-6 days. After 10 day survival, the dogs were reanesthetized with alpha chloralose (100 mg/kg IV), and both sympathetic and parasympathetic stimulation were repeated to determine the degree of denervation. If any stimulation-induced change in the heart rate or electrocardiogram (P wave shift) occurred indicating incomplete denervation, the animal was not used in the study (two dogs). The pulmonary vein fat pad (PVFP) from successfully denervated hearts (five dogs) were then removed, and the ganglia were used for HRP injections.

B. Bilateral cardiac parasympathectomy:

Six dogs of either sex were used for parasympathetic

denervation. Selective parasympathetic denervation was accomplished according to the method of Randall and Ardell (1985). The chest was entered through a thoracotomy in the right fourth intercostal space followed by identification and stimulation of the right and left thoracic vagi and the corresponding ansae of the right and left stellate ganglia. The denervation protocol proceeded in a rostral codal direction and included dissection of the pericardial reflection distally along the dorsal surface of the superior vena, then dissection of all the adventitia around the superior vena cava followed by tying and cutting the azygos vein. The dorsal atrial tissues between the right and left pulmonary veins, including the ventral surfaces of the descending right pulmonary artery. After denervation was completed, electrical stimulation of the vagal and sympathetic nerves were repeated to verify that chronotropic responses to vagal stimulation was completely abolished while chronotropic responses to sympathetic stimulation was intact. After a 10 day survival, the dogs were reanesthetized with alpha chloralose (100 mg/kg IV), and tested to determine the degree of denervation. Only PVFP from dogs with successful selective parasympathetic denervation were removed and dissected for their ganglia (five dogs). All experimental procedures were conducted in strict accordance with the standards established by the American Physiological Society for the care and use of

laboratory animals.

Methods for HRP iontophoresis:

The PVFP were placed immediately in cold Krebs solution, equilibrated with 95% O_2 and 5% CO_2 mixture, and maintained at 20 °C until a ganglion with its associated piece of atrial muscle was removed.

The piece of atrial muscle containing the ganglion was transferred to a tissue bath and attached to a small piece of silicon rubber to ensure the stability of the ganglionic neurons during the injections. The tissue bath was maintained at 35.0 ± 0.5^{0}C and superfused with Krebs solution with the following contents (in mMol/L): NaCl, 117; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; glucose, 11.5. The solution was aerated with 95% O_2 and 5% CO_2. The pH was 7.35±0.04. Krebs solution was prepared two hours before each experiment using double distilled, de-ionized water.

Transmembrane potentials were measured using ultrafine glass microelectrodes (50-80 MΩ) filled with a 5% filtered solution of HRP (Sigma type VI) in 0.5M KCl buffered to pH 7.6 with 0.05 Tris buffer. Successful intracellular penetration of a cell was indicated by a sudden negative shift in the monitored potential, stable resting membrane potential for 5 minutes and responses to intracellular stimulation. HRP solution was iontophoretically injected into the neuron using 100 msec depolarizing pulses of 7.5 nA

at 3 Hz for 4 minutes.

Two hours was allowed for the enzyme to diffuse throughout the dendritic tree and axons of the injected neuron. The whole piece of tissue along with the strip of silicon rubber was removed from the tissue bath and incubated with a solution of 0.2M Tris buffer, 10 mg p-phenylenediamine dihydrochloride (Sigma), 20 mg pyroatechol (Sigma) and 10 ml 30% hydrogen peroxide for 20 minutes at room temperature. The tissue was rinsed in saline and fixed in 4% paraformaldehyde and 4% glutaraldehyde in phosphate buffer. The ganglia were then dehydrated in a series of 50%, 70%, 95% and 100% ethanol and cleared in 8% methyl-benzoate in ethanol. A total of 26 successfully labelled neurons were studied (15 control, 5 parasympathectomy, and 6 total denervation). Only one neuron was labelled in each ganglion to avoid any dendritic overlapping. Only successfully injected neurons, characterized by dark somas with well defined smooth contours, no extracellular HRP deposits, and a full dendritic appearance were traced and measured.

Injected cells were examined under the microscope and traced using a camera lucida drawing attachment. Six parameters were measured on the tracings: somal area, number of primary dendrites, total length of primary dendrites, mean length of primary dendrites, number of branching points, and longest dendrite. A computerized video image

analysis system was used to perform these measurements.

Primary dendrites were defined as processes that emerged directly from the soma, while secondary and tertiary dendrites were those that emerged from the primary or secondary dendrites. The maximum length of primary dendrite was the distance between the origin of the dendrite from the soma and its furthest point. Branching points gave rise to secondary or tertiary dendrites which were at least 5 μm in length.

ANOVAs were performed to determine whether there were any statistically significant differences between the means or variances of each parameter.

Methods for video image analysis of somatic profiles:

Three sections 2μm thick were cut from each ganglion. The sections were 20 μm apart and were stained with toluidine blue (Fig. 4A). The nucleated cells in each section were traced using a camera lucida attachment (Fig. 4C). The nucleated profiles were then retraced and filled with ink, allowing a minimum distance of 1 mm between adjacent profiles to permit accurate measurement of each soma (Fig.4D). Areas of the filled profiles were measured by a video image analysis system using NIH Image installed on an Apple IIcX computer and a MOS Javelin Solid State video camera (Javelin Electronics; Torrance, CA). All images of filled profiles were digitized under stabilized illumination source. Individual measurements and mean value

of somal areas were calculated. Cumulative frequency graphs of the distribution and percent distribution of somal areas were obtained. ANOVAs were performed to determine if there were any statistically significant differences between the groups' areas or variances.

Results

HRP-filled neurons:

Table 1 summarizes the data on the HRP-injected ganglionic neurons. ANOVAs comparing the mean values of each parameter across groups found no significant differences. Interestingly, an F-test comparing the variance in the control group was significantly higher than in the total denervation group in the soma area (Table 2). A scatterplot of the areas of all the injected cells is found in Figure 1A. The smaller variance in the total group is readily apparent.

The principal neurons of canine cardiac ganglia were multipolar neurons and displayed a wide range of interneuronal variation in both size and degree of dendritic complexity (Figs. 2,3). Somas were usually ovoid or round with a smooth outer surface and occasional, short perikaryal processes (Figs. 2,3). Axonal profiles, identified by their smooth, non-beaded contour and absence of branches or spine-like processes, left the soma and exited the ganglion

without giving off any collaterals. Of all examined neurons only three had no processes that could be identified as axons (Fig.3 F,G). Variations in the dendritic configuration were evident within every group. The dendrites emerged from the soma in different patterns: from one pole (Fig.3 A,C,H), from opposite poles (Fig. 3 E), or from all sides of the soma (Fig. 3 G). However, the sample size was too small to conclude whether such patterns were consistent enough to classify the neurons. The dendrites appeared consistently beaded with varicosities and slender intervaricose segments exhibiting many spine-like processes (Fig. 2 D). Dendrites usually had a wide base close to the soma and then tapered off as they divided into secondary and tertiary branches. The length of the dendrites varied from a few microns to several hundred microns (Table 1).

Toluidine-blue somal area measurements:

In order to obtain a larger sample size, nucleated somas were measured from these three groups using a video image analysis system. The Pearson Chi-squared test of normality indicated the distribution of the sizes of ganglionic neurons was not normal (321.4, 4d.f.) and was positively skewed toward the small size. This larger sample of nucleated somas had a large size variation (128 to 2093 μm^2) but, like the HRP study, showed no significant changes in mean or median values after selective or total denervation, as determined by ANOVA. This was true despite

the fact that after denervation some ganglia, like the one shown in Figure 4B were severely affected, showing signs of degeneration such as vacuolation and separation from the satellite cells. However, in agreement with HRP-injected somal areas, F-tests comparing the variance between different groups found that in control animals the variances were significantly higher than in both of the denervated groups (Table 3). When frequency graph distribution for somal areas for each animal were done (Fig. 5), the variability between the control animals and the overlap of the distributions between animals of the other experimental groups were evident.

Discussion

The present results demonstrate that canine cardiac neurons are morphologically one population with large variation in somal area and dendritic complexity. These results also show that, in general, the ganglionic neurons do not depend on preganglionic innervation for maintenance of their morphological characteristics. A few neurons were severely affected by the denervation, but they did not change the overall population characteristics. What did appear to change was the amount of variability between animals. This variability was significantly reduced in the denervated groups.

Cardiac ganglia: one or several neuronal populations?

Electrophysiological studies on cardiac neurons have been conducted by several investigators, using different species such as dog (Xi et al., 1991) and rat (Selyanko, 1992), and have indicated that there are functionally more than one type of neuron. Xi et al. (1991) reported that canine cardiac neurons consist of heterogenous populations of multipolar neurons, divided electrophysiologically into three functional groups: single, repetitive, and nonfiring neurons that are morphologically different, especially regarding their somal sizes. These investigators also suggested that a large number of intraganglionic interneurons may exist in addition to the classical, small, intensely fluorescent (SIF) cells. However, in the present study when large samples of somal areas were analyzed, no morphologically different subpopulations could be detected, suggesting the existence of large morphological overlap between these functionally distinct groups.

The inability to identify the axonal profiles in a few neurons (12%) in this study supports the suggestion that some of the canine cardiac neurons are ganglionic interneurons that may be intraganglionically active (Xi et.al., 1991), although an ultrastructural study of these specific neurons is needed to prove their interneuronal connections.

Importance of preganglionic innervation:

In the present study the average overall morphological characteristics of canine cardiac neurons were not dependent upon intact preganglionic innervation. However, cardiac ganglia can be regulated through the connections with the CNS in a manner that creates a high degree of variability between animals. Although the factors involved in determining such variability are unclear, it may be that age, sex, heart size or blood pressure are among the factors that determine the mean or median neuronal size that exists in a specific dog. Clearly, a more detailed study is needed to determine such factors.

To explain the reduction of variability between dogs after denervation seen in this study is difficult, but it is possible that denervated canine ganglionic neurons may become exclusively dependent on connections with inter- or intra- ganglionic neurons or on factors derived from their cardiac target for maintenance of their shape, leading to less variability between dogs. The observation that the dendritic complexity of neurons of the submandibular parasympathetic ganglia is correlated with the size of the target (Snider, 1987) suggests that the cardiac target tissue may be important for the maintenance of the morphological properties of canine cardiac neurons.

The observation that peripheral autonomic ganglionic neurons can survive with no apparent morphological changes

after preganglionic denervation is in agreement with previous studies. In humans, heart or heart-lung transplantation causes total denervation of preganglionic input to heart or lungs. The parasympathetic ganglionic neurons in the heart and lungs are apparently morphologically unaltered (Rowan and Billingham, 1989, Springall et al., 1990). However, significant neurochemical changes in phenotype from typical cholinergic parasympathetic neurons to tyrosine hydroxylase and neuropeptide Y positive neurons have been reported in respiratory tract ganglia after heart-lung denervation (Springall et al.,1990), suggesting that denervation does cause more subtle changes that are not found using light microscopy only and indicating the need to investigate the issue of transneuronal degeneration using different approaches such as immunocytochemical and ultrastructural techniques.

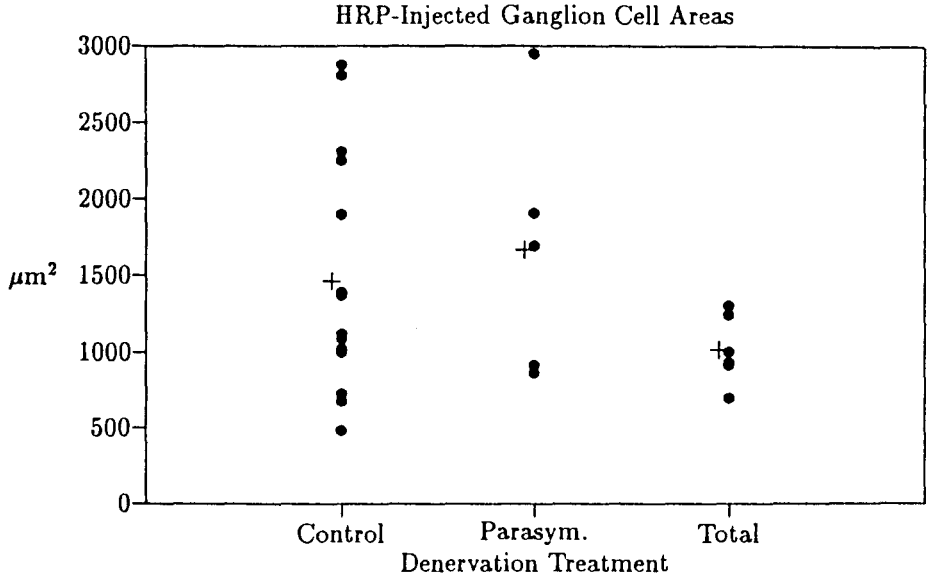
In conclusion, although a few neurons in the canine cardiac ganglia were severely affected by the denervation, they did not change the overall population characteristics. However, significant changes were seen in the variability between the animals after denervation indicating that there were some subtle changes that may be detected by a more sensitive technique as the ultrastructure.

Figure 1.

A: Scatter plot of individual somal areas for all HRP labelled neurons. Each circle represents one cell, and the (+) indicates the mean of each group. The smaller variance in the total group is obvious.

B: Scatter plot of mean ganglion cell areas measured by video image analysis system. Each circle represents the mean of soma area of one dog. Although the mean (+) is not affected by the denervation, the variation between the denervated dogs is significantly lower than between control dogs.

A


HRP-Injected Ganglion Cell Areas
3000
2500
2000
μm²
1500
1000
500
0
Control
Parasym.
Total
Denervation Treatment

B

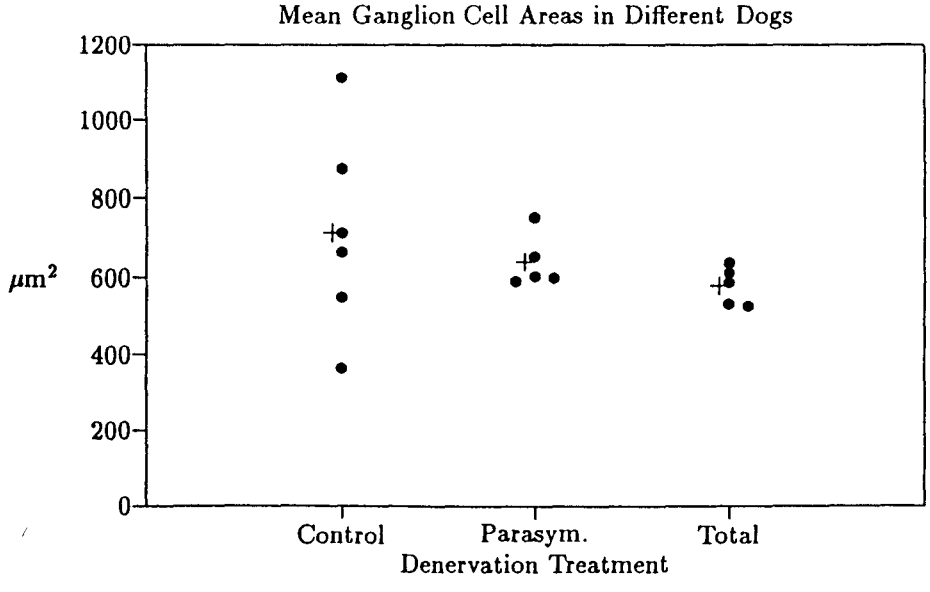
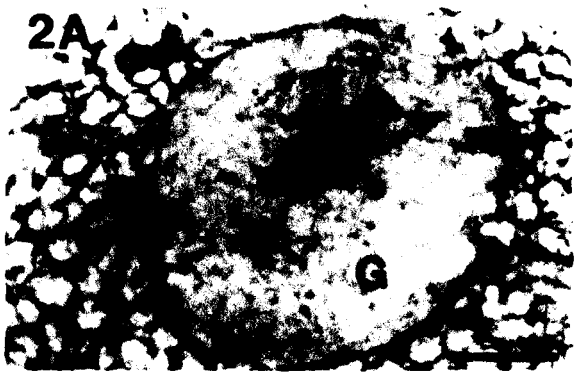
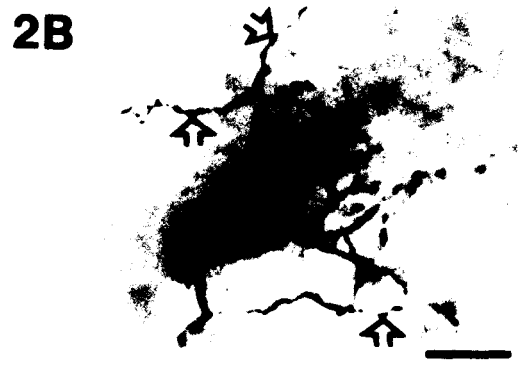

Mean Ganglion Cell Areas in Different Dogs
1200
1000
800
μm²
600
400
200
0
Control
Parasym.
Total
Denervation Treatment

Figure 2.

Photomicrographs of two HRP labelled neurons in canine cardiac ganglia. (Camera lucida tracings of the same neurons are illustrated in Fig. 3 D,G). Figs. 2 A,C show low power views of whole ganglia (G) as they are surrounded by the fatty tissue (F) of the pulmonary vein fat pad. Figs 2B and D show high magnification views of the same respective neurons, displaying multipolar dendritic arborizations, with dendritic varicosities (open arrows), branches, and spine-like processes (arrows). Although the axonal profile of the cell in A was not in the plane of focus, it still can be seen leaving the ganglion (arrowheads). No axon could be identified for the cell in C. Scale bars = 100 μm in A,C and 25 μm in B,D.


2A
G


2B

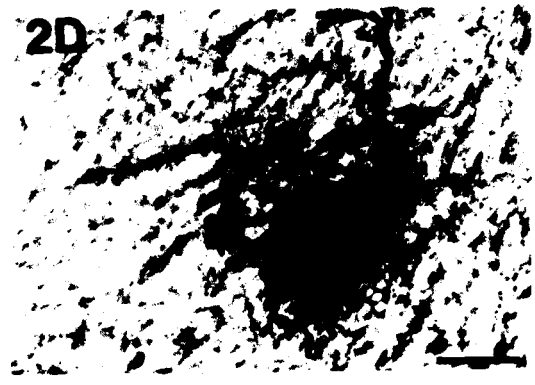

2D

Figure 3.

Camera lucida tracings of nine neurons of canine cardiac ganglia. The neurons are representative of three different groups: control (A,B,C), parasympathetic denervation (D,E,F), and total denervation (G,H,I). Somal size as well as degree of dendritic complexity vary highly within each group. Primary dendrites arise either from a pole of the soma (A,C,H), from opposite poles (B,E,F,I) or simply leave the soma in a radiate form (D,G). Asterisks indicate axonal profiles. Scale bar = 100 μm.

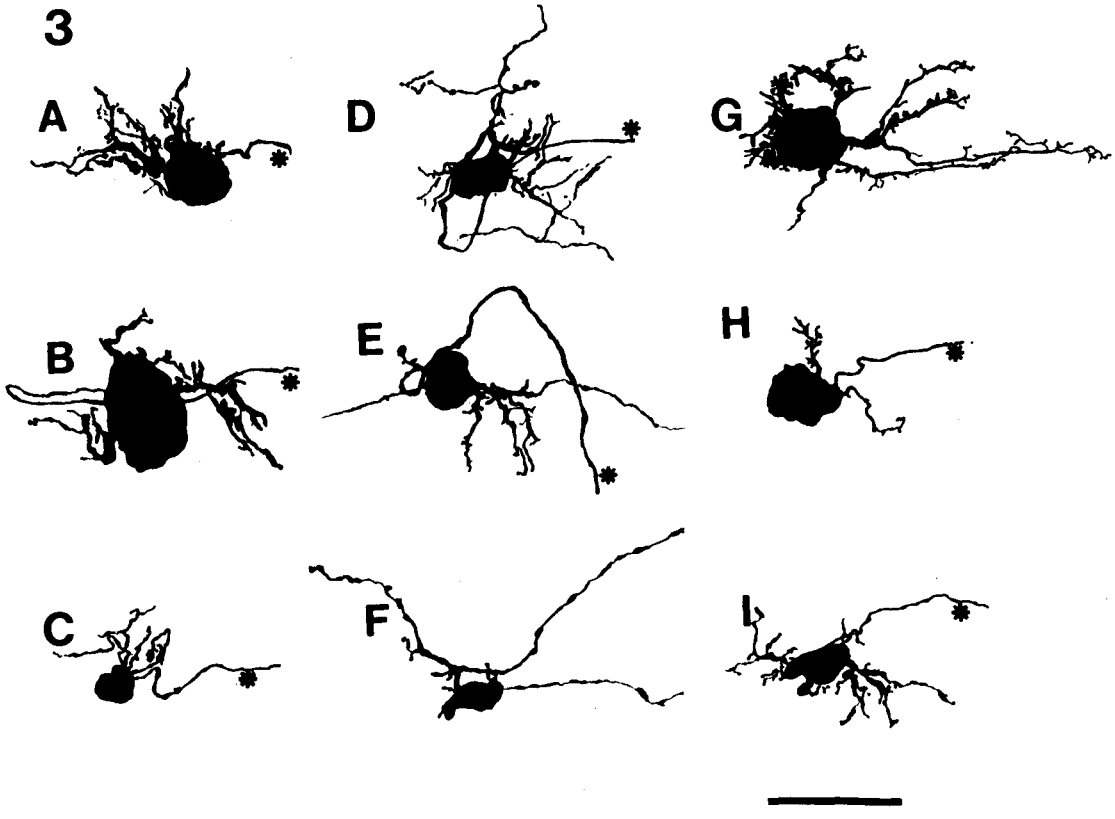

3
A
D
G
B
E
H
C
F
I

Figure 4.

A. Photomicrograph of 2 μm thick section stained with toluidine blue of control ganglion, showing large variation of somal sizes with a few very large neurons (arrows), few very small neurons (open arrows), and many intermediate neurons. B. Example of a severely affected ganglion, as occasionally seen after denervation. Cells are shrunken, vacuolated, and separated from their satellite sheaths and also separated from each other. C. Camera lucida drawing of nucleated somas of cardiac ganglion in A. D. Separated and ink-filled tracings of the same neurons, providing the image that was measured by the image analysis system. Scale bars = 50 μm.

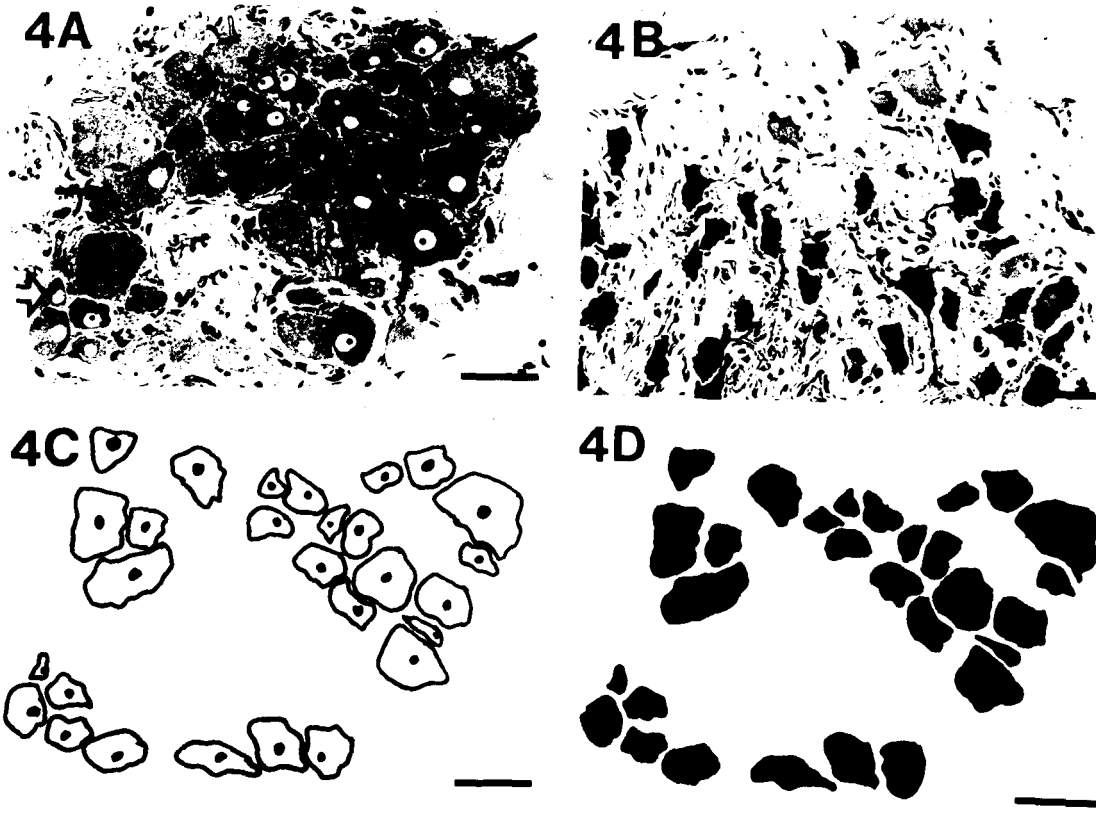

4A
4B
4C
4D

Figure 5.

Frequency graphs of the distribution of somal areas of all individual animals in the three groups. The variation between the control animals is evident (A), while the somal ares in the animals of both of the denervated groups (B,C) are closely overlapping.

Cardiac Ganglia Cell Area Distributions

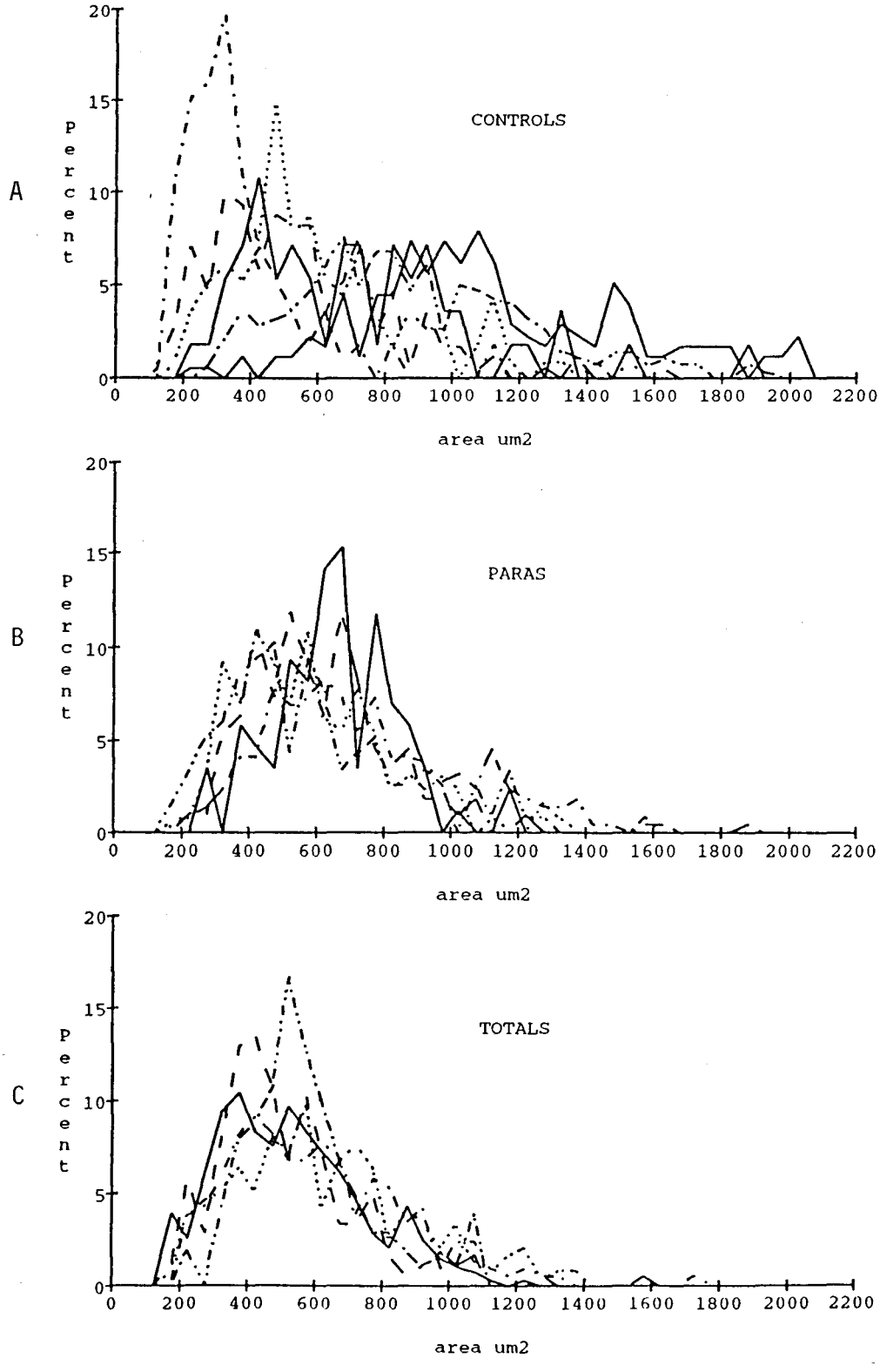


Table 1.

Statistical analysis of the six morphological parameters of HRP-injected neurons in the three different groups (c = control, p = parasympathectomy, and t = total denervation). The six parameters are: somal area, number of primary dendrites (Number of dendrites), total length of primary dendrites (Total dendrite length), mean length of primary dendrites (Mean dendrite length), number of branching points, and longest dendrite. The values are represented as Mean ± SEM. ANOVA was performed comparing all three groups in each category. No significant differences ($P < 0.05$) were found. The two denervation groups were then combined into one denervation group (P+T) and ANOVA was performed to compare this group to controls. No significant differences ($P < 0.05$) were found.

Table 1

Statistical analysis of the six morphological parameters of HRP-injected neurons in the normal and denervated ganglia

	Soma Area μm^2	Number of Dendrites	Total Dendrite Length μm	Mean Dendrite Length μm	Number of Branching Points	Longest Dendrite μm
c (n=15)	1470±198	5.6±4.9	457±40	86±7	10±1.4	154±13
p (n=5)	1667±380	6.0±1.5	403±109	68±4	17±5.6	156±37
t (n=6)	1018±92	4.8±0.7	371±69	105±10	12±3.4	165±19
ANOVA F(2,23)	1.31	0.42	0.56	2.65	1.47	0.07
P VALUE	0.289	0.659	0.579	0.09	0.250	0.928
P+T=11	1314±198	5.4±0.77	586±59.5	88±8.3	15.0±3.1	161±18.8
ANOVA F(1,24)	0.30	0.07	1.07	0.03	1.73	0.08
p value	0.59	0.787	0.312	0.854	0.200	0.776

Table 2.

Statistical analysis using the F test comparing the variance of control group, selective parasympathetic denervation and total denervation of HRP-injected neurons. Statistically significant differences between the control and the total denervation is evident (Asterisk indicates statistical significance with $P < 0.05$).

Table 3.

Statistical analysis using F test comparing the variance in both control group and denervated groups of traced somatic profiles indicated that variance was significantly reduced in both of the denervated groups. (Asterisk indicates statistical significance with $P < 0.05$).

Table 2

Somal Areas of HRP-Injected Neurons

	Variance	N	F(x,y)	P
Control	592013	15		
Parasym	725609	5	1.2256 (4,14)	0.6883
Total	50865	6	11.63 (14,5)	0.0134*

Table 3

Somal Areas Measured by Image Analysis

	Variance	N	F(x,y)	P
Control	67489	6		
Parasym	4534	5	14 (5,4)	0.0216*
Total	2407	5	28.03 (5,4)	0.0065*

CHAPTER V

ULTRASTRUCTURAL ORGANIZATION OF NORMAL AND DENERVATED CANINE CARDIAC GANGLIA

ABSTRACT

This study is designed to test the hypothesis that canine cardiac neurons are integrating centers which receive sympathetic, parasympathetic and intraganglionic inputs.

The results demonstrate that canine cardiac ganglia are predominantly parasympathetic. They receive vagal innervation but lack sympathetic innervation. The preganglionic innervation consists of boutons with clear vesicles and boutons with mixed clear and dense-cored vesicles. Both types of boutons make their synaptic contacts on the dendritic shafts and spines, with the majority on the shafts. No synapses were seen on the somas. However, dendro-dendritic contacts are seen regularly in the ganglia both, before and after cardiac denervation. Such contacts may be sites of intraganglionic communication between the neurons and may add to the integrating ability of these neurons.

Introduction

The ultrastructure of cardiac ganglia of the normal mammalian heart has been the subject of many investigations (Yamauchi, 1973; Kaye, 1984; Moravec and Moravec, 1987). In mammals the nature of preganglionic innervation to these ganglia is conflicting. Mostly the terminals are cholinergic: boutons with clear vesicles, and boutons with both clear and large dense-cored vesicles. (Shvalev and Sosunov, 1985; Papka, 1974, 1976; Wong et al., 1987). However, small dense-cored vesicles characteristic of sympathetic innervation have been seen regularly by Shvalev and Sosunov (1985) in the three animals investigated: rat, rabbit, and guinea pig. In addition, several physiological reports suggests complex functions for canine cardiac ganglia. Physiological evidence suggests that the pulmonary vein fat pad (PVFP), which controls the sinoatrial node, contains not only parasympathetic postganglionic neurons but also some sympathetic and afferent neurons as well (Armour and Hopkins 1990, Ardell et al., 1991). In addition, Xi et al. (1991), using intracellular injection of HRP in cardiac ganglia of the dog, reported that almost half of the injected neurons did not have distinguishable axons and suggested that they may be intraganglionically active interneurons. However, the ultrastructural organization of these canine cardiac ganglia has not been investigated.

The effect of cardiac denervation on the cardiac ganglia is of interest because, in man, heart transplantation or heart-lung transplantation produces total cardiac denervation that may cause significant changes in phenotype of parasympathetic neurons (Rowan and Billingham, 1988, Springall et al., 1990).

Denervation studies on non-human primates done by Tay et al. (1984) and Wong et al. (1987) showed that both unilateral and bilateral vagotomy caused early widespread glycogen accumulation in ganglionic neurons, followed by intense darkening of the dendrites in 10% of the principal ganglionic neurons, indicating the occurrence of transneuronal degeneration in the cardiac ganglia.

Although both the pre- and postganglionic pathways to the cardiac ganglia in PVFP have been interrupted surgically in dogs (Randall et al., 1991), the histological and ultrastructural changes in PVFP after preganglionic denervation have not been investigated and an attempt to study such effects will be presented in this study. This study will also investigate the normal ultrastructural characteristics of principal ganglionic neurons, SIF cells and the preganglionic terminals within canine cardiac ganglia.

Materials and Methods

1. Methods for normal ultrastructural organization of the cardiac ganglia:

Five dogs of either sex were anesthetized with sodium pentobarbital (30 mg/kg i.v.), and the PVFP was removed and placed in fresh fixative of 4% paraformaldehyde, 3% glutaraldehyde in 0.1 M Millonig's phosphate buffer, pH 7.4, for 24 hours. Three individual ganglia were removed from the PVFP under the dissecting microscope and placed in fresh fixative for another 24 hours, then osmicated in 1% osmium tetroxide in Millonig's buffer, dehydrated in ethanol and embedded in Epon. Each ganglion was sampled for ultrastructural investigation at two levels, 30 μm deep from the surface and 70 μm from the surface. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under the electron microscope (Hitachi H600). At high magnification of 25,000 X, all somas and dendrites were carefully examined for synaptic contacts. Synapses were identified by the following criteria: clear synaptic cleft with post synaptic density and a minimum of three synaptic vesicles aggregating close to the synaptic site at the presynaptic terminal, with or without presynaptic density. Synaptic contacts were counted to determine the frequency of both somatic and dendritic synapses. Adrenergic and cholinergic boutons and synapses were counted and the

frequencies of both boutons and synapses of either type were determined. The number of somatic profiles showing signs of transneuronal degeneration (defined in this study as any somatic profile that has one or more of the following characteristics: 1- appearance of intracytoplasmic vacuoles, 2- dilatation of endoplasmic reticulum, 3- separation from surrounding satellite cells, 4- accumulation of large aggregation of intracytoplasmic glycogen granules) was counted.

2. Methods for investigating small intensely fluorescent (SIF) cells.

Five dogs were anesthetized with sodium pentobarbital (35 mg/Kg, IV) and the same steps as above were followed until the removal of the PVFP, which was washed in Ringer's solution for 30 seconds. To ensure fast fixation of the ganglia embedded inside the fat pad, 2-3 cc of fixative (2% paraformaldehyde, 2% glutaraldehyde, 4% polyvinylpyrollidine, 0.1% CaCl buffered to pH 7.3 with 0.67 sodium cacodylate at room temperature) were injected into the fat pad. The PVFP was put in the same fixative for 18 hours, after which individual ganglia were removed from the fat pad using a dissecting microscope, placed in 50% glycerol in H_2O, and examined under a fluorescent microscope to identify the highly fluorescent monoaminergic SIF cells. Ganglia which contained SIF cells were photographed to identify the exact location of SIF cells in the ganglia so

that ultrathin sections could be cut from that area for electron microscopic examination of the SIF cells. Ganglia were removed from glycerol and rinsed in several changes of cacodylate buffer for one hour, post-fixed in 1% osmium tetroxide in 0.M cacodylate buffer (pH 7.3) for 90 minutes at 4^0C. Ganglia were then dehydrated and embedded in Epon. Thick sections (1 mm) were cut and examined at the light level until SIF cells were identified, then 10-15 semiserial ultrathin sections were cut, stained and placed on one-hole grids coated with formvar. Sections were examined for any synaptic contacts. At the ultrastructural level the types of SIF cells, types of cytoplasmic vesicles, synaptic contacts, as well as the relationship between SIF cells and ganglionic blood vessels, were investigated. Two individual cells of type II SIF cells and two groups of type I SIF cells were examined.

3. Methods for electron microscopic examination of the synaptic vesicles in canine cardiac ganglia after 5-hydroxydopamine administration.

For ultrastructural labelling of adrenergic terminals, two dogs were anesthetized with sodium pentobarbital (30 mg/kg IV), then injected with 50 mg/kg 5-hydroxydopamine IV (a method described by Tranzer and Thoenen, 1967). The dogs were sacrificed 10 minutes after the injection and three areas were removed: vas deferens and/or part of spleen capsule and PVFP. All the tissues were processed for

regular ultrastructural examination, with spleen capsule, which contains pure adrenergic terminals, serving as positive control, and vas deferens, which contains both adrenergic and cholinergic terminals, serving as both positive and negative controls for the cardiac ganglia.

4. Methods for selective and total cardiac denervation:

A. Total cardiac denervation

Seven dogs of either sex were anesthetized with sodium pentobarbital (30 mg/kg i.v.), supplemental doses of same anesthetic were administered as required. After intubation respiration was maintained with a positive-pressure respirator. Under sterile conditions the right fourth intercostal space was opened, and the pericardium exposed by retracting the lung medially and caudally. The left thoracic vagus, right thoracic vagus and left and right stellate ganglia including the two ansae branches (anterior and posterior), were identified and freed from surrounding tissue. Electrical stimulation was done to ensure the identification of the structures. A bipolar electrode was used to stimulate the isolated nerves (10 Hz, 5 msec, 5V for sympathetic stimulation and 20 Hz, 5 msec, 5 V for parasympathetic stimulation) while recording heart rate and electrocardiographic responses to stimulation using lead II electrocardiogram. The pericardium was opened, and all nerves were cut along their intrapericardial pathways around the adventitia of the great blood vessels. The first stage

of denervation started by cutting the ventrolateral cardiac nerve, then stripping all the adventitia at the bifurcation of the pulmonary artery and its branches. In the second stage, all nerves between the aorta and the main pulmonary artery were cut. Finally, all adventitia around the superior vena cava were dissected and cut, and the azygos vein was tied and cut. After denervation was complete, the stimulation procedure was repeated to ensure total denervation of the heart. The pericardium was closed by sutures, the lungs inflated, and the chest closed in layers. The chest was aspirated to create negative pressure, and the endotracheal tube was removed when the dog started to breathe. An analgesic therapy and antibiotics were administered post-operatively for 3-6 days. After 10 day survival, the dogs were reanesthetized with alpha chloralose (100 mg/kg IV), and both sympathetic and parasympathetic stimulation were repeated to determine the degree of denervation. If any stimulation-induced change in the heart rate or electrocardiogram (P wave shift) occurred indicating incomplete denervation, the animal was not used in the study (two dogs). The pulmonary vein fat pad (PVFP) from successfully denervated hearts (five dogs) were then removed, and the ganglia were used for HRP injections.

B. Bilateral cardiac parasympathectomy:

Six dogs of either sex were used for parasympathetic denervation. Selective parasympathetic denervation was

accomplished according to the method of Randall and Ardell (1985). The chest was enetered through a thoractomy in the right fourth intercostal space followed by identification and stimulation of the right and left thoracic vagi and the corresponding ansae of the right and left stellate ganglia. The denervation protocol proceeded in a rostral codal direction and included dissection of the pericardial reflection distally along the dorsal surface of the superior vena, then dissection of all the adventitia around the superior vena cava followed by tying and cutting the azygos vein. The dorsal atrial tissues between the right and left pulmonary veins, including the ventral surfaces of the descending right pulmonary artery. After denervation was completed, electrical stimulation of the vagal and sympathetic nerves were repeated to verify that chronotropic responses to vagal stimulation was completely abolished while chronotropic responses to sympathetic stimulation was intact. After a 10 day survival, the dogs were reanesthetized with alpha chloralose (100 mg/kg IV), and tested to determine the degree of denervation. Only PVFP from dogs with successful selective parasympathetic denervation were removed and dissected for their ganglia (five dogs). All experimental procedures were conducted in strict accordance with the standards established by the American Physiological Society for the care and use of laboratory animals.

5. Data analysis

Different types of boutons and synaptic contacts were counted and the percentage of different types were calculated. Bouton and synaptic density were calculated and groups were compared using mean values ± the standard error of the mean (SEM).

Results

Normal ultrastructure of cardiac ganglionic neurons:

The canine cardiac neurons of the PVFP showed typical features of autonomic neurons. Although the neurons varied greatly in size, their ultrastructural features were not different. They all had centrally or eccentrically located nuclei and one or two prominent nucleoli (Fig. 1A). Their cytoplasm contained numerous mitochondria and large collections of ribosomes and rough endoplasmic reticulum (RER) that occasionally were organized in stacks to form Nissl substance (Fig. 1 A,C). The Golgi apparatus was well developed and usually surrounded by several vesicles of different sizes. Lysosomes of spherical shape were commonly seen and were either electron-lucent or electron-opaque with lipofuscin like granules.

All somatic and dendritic profiles were covered by one or more layers of satellite processes except where synapses or dendro-dendritic or SIF cell contacts were made. The

relationship between the satellite processes and the neuronal components were regular and smooth with no wide separation between them (Fig. 1A,B). The somatic profiles showed a few somatic spines, but no somatic or perisomatic synaptic contacts were seen on the soma or its spines (Fig. 1B).

The dendrites characteristically had an irregular shape with many appendages and spines that arose either from the appendages or directly from the shaft of the dendrites (Fig. 2A). All synaptic contacts were seen on the dendritic trees, with the majority of them on the shaft and the rest on the spinous processes (Fig. 2A,B). The density of synaptic contacts (mean ±SEM) was 3.4±1.5 synapses/10.000μm^2, whereas the density of boutons, whether making synapse or not, was 8.8±3.8 /10.000μm^2 (Table 1). All boutons contained clear vesicles of 40-50 nm in diameter that were spherical or elongated in shape (Fig. 2 A,B,C,). However, in addition to the small clear vesicles, a large number of these boutons (40.83% ±8.01) contained a few, large, dense-core vesicles (70-80 nm) as well. None of the presynaptic boutons were of the adrenergic type with small dense-core vesicles. A common profile seen in the canine cardiac ganglia was a large multivesicular body that contained a large number of clear vesicles, granular vesicles, mitochondria, and several large myelin bodies (Fig. 3). This profile, which is considered characteristic

of afferent nerve terminals, continued to exist after both selective and total cardiac denervation.

After pretreatment with the adrenergic marker (5-hydroxydopamine), no identified adrenergic boutons were seen in these ganglia (Fig. 4A), although such treatment clearly allowed identification of adrenergic boutons in the spleen and vas deferens (Fig. 4B,C).

Although the synapses were of the axodendritic type and the presynaptic elements were typical of cholinergic boutons, on some occasions atypical bouton-like structures which contained less numerous vesicles and mitochondrial profiles and possibly fragments of RER were seen. These bouton-like structures were in contact with the dendritic spines, with some vesicles close to what appears to be an active synaptic zone (Fig. 2C). Such structures could be sites of dendrodendritic synaptic contacts.

Another common feature seen in the cardiac ganglia was the attachment plaque (Fig. 2 b,c) that consists of symmetrical densities along the cytoplasmic surface of the opposing dendritic membranes. The two cytoplasmic membranes were separated by a space of 18-20 nm. Attachment plaques can appear in multiples of two or three or as a single long structure. Although most attachment plaques were found between dendritic profiles in association with synaptic contacts, a few were seen between the membranes of satellite cells and the ganglionic neurons.

SIF cells were seen in 55% of canine cardiac ganglia as identified by light microscopy. Ultrastructurally, SIF cells were identified by their numerous large dense-cored vesicles that were scattered in the cytoplasm. The vesicles were about 100-150 nm in diameter with a dark dense core that occupies most of the vesicle (Fig. 5 A,B). Type I SIF cells appeared in aggregates of 5 to 10 small non-branched neurons that were closely related to blood vessels (Fig. 5A). These blood vessels were fenestrated at the sites of close approximation of the endothelium and the SIF cells (Fig 5B). In this area the SIF cell lost its covering of satellite processes and came in direct contact with the fenestrated endothelium, suggesting a secretory output into the blood supply of the cardiac ganglion.

Type II SIF cells were always found as single cells that were scattered among the principal ganglionic neurons. They were covered with satellite processes, except occasionally, where they came in close contact with the principal ganglionic neurons (Fig. 5C) or with preganglionic terminals (Fig. 5D). However, no synaptic contacts between the SIF cells and the ganglionic neurons were seen. However, this may be due to the difficulty in sampling these cells.

Effect of preganglionic denervation on the ultrastructure of the ganglia:

After 10 days of denervation, boutons or synaptic specializations were seen only rarely in the cardiac ganglia. After total denervation, the bouton density dropped from 8.8±3.8 to 0.12 ±0.14/10.000μm^2, while the synaptic density dropped from 3.4±1.5 to 0.01 ±0.32/10.000μm^2 (Table 1). After parasympathetic denervation the bouton density dropped to 0.06±0.10 /10.000μm^2, and the synaptic density was zero (Table 1). The afferent terminals did not disappear after preganglionic denervation and their morphological characteristics were not affected by the denervation. However, they were less commonly seen. Similarly, several myelinated axonal profiles continued to exist after denervation.

Following 10 days of preganglionic denervation, a gradation of transneuronal ultrastructural changes often occurred in the principal ganglionic neurons and in their relationship to their satellite cells. Most principal ganglionic neurons appeared normal except for some increase in the cytoplasmic glycogen content. Mildly affected neurons showed increased electron density of the cytoplasm, with an increase of free ribosomes and the absence of any Nissl bodies. Some small empty vacuoles in the periphery of the cytoplasm were observed (Fig. 6B) along with very large aggregations of glycogen particles (Fig. 6A). The severely affected neurons had a markedly dilated endoplasmic reticulum leading to a vacuolated appearance of the neuron

(Fig. 7A) The nucleus seemed to be highly convoluted but with normal content and an intact nuclear membrane (Fig. 7A). Other severely affected neurons lost their smooth regular contour, and their cytoplasm became very dense and vacuolated. However, their most characteristic feature was a separation from the satellite processes and degeneration of the dendrites (Fig. 7B,C). After total and parasympathetic denervations, signs of transneuronal degeneration were noted in 7.3±3.6% and 9.7±7.0% of the total somatic profiles, respectively.

Discussion

Preganglionic innervation of canine cardiac ganglia:

The results demonstrate that canine cardiac ganglia are predominantly parasympathetic. They receive vagal innervation but lack sympathetic innervation. The preganglionic innervation consists of boutons with clear vesicles and boutons with mixed clear and dense-cored vesicles. Both types of boutons make their synaptic contacts on the dendritic shafts and spines, with the majority on the shafts. No synapses were seen on the somas. The cholinergic nature of preganglionic innervation of the cardiac ganglia shown in this study is in agreement with previous anatomical reports indicating the consistent absence of fluorescent adrenergic terminals within the

cardiac ganglia of monkey, rabbit, guinea pig, and dog (Osborne and Silva 1970, Angelakes et al., 1963, Dahlström et al., 1965, Ehinger et al., 1968, and Malor et al., 1974). However, other reports have shown that cardiac ganglia in rat, cat, guinea pig and mice do receive adrenergic fibers (Jacobwitz 1967). Furthermore, Papka (1976) reported that cholinergic terminals make axosomatic synapses while adrenergic terminals make axodendritic synapses on the neurons of the cardiac ganglia of rabbit. In addition, cardiac ganglia of rat, rabbit and guinea pig have been reported to receive cholinergic, adrenergic and afferent synaptic contacts, with the adrenergic synaptic contacts seen only on the dendrites (Shvalev and Sosunove 1985). None of these studies, however, attempted to quantify the percentage of adrenergic boutons in relation to the total number of identified boutons. Without quantification it is difficult to determine whether the conflicting results are due to the rare presence of adrenergic terminals which are easily missed when sampling the tissue or due to high variability between different ganglia or animals.

The canine cardiac ganglia have been the subject of similar conflicting reports regarding their electrophysiological properties. For example, extracellular recordings of canine ganglia find that stimulation of sympathetic nerves, parasympathetic nerves, or limited regions of the heart will activate the principal ganglionic

neurons, indicating that canine intracardiac ganglia can be modulated by sympathetic, parasympathetic and afferent terminals (Gagliardi et al., 1988, Armour and Hopkins 1990, Ardell et al., 1991). Intracellular recordings of canine intracardiac neurons have shown them to respond to Ach through nicotinic and muscarinic cholinergic receptors (Xi et al., 1991, Xi-Moy et al., 1993); no similar responses to adrenergic agonists have been investigated.

The present ultrastructure study indicates that terminals typical of sympathetic nerve endings are absent on these neurons. It is difficult to explain why we could not see any sympathetic terminals when extracellular recordings show that sympathetic nerves can influence the ganglia. It might be that some of the ganglionic neurons are sympathetic or that they may even have a dual sympathetic and parasympathetic nature and are capable, through atypical synaptic effects ,e.g., dendro-dendritic contacts, of producing the sympathetic effects seen by Yuan et al.(1991). Supporting this idea, in the immunocytochemical study of this dissertation some of the ganglionic neurons stained positively for TH and DBH.

Location of synaptic contacts:

The present study shows that the canine intracardiac neurons receive all their synaptic contacts on their dendrites. However, there is considerable variation in the type and location of these contacts on the dendritic tree.

The synaptic contacts were seen on large shafts, indicating close proximity to the soma and suggesting that they would have powerful influence on the physiological response of the neuron. Other contacts were seen on small shafts and on dendritic spines of different shapes and sizes, indicating that a larger summation is needed for these synaptic contacts to cause stimulation of the neuron. In addition, both symmetrical and asymmetrical synaptic contacts were seen. Although these specialized contacts were not quantified, their existence indicates that the cardiac neurons are not a simple monosynaptic relay station but, rather, have high integrating capabilities. In agreement with the ultrastructural variability of canine intracardiac neurons, a variety of electrophysiological characteristics suggest considerable complexity, with an array of different fast and slow excitatory and inhibitory responses involving spatial summation (Xi et al.1991; Xi-Moy et al. 1993).

Intraganglionic neuronal contacts:

The dendro-dendritic contacts seen regularly in the ganglia both, before and after cardiac denervation may be sites of intraganglionic communication between the neurons and may add to the integrating ability of these neurons. Although these contacts are not classic synaptic contacts, they still could be sites of chemical interaction between the neurons (Matthews 1983). Similar dendro-dendritic synapses have been suggested in other autonomic ganglia,

e.g., in rat superior cervical ganglia (Elfvin 1971; Kondo et al. 1980). Also, the neurons of autonomic ganglia have been shown to form synaptic contacts upon each other in cell culture (Rees and Bunge 1974). Electrophysiological studies are needed to determine the possible synaptic function of these dendro-dendritic contacts.

Attachment plaques were quite common in the canine cardiac ganglia. This non-synaptic contact usually involved dendrites, spines and presynaptic terminals in different combinations within the ganglia. Although the function of such symmetrical attachments is not clear, a mechanical supportive role has been suggested in the superior cervical ganglion (Matthews and Raisman 1969, Matthews 1983). The large number of these plaques in canine intracardiac ganglia may serve to stabilize synaptic contacts because of the need for greater mechanical stability in a beating heart.

Afferent terminals in the cardiac ganglia:

Accumulations of a large numbers of vesicles, mitochondria, and lamellated dense bodies (myelin bodies) are the distinguishing features of afferent nerve terminals of peripheral sensory receptors (Böck and Grogas 1976; Chiba 1972). The existence of afferent terminals in control dogs and the persistence of these terminals after total cardiac denervation suggest the existence of intracardiac afferent reflexes. In agreement, Ardell et al. (1991) demonstrated that canine cardiac ganglia of chronically decentralized

hearts can be activated by touching specific areas of the heart. In addition, Moravec and Moravec (1987) demonstrated that some cardiac neurons in rat project peripherally to the atrioventricular node where they form sensory capsule-like bodies. They also proposed that some intracardiac neurons may have a sensory function and may be involved in a proprioceptive feedback loop in the heart similar to that in the gut, which might explain the autorhythmic phenomena of the totally denervated heart.

SIF cells in canine cardiac ganglia:

The existence of SIF cells in only 55% of the ganglia adds to the variable morphology of the ganglia. The functional role of SIF cells in canine cardiac ganglia is not clear, but the fact that type II SIF cells receive preganglionic cholinergic innervation and have close contact with the principal ganglionic neurons suggests that these cells can play the role of interneurons. As interneurons, type II SIF cells may have an inhibitory feed-forward action on the principal neurons following preganglionic stimulation (Yamauchi 1975). Type I SIF cells, on the other hand, may have an endocrine or chemoreceptive function within the ganglia. As endocrine cells, Type II cells can affect the neurons by diffusion or by influencing the fenestrated blood vessels lying next to them (Mekhail et al., 1990). However, the exact roles played by SIF cells in canine cardiac ganglia will require further investigation.

Transneuronal degeneration:

Transneuronal degeneration of some principal neurons was evident in this study after 10 days of total or selective preganglionic denervation. Such a phenomenon is more conspicuous in the central nervous system. Among the signs of such trans-synaptic degeneration are shrinkage of the nucleus, increased electron density of cytoplasm, swollen mitochondria, dilated rough and smooth endoplasmic reticulum, darkened dendrites, and an increased amount of free ribosomes (Knyhár-Cisillik et al.,1989). The extent of these changes depends on several factors including age, species, size of the neuron and extent of denervation (Matthews et al.,1960, Wiesel and Hubel, 1963, Knyhár-Cisillik et al.,1989).

Similar changes have been reported in autonomic ganglia. For example, preganglionic denervation of the superior cervical ganglia in rabbit caused shrinkage of the cells and reduced the cross-sectional area of the neuron (Hamlyn 1954). In addition, ultrastructural studies on denervated cardiac ganglia of non-human primates showed early, wide-spread glycogen accumulation in ganglionic neurons. This was followed by intense darkening of the dendrites and vacuolization in 10% of the principal ganglionic neurons, indicating the occurrence of some transneuronal degeneration in cardiac ganglia (Tay et al., 1984; Wong et al., 1987).

In the present study, the appearance of glycogen in many of the neurons after denervation was striking. Glycogen particles were rarely seen before denervation. The cellular mechanism causing this increase of glycogen is not clear. Perhaps deafferentation causes a decrease in the metabolic rate of stressed neurons, allowing glycogen to accumulate (Ibrahim et al. 1968). Glycogen accumulation may be triggered by withdrawal of a factor released from the afferent terminals (Wong et al. 1987). Deafferentation may lead to a Ca^{2+} influx that causes activation of calmodulin, which indirectly activates glycogen synthase that will lead to increase in glycogen accumulation (Maxwell et al. 1990).

Vacuolization of the cytoplasm to different degrees and occasional total vacuolation and degeneration of some dendrites, were seen after total and selective denervation. Vacuoles are probably widened saccules of endoplasmic reticulum. Whether these vacuolated neurons will survive or eventually degenerate awaits further study.

Increased intercellular spaces and wide separations between the affected principal ganglionic neurons and the surrounding satellite cells were usually seen after denervation. Similar observations were reported in superior cervical ganglia after preganglionic sympathectomy (Chang et al. 1976), but only a slight ruffled appearance of the plasma membrane was seen in the denervated cardiac ganglia of monkey (Wong et al. 1987). Unlike the monkey (Wong et

al. 1987), canine satellite cells did not show signs of reactivity and phagocytosis. However, the increased separation between the satellite cells and the principal ganglionic neurons would be expected to interrupt the blood-ganglion cell barrier formed by these cells (Ten Tusscher et al. 1989). Whether this barrier change is the cause or the result of cellular changes following deafferentation is unclear.

Several other causes for transneuronal degeneration have been suggested, such as depression of nucleic acid metabolism (Cook et al., 1951), dependence of these neurons on incoming trophic factors, indirect cellular reactions to degenerating terminals (Tay et al., 1984), and voltage dependent synaptic activity (Benshalom, 1989). Although these factors may all contribute to the observed degeneration, it is not clear why only a small number of the neurons become affected by the denervation. It may be that the dendrodendritic contacts are important to neuronal survival, in that neurons with fewer contacts may be more susceptible to degeneration.

In conclusion, canine cardiac ganglia have been shown to receive parasympathetic and afferent terminals and do not receive any sympathetic terminals. Although the parasympathetic terminals are mainly extracardiac in origin, the afferent terminals were partly of intracardiac origin. The possibility of functional intracardiac contacts is

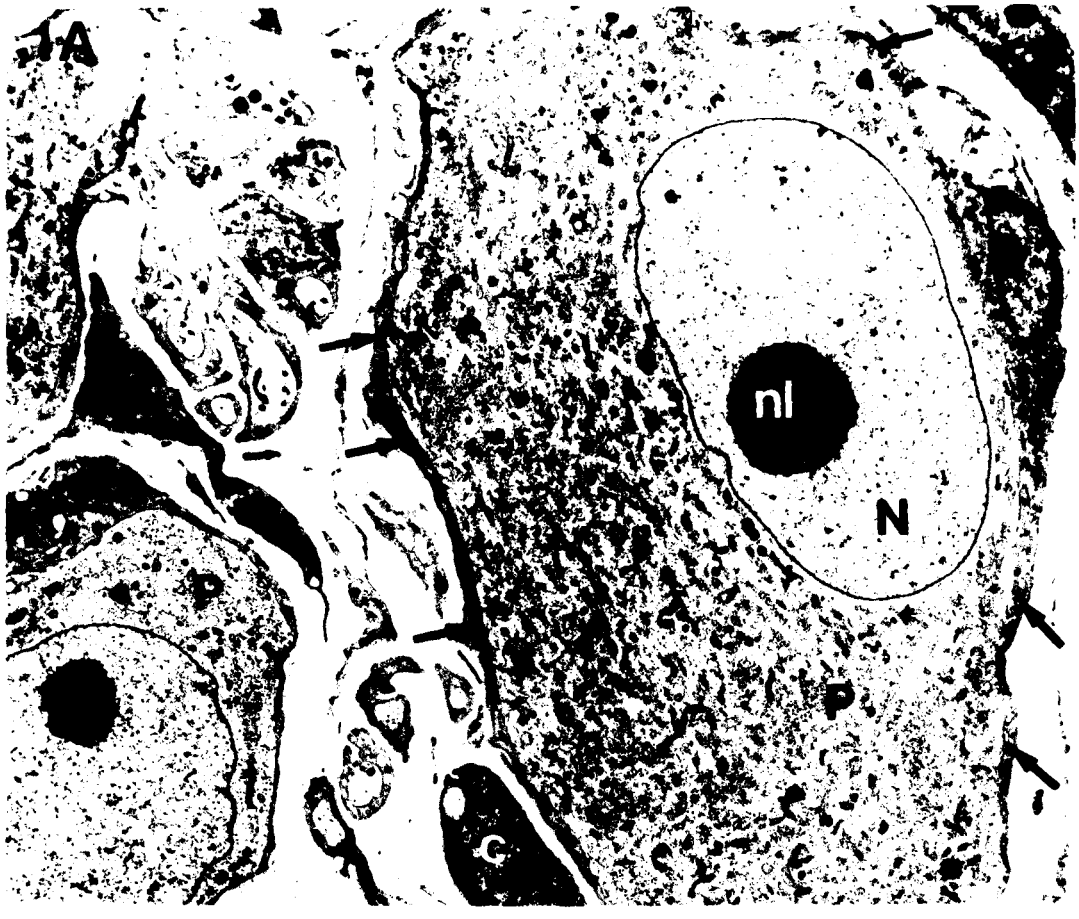
suggested by dendrodendritic synaptic contacts. In addition, SIF cells, both type I and type II, can influence the intracardiac neurons. Signs of transneuronal degeneration occur in some of the principal neurons, but the eventual degeneration or survival of these neurons needs further investigation.

Figure 1.

A. An Electron micrograph at low magnification showing two nucleated principal ganglionic neurons (P). The nucleus (N) is spherical in shape, shows few indentations, and contains homogenous karyoplasm and a prominent nucleolus(nl). Each perikaryon is completely ensheathed by satellite cells (S) sending their processes (arrows) to cover the somatic profile. Many axonal profiles can be seen surrounded by Schwann cells (C). X 6000.

B. Higher magnification of a somatic profile with somatic spines (open arrows) on which no synapses occur. Solid arrows show the satellite processes as they completely cover the principal ganglionic neuron. X 13,000.

C. Nissl bodies (N) as seen in some of the neurons before denervation. They consist of stacks of 3 to 8 saccules which are usually in close relation to the mitochondria (M). X 13,200.


A
nl
N
P
P
C


Bo


M

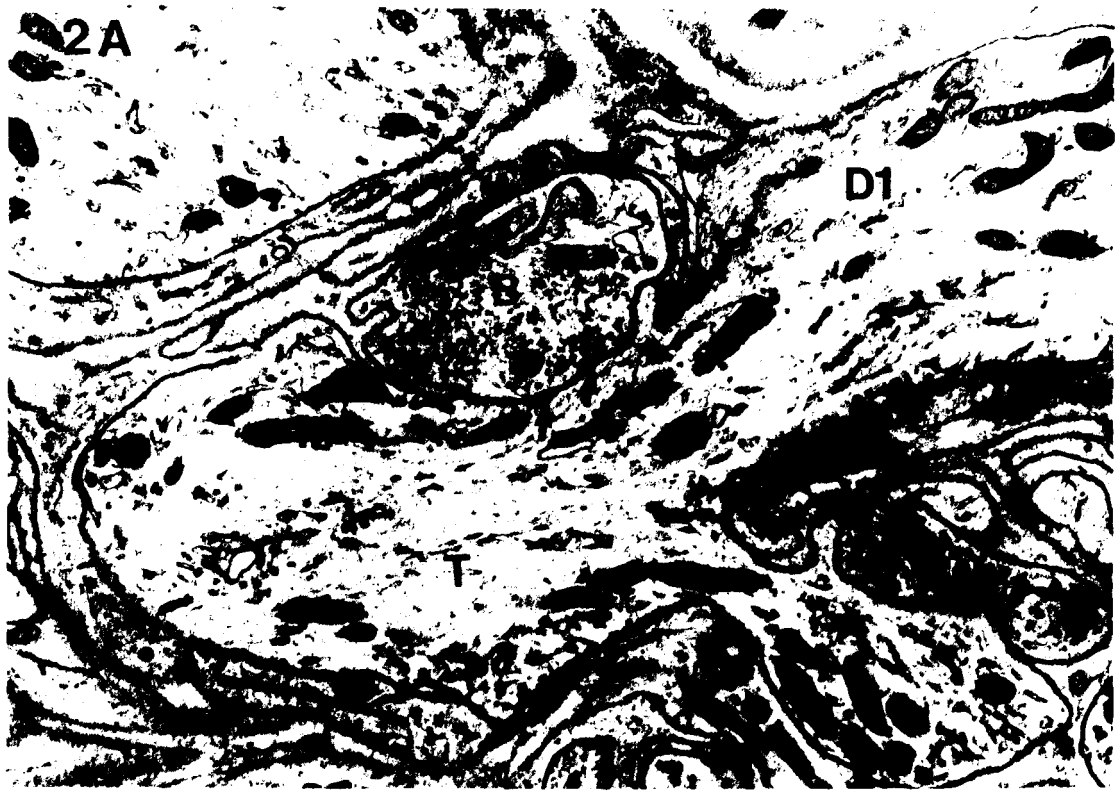
Figure 2.

A. A dendritic profile (D1) of a ganglionic neuron showing a spine (arrowhead) and an appendage (A) that also shows a spinous process (open arrow). Mitochondria (M) and microtubule (T) are seen in both the dendrite and the appendage but not in spines, which only contain a small amount of filamentous material. Synaptic contact of a preganglionic bouton (B) can be seen mainly on the shaft of the dendrite with several focal dense points (arrows). The appendage and its spine seem to be in close contact with possibly another dendrite (D2) without any satellite processes separating them. X 15,000.

B. A commonly seen configuration of a symmetrical synaptic contact between a preganglionic bouton (B) and a dendritic process (D1) and its spine (arrowhead). An attachment plaque can be seen between postsynaptic profile (D1) and another dendritic profile (D2) that in turn is forming attachment plaques with a third dendritic process (D3). Notice that all neurite elements are covered by several layers of satellite processes except in areas devoted to synaptic contacts or attachment plaques. X 24,000.

C. A synaptic configuration that was seen less commonly, suggesting a dendrodendritic synapse. The presynaptic element (B) has very few clear synaptic vesicles and

contains elements that are characteristic of dendritic profiles such as small profiles of RER and attachment plaques. Three areas of specialization could be detected in this contact: an asymmetrical synaptic contact on the apex of the spine (black arrowhead), another one at the neck of the spine (open arrowhead), and a long attachment plaque (short arrows) between the bouton and the shaft of the dendrite. A subsurface cisterna (large arrow) that consists of one smooth layer that faces the inner side of the plasma membrane and a ribosomal layer facing the inside of the cell can be seen. X 30,000.


2A
D1
B
T


2C

Figure 3.

Afferent terminal seen in a totally denervated heart. The terminal is characterized by a heterogeneous group of vesicles (small arrows), mitochondria (M), and myelin bodies (large arrows). X 29,000.

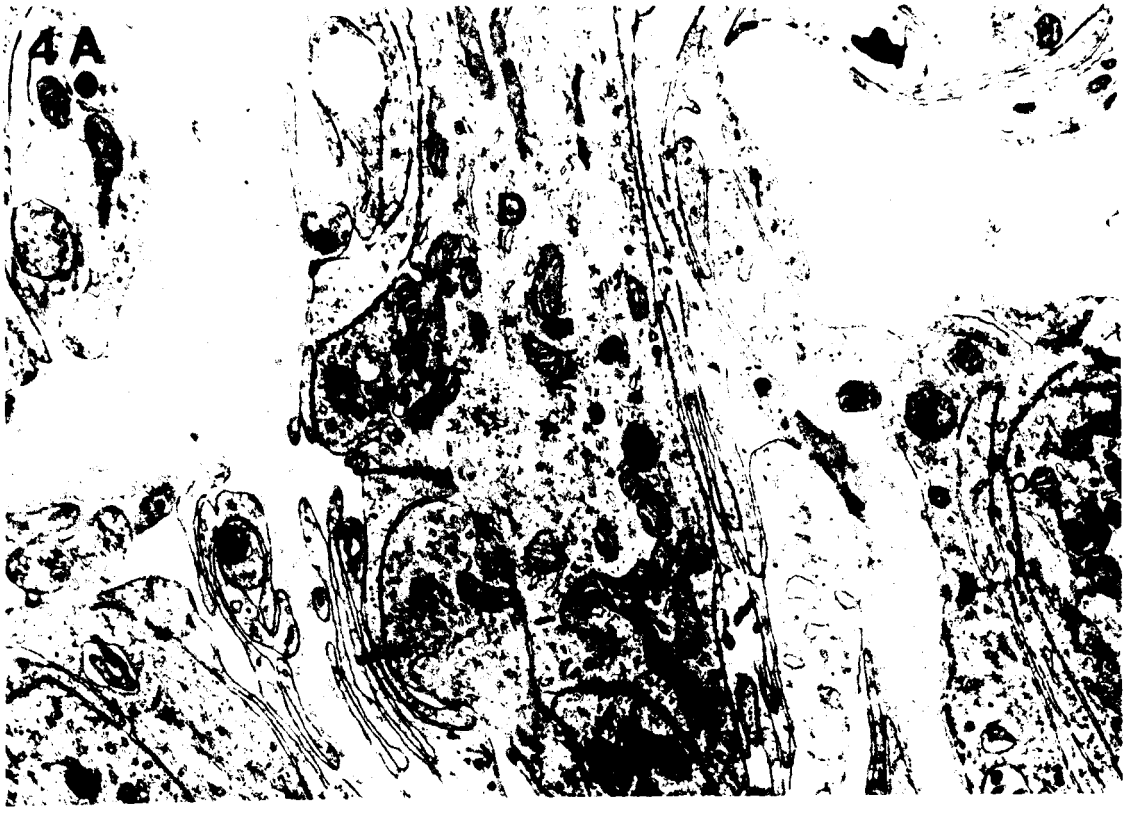

3

Figure 4.

A. Dendritic profile (D) receiving a synaptic bouton (B) in canine cardiac ganglion after administration of 5 hydroxydopamine. The ultrastructural marker did not have any effects on the contents or the shape of synaptic vesicles (arrow) in the boutons of the ganglion. X 29,000.

B. The adrenergic terminals of the spleen of the same dog are heavily labelled with extremely osmiophilic dense-core vesicles (arrows). X 42,000.

C. The vas deferens of the same dog contains both clear cholinergic (arrows) and small adrenergic dense core vesicles (open arrow) boutons. X 30,000.


4A
D


4B


4C

Figure 5.

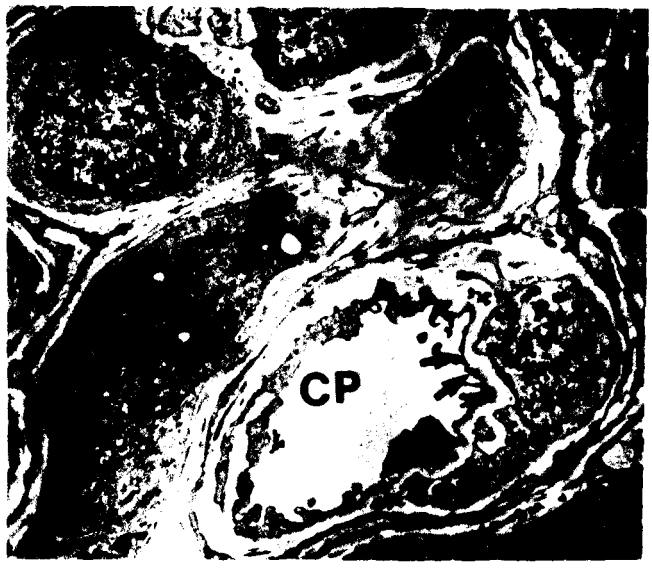
A. Type I SIF cells as seen in close contact with a capillary (CP) in a cardiac ganglion. Three SIF cell profiles (1,2,3) surround the capillary. One of the profiles (3) is in direct contact with the fenestrated capillary endothelium (arrows). X 7000.

B. High magnification of SIF cell profile 3 in Fig. A. It shows a satellite cell process (open arrows) stopping as the SIF cell comes into direct contact with the fenestrated endothelium (solid arrows). Here the basal lamina is the only barrier between the neurosecretory cell and the blood supply of the ganglion. X 14,300

C. Type II SIF cell (S) in direct contact (arrows) with a principal ganglionic neuron (P) without synaptic specialization. X 37,400.

D. A cholinergic bouton (B) in close contact with a type II SIF cell (S). Notice that the whole membrane of the SIF cell is separated from the neuropil by satellite processes except where the SIF cell sends flanges around the bouton. The inset depicts a higher magnification of that contact showing a small density (arrow) at the line of contact between the bouton and the cell. The aggregation of vesicles close to this contact suggests the possibility of

synaptic specialization. X 15,200. (Inset: X 28,900.)


CP


5B


S
P

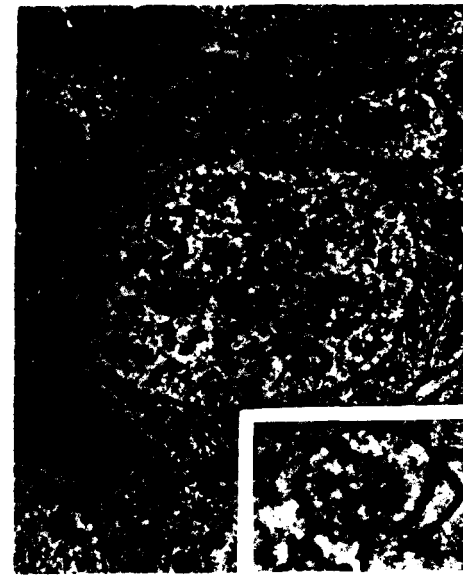


Figure 6.

A. An electron micrograph of a denervated neuron (P), showing a very common reaction consisting of the peripheral accumulation of particles (G) the size of glycogen (17-30 nm in diameter). These particles extend into the dendrite (top left). However, no particles are seen in the satellite cell (S). The inset is a high magnification of glycogen granules. X6800. (Inset: X 42,500.)

B. An example of the vacuolization of ganglionic neurons. Small vacuoles can be seen in the middle of the neuron, whereas large vacuoles (V1,V2) are typically close to the periphery of the neuronal profile. The inset shows that such vacuoles are not membrane limited. Notice that at this stage of reaction to denervation the satellite cells are still in close contact with the neuron and only a few separations can be seen (arrows) with some pyknotic material inside them. X 4200. (Inset: X 13,600.)

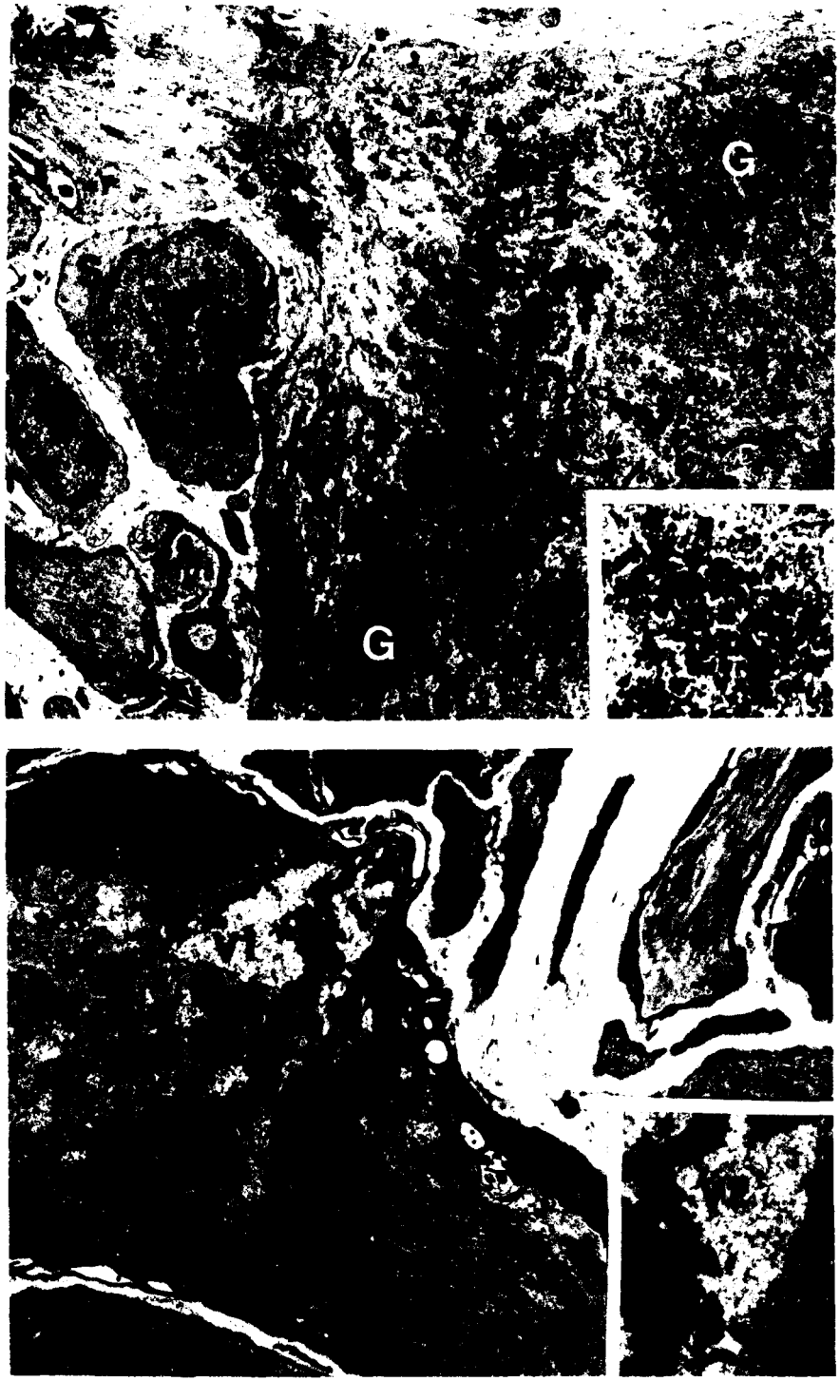
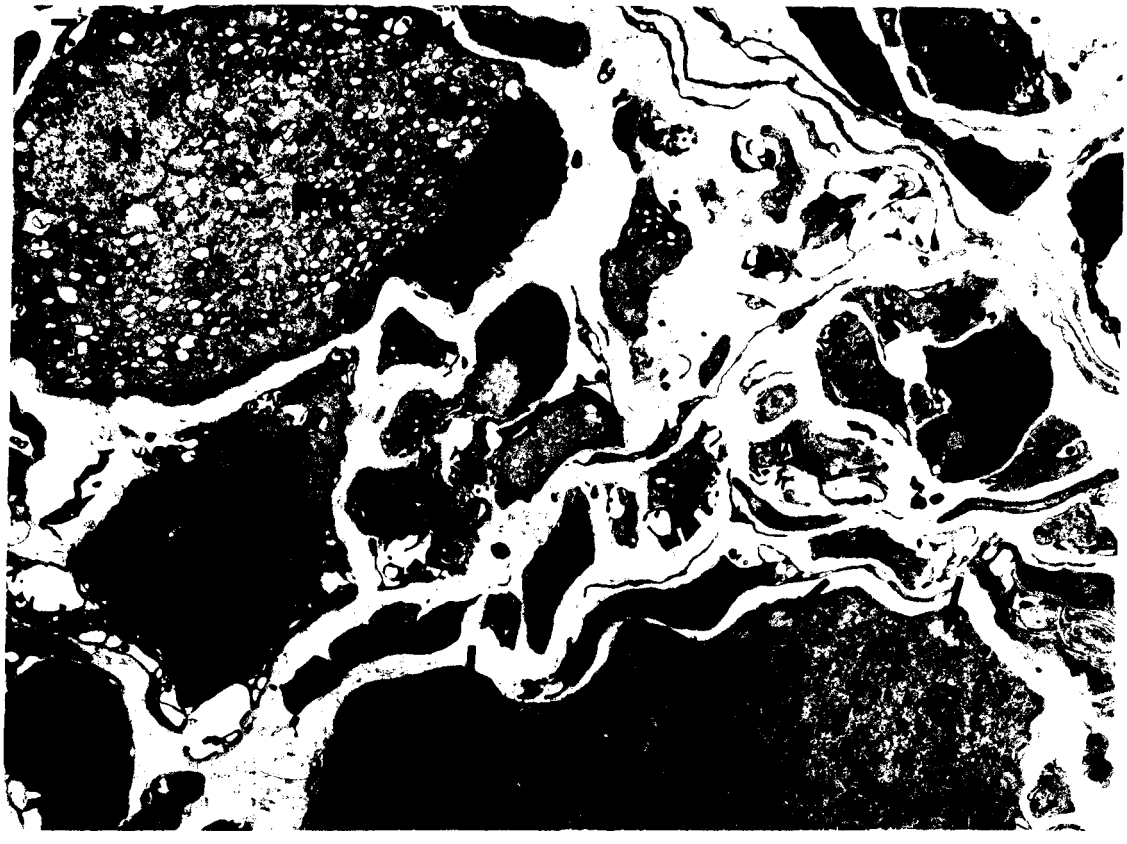

G
G

Figure 7.

A. Low power electron micrograph to show the gradation of response to denervation. The upper neuron (P1) shows severe cytoplasmic vacuolation, probably due to dilatation of its endoplasmic reticulum; its nucleus (N) is highly convoluted. The lower neuron (P2) does not seem to be affected by the denervation except in the formation of small, scattered vacuoles (arrows) near the plasma membrane. The dendritic profile (D) in between the neurons shows considerably more separation from its satellite processes. X 4000.

B. Example of severely affected neuron with a completely degenerating dendrite (D) that is totally vacuolated with no identifiable organelles. X 3500.

C. Another degenerating dendrite (D) is surrounded by either a macrophage or an active satellite cell. The other two neuronal profiles show the typical separation (arrows) between the neurons and the satellite processes and the usual increase in electron density of these neurons. X 3800.


7


7B


7C

Table 1.

The table summarizes the synaptic density per 10.000 μm^2 and the bouton density in the three groups of dogs. Notice that virtually all cholinergic boutons disappear after denervation.

Table 1

Synaptic and bouton density in cardaic ganglia

Group	Synaptic Density (mean ± SEM)	Bouton Density (mean ± SEM)
Control	3.4±1.5	8.8±3.8
Total Denerv.	0.0±0.32	0.12±0.14
Parasymp. Denerv.	0	0.06±0.10

CHAPTER VI

GENERAL DISCUSSION

The purpose of this study was to identify the anatomical features of canine cardiac ganglia with and without intact connections to the central nervous system. The experiments addressed a number of questions:

Are these ganglionic neurons purely cholinergic or do they contain some adrenergic and peptidergic cells?

The results of this study show that canine cardiac perikarya stained positive for both cholinergic (ChAT) and adrenergic (TH,DBH) synthesizing enzymes. The ChAT enzyme could be detected without the need for pretreatment of the ganglia with colchicine, indicating that ganglionic neurons contain high concentrations of the enzyme. On the other hand, the adrenergic enzymes (TH and DBH), as well as other tested peptides (NPY, VIP, SP, galanin) could not be detected in the principal neurons without prior colchicine treatment. It is very interesting to see the expression of all these enzymes and peptides after colchicine treatment, but, presuming this is not an artefact due to the effects of

colchicine, the appearance of these peptides indicates a very complex system within the cardiac ganglion. The possible existence of a complex intrinsic cardiac neural network is in agreement with other immunocytochemical studies that have shown that some cardiac ganglionic neurons of the rat may be adrenergic since some neurons stain positive for TH, DBH and NPY; other cardiac neurons appeared to be sensory neurons because they formed sensory, capsule-like bodies in the atrioventricular node (GU et al., 1984; Moravec and Moravec, 1984, 1987, 1989, 1990). In addition, there is physiological evidence that suggests that canine cardiac neurons contain not only parasympathetic neurons but also sympathetic and some afferent neurons as well (Yuan et al.,1991, 1993) and that these neurons become activated upon stimulation of sympathetic nerves (Armour and Hopkins 1990; Gagliardi et al. 1988).

If the multipotential abilities of canine ganglionic neurons are genuine, one would expect to see positive staining for these enzymes and peptides in the terminals of these neurons after total cardiac denervation of dogs, but recent work has shown that, although VIP positive fibers exist after denervation, there is a 98% decrease in the NE levels in the canine heart (Anderson et al., 1992). Also, cardiac ganglia that survive cardiac denervation have been shown to function in a way similar to parasympathetic neurons, i.e., they decrease AV nodal conduction, heart

rate, and atrial contractility (Priola, 1980). A method to resolve the conflict will be to study canine cardiac neurons using the in situ hybridization techniques to see whether these neurons express mRNA for these peptides; furthermore, local injection of colchicine can be used to investigate whether colchicine can trigger the genes for these peptides.

What is the role of SIF cells in the ganglia?

About one half of the canine cardiac ganglia contained SIF cells. Smaller neurons that resembled type II SIF cells were sparse but stained positive for TH, VIP, 5-HT and galanin. Ultrastructurally, type II SIF cells make direct non-synaptic contact with the principal ganglionic neurons and with the fenestrated blood vessels within the ganglion. This gives the SIF cells some functional importance since they can secrete their products directly into the microcirculation of the ganglia or directly into the intercellular space of neighboring principal neurons, which may affect the sensitivity of the principal neurons. For example, secreted catecholamine may generate catecholamine-mediated postsynaptic inhibitory potentials (slow IPSP's) (Dun and Karczmar 1981b) or perhaps modify the presynaptic input to these neurons. Also, in the ciliary parasympathetic ganglion, 5-HT causes depolarization of the ganglionic cells due to increased membrane permeability to sodium and potassium while causing depression of ACh release

from the presynaptic terminals (Tatsumi and Katayama 1987). On the other hand, in the vesical pelvic parasympathetic ganglia, 5-HT causes an increased release of ACh from preganglionic nerve terminals by acting on the presynaptic 5-HT_1 receptors (Nishimura and Akasu 1989).

How homogeneous are the principal ganglionic neurons in their morphological characteristics?

The findings of this work indicate that ganglionic neurons of PVFP vary considerably in somal size and in their dendritic trees, but that they do not fall into distinctly different subpopulations according to their size only. Whether there is functional importance to the obvious variations in size, shape or architecture of the neurons is not yet clear. Functionally these neurons have three subpopulations (S,N, and R) as reported by Xi et al. 1991. This is similar to the enteric nervous system that has two electrophysiological types of neurons, the S-neurons which are characterized by fast EPSPs and the AH-neurons that have fast EPSPs and long after-hyperpolarization. However, these two types have different morphological characteristics and a lot of variability is seen within each type (Lees 1986; Bornstein et al. 1983).

Do these neurons receive their preganglionic innervation from both sympathetic and parasympathetic limbs of the

autonomic nervous system? Are the synaptic contacts somatic or dendritic ? Are they partly innervated by intraganglionic neurons?

To test for the presence of any sympathetic terminals, three methods were used 1: ultrastructural study of normal cardiac ganglia, 2: administration of 5-hydroxydopamine as an ultrastructural adrenergic marker, 3: selective parasympathetic preganglionic denervation. The results indicate that all the synaptic contacts are made on the shafts and spines of the neuronal dendritic arborization. Labelling with the adrenergic marker indicated that all the preganglionic terminals are cholinergic or peptidergic in nature, with no boutons typical of adrenergic sympathetic terminals ending on these ganglionic neurons. Sympathetic terminals might have been expected because of the fact that TH positive fibers were seen with light microscopy in the ganglia. Perhaps the presence of the TH enzyme in the fibers is unaccompanied by any monoamine synthesis, or perhaps the TH positive fibers are too few to be seen routinely in the sampling for ultrastructural study.

In addition, selective parasympathetic denervation of the heart leads to total disappearance of almost all classical terminals, indicating their extracardiac origin. The absence of such classical terminals after parasympathetic denervation suggests that the intraganglionic interneurons hypothesized by Xi et al.(1991)

do not have any classical axonal terminals and that they perform their interneuronal function through dendro-dendritic contacts. In support of that concept the dendrites of principal ganglionic neurons in the present study showed a remarkable degree of direct dendritic contacts with other dendrites or spines. Some of these contacts might be synaptic in nature. Similar observations have been made in the superior cervical ganglia of rat where about one third of the neurons show dendritic varicosities that contain aggregations of vesicles and make direct contact on non-labelled dendrites that show synaptic specialization, indicating the possibility of dendrodendritic synaptic connections (Kondo et al.1980).

Does transganglionic degeneration takes place after 10 days of cardiac denervation?

Most principal ganglionic neurons do not change in somal size or dendritic configuration in any significant way after 10 days of preganglionic denervation. However, there are significant ultrastructural changes in about 7-9% of the neurons in the form of dilated vacuoles in the cytoplasm and degenerated dendritic profiles and an increase in glycogen content. This is in agreement with denervation studies on non-human primates (Tay et al., 1984, Wong et al., 1987) using unilateral and bilateral cervical vagotomy as a means to partial and total cardiac parasympathetic

denervation. Whether the stressed neurons will survive or not, and whether the apparently normal neurons will function in a normal way that does not depend on the central nervous system, still requires further investigation.

Are there any morphological features that suggest the presence of a local cardiac reflex?

The ultrastructural study of the PVFP ganglia has revealed two interesting characteristics of these ganglia. The first is the existence of myelinated axonal profiles before and after total cardiac denervation. Similar observations were reported in human hearts after 12 years of transplantation (Rowan et al. 1988). The second is the existence of structures typical of afferent terminals that ,also, continue to exist after cardiac denervation. These observations indicate that the ganglia of the PVFP, or those of some other ganglionic plexi in the heart are afferent in nature and that cardiac afferent reflexes may be functional, giving an anatomical explanation for the physiological response of chronically decentralized cardiac ganglia to sensory stimulation of the heart (Ardell et al. 1991).

In summary, the principal ganglionic neurons contain acetylcholine and many of them contain the synthetic enzymes necessary for adrenalin synthesis in addition to expressing several peptides. The ganglia contain afferent terminals that originate from intracardiac neurons and possibly from

extracardiac neurons. There are 5 possible inputs that can influence the state and excitability of the principal ganglionic neurons:

1- Parasympathetic preganglionic input to the dendritic tree of the principal ganglionic neurons may be purely cholinergic or mixed cholinergic and peptidergic.

2- Afferent collaterals of intracardiac origin may innervate the ganglia and, in so doing, form local cardiac afferent reflexes.

3- Dendritic contacts with other neurons that may be principal ganglionic neurons or interneurons are quite numerous and may be functionally active.

4- Direct contact with type II SIF cells may influence neuronal excitability or the preterminal release of neurotransmitters.

5- Indirect contact with type I SIF cells through the release of SIF cell products into the intercellular space or into the microcirculation of blood vessels that supply the neurons may also influence neurons or preterminal activity.

The results of these studies suggest that the organization of the canine cardiac ganglion is complex and that the ganglion is not a simple monosynaptic relay station but, rather, a complex neuronal structure that can survive and maintain a relatively normal internal structure, without intact connections with the central nervous system, but with

an intact potential of modulating cardiac activities.

The effect of colchicine treatment on the interpretation of immunocytochemical staining results:

Colchicine is known to be a microtubule synthesis inhibitor that causes blockage of axonal transport leading to accumulation of amine storage granules in sympathetic somas (Dahlström 1968) and accumulation of acetylcholine esterase enzymes in axons proximal to the site of injection (Krentzberg 1968). Colchicine has been routinely used to study the anatomical distribution of neurotransmitters and different peptides throughout the nervous system. It enhances immunostaining by increasing the amount of peptides and transmitters in the soma, through prevention of their transport from the perikaryon down to the axon terminals. However, this treatment fails to take into account the injurious effects of colchicine on cells and that neurons have a differential susceptibility to colchicine's toxicity. For example, colchicine causes selective degeneration of the granule cell layer of the hippocampal dentate gyrus without affecting the pyramidal cells, degeneration of Purkinje cells and granular cells of the cerebellum, and degeneration of granule cells of the olfactory bulb and most cells of the striatum (Goldschmidt and Steward 1982). Colchicine can cause selective degeneration of the cholinergic neurons in the striatum and medial septal nucleus even with a small

dose of 10 μg (Peterson and McGinty, 1988), and can cause a 60% decrease in levels of ChAT, GABA, and dopamine in striatal neurons (Morelli et al. 1980).

Cortès et al.(1990) reported that colchicine can differentially modify the synthetic activity of neurons, causing an increase in galanin mRNA and depletion of ChAT mRNA in the basal forebrain and striatum, a decrease of TH mRNA in the substantia nigra, and an increase of TH mRNA in the locus coeruleus. Similar results have been seen in the hypothalamic paraventricular nucleus where neurons with VIP mRNA and neurotensin mRNA could only be detected after colchicine treatment (Ceccatelli et al. 1991).

Other types of injury that disrupt transport between neurons and their targets can cause modification of peptide and neurotransmitter expression similar to those seen after colchicine treatment. For example, in the neurons of dorsal root ganglia after crushing of their peripheral processes. These are complex changes in peptide expression leading to an increased number of cell bodies expressing galanin and VIP, a decrease in the number of SP immunoreactive neurons, and no effect on CGRP amount (Villar et al 1989).

CHAPTER VII

SUMMARY

The experiments of this dissertation were designed to examine the immunocytochemical and ultrastructural organization of neurons of the canine cardiac ganglion. The findings of this study were:

1) Canine cardiac ganglia contain large numbers of neuronal perikarya that stain positively for both cholinergic (ChAT), and adrenergic (TH,DBH) synthesizing enzymes. Neurons also express NPY, VIP, SP and galanin. The ganglia contain fibers that stain positively for TH, SP, galanin and NPY.

2) SIF cells stain positively for 5HT, galanin, TH and VIP.

3) The neurons are morphologically one population, with large variations in somal area and dendritic complexity.

4) After 10 days of preganglionic denervation, ultrastructural signs of transneuronal degeneration appear in 7-9% of the neurons, but the denervation does not affect the overall morphological characteristics of the ganglionic

neurons.

5) After 10 days of preganglionic denervation, the variability in somal area between dogs is significantly reduced.

6) The cardiac ganglion contains afferent terminals that do not disappear after denervation, indicating their intracardiac origin.

7) The ganglionic neurons receive cholinergic and peptidergic terminals on the shafts and spines of their dendrites. All such terminals disappear after cardiac denervation.

8) No adrenergic terminals were seen in the ganglia.

9) Dendro-dendritic contacts occurred frequently in the ganglia and could be the site of synaptic contacts.

10) Both type I and type II SIF cells were seen in 55% of the ganglia. Type I SIF cells were seen close to fenestrated capillaries. Type II SIF cells were seen receiving cholinergic synaptic contacts on their soma; these cells were also seen in direct contact with the principal ganglionic neurons.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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