# THE OTIC GANGLION AND ITS CONTROL OF THE PAROTID GLAND

by

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To my parents

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## ABSTRACT

FACULTY OF MEDICINE

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# THE OTIC GANGLION AND ITS CONTROL OF THE PAROTID GLAND

by Doctor Bara Ahmed Kamel Al-Hadithi

The precise localisation of the rat otic ganglion and its neural connections, particularly those related to salivation, have been established by means of dissection and from a study of histological sections taken serially through the head.

The otic ganglion lies medial to the pterygopalatine branch of the internal carotid artery and beneath the trigeminal ganglion from which it is separated by the sphenoid bone. There is no direct connection between the glossopharyngeal nerve and the otic ganglion, as is the case in man. However, a neural connection between the facial nerve and the otic ganglion and a further connection linking the glossopharyngeal with the facial nerve through which the salivatory axons may pass to reach the otic ganglion is described. A comparison is made between the neural pathways involved in parotid secretion in the rat with those believed to exist in man.

Electron microscopy was used to examine the preganglionic nerve terminals in the otic ganglion and the postganglionic nerve terminals in the parotid gland. The majority of nerve terminals observed were morphologically similar to the cholinergic terminals reported by other workers.

Immunofluorescence, immunoperoxidase and immunogold techniques were used to look for the neuropeptides substance P (SP), vasoactive intestinal polypeptide (VIP) and calcitonin-gene-related peptide (CGRP) in both the postganglionic neuronal cell bodies in the otic ganglion and in the axonal terminals of the parotid gland. Both SP and VIP were found in neurones which were positive for acetylcholinesterase activity and which projected to the parotid gland. At the ultrastructural level SP and VIP were found to be colocalised in the same nerve terminals and the immunogold studies suggested that these two peptides may also be present in the same synaptic vesicles. The significance of these results is discussed.

# CHAPTER ONE

# INTRODUCTION AND AIMS OF THE STUDY

# INTRODUCTION

Classical knowledge about the involvement of the autonomic nervous system in the control of secretion from the salivary glands is based largely on the results of dissection, retrograde degeneration and electrical stimulation studies carried out in the early part of the 20th century (Kohnstamm, 1902; Yagita, 1910; Miller, 1913; Magoun and Beaton, 1942; Wang, 1943).

The preganglionic parasympathetic neurones to the parotid gland were located in the inferior salivatory nucleus and their axons travelled in the tympanic branch of the glossopharyngeal nerve. The otic ganglion was the relay station where these axons synapsed and the postganglionic cholinergic fibres originated. The postganglionic parasympathetic fibres passed from the otic ganglion along the auriculotemporal nerve to the parotid gland.

The preganglionic parasympathetic neurones to the submandibular and sublingual salivary glands were located in the superior salivatory nucleus. The axons of these cells travelled in the intermediate part of the facial nerve (nervus intermedius) and accompanied the chorda tympani before it joined the lingual nerve. The cholinergic postganglionic parasympathetic neurones to the submandibular and sublingual glands were located in the submandibular ganglion or in small ganglionic masses on the surface of these glands.

The preganglionic sympathetic neurones to the salivary glands were found in the intermediolateral columns of the upper thoracic segments of the spinal cord. They relayed in the superior cervical ganglion where the noradrenergic post ganglionic sympathetic fibres originated. These fibres accompanied the arteries to the salivary glands.

The classical view summarised above is being modified as a result of research undertaken im the last 20 years. The us~~e~~age of more sophisticated electrophysiological methods (Hellekant, 1971: Hellekant and Kasahara, 1973; Donaldson, Mitchell, and Templeton, 1984) and enzyme histochemical methods (Hiura, 1977; Contreras, Gomez & Norgren. 1980; Mitchell and Templeton, 1981) have shown that the preganglionic parasympathetic salivary neurones in the rat are located in an uninterrupted column of cells in the medullary reticular formation of the brainstem. The salivatory neurones which innervate the submandibular and sublingual glands occupy a rostral position within this column whereas those which supply the otic ganglion lie more caudal and dorsal (Contreras, Gomez and Norgren, 1980).

Knowledge about the neuronal pathways to the salivary glands is also undergoing change. It seems that the secretory fibres to the parotid gland are carried not just in the glossopharyngeal nerve but also in the seventh cranial nerve. This has been found to be the case in many mammals including man (Diamant and Wiberg, 1965). In the dog some secretory fibres to the parotid gland come from the facial nerve and many of the postganglionic parasympathetic fibres pass along the internal maxillary artery to reach the gland (Emmelin, Garrett and Holmberg, 1968, Garrett and Holmberg, 1970, 1972, Holmberg, 1971, 1972 a,b,c,d).

In addition to those secretory fibres classically described as travelling in the chorda tympani nerve secretory fibres have been found in the lingual branch of the fifth cranial nerve in the rat, monkey and man (Hellekant and Kasahara, 1973, Laage-Hellman and Stromblad, 1960, Wiberg, 1972). In rat the exact location and relations of the otic ganglion and the route which the preganglionic parasympathetic axons take to reach it are unknown. The classical description of the control of the salivary gland secretion by cholinergic parasympathetic and noradrenergic sympathetic nerves is also being modified in the light of recent work. The neuropeptide substance P has been found in rat salivary glands (Robinson, Schwartz & Costa , 1980; Brodin & Nilsson, 1981, Gallacher, 1983, Ekströhm and Wahlestedt, 1982) and has been shown to stimulate salivary secretion (Leeman and Hammerschlag, 1967; Chang and Leeman, 1970; Gallacher, 1983; Ekström and Wahlestedt, 1982; Brown and Hanley, 1981) which is not affected by blockade of $\alpha$-adrenergic, $\beta$-adrenergic or muscarinic receptors.

Vasoactive intestinal polypeptide (VIP) is another neuropeptide found in rat salivary glands (Wharton et al, 1979; Uddman et al, 1980) which when injected intravenously causes salivary secretion from the rat parotid gland. This secretion is not affected by blockade of $\alpha$-adrenergic, $\beta$-adrenergic or muscarinic receptors (Ekström, Månsson & Tobin 1983).

The presence and effect on secretion of substance P , VIP and possibly other neuropeptides raises the possibility of their involvement in the autonomic control of the parotid gland in addition to the classical neurotransmitter control by acetylcholine and noradrenaline .

The present thesis will concentrate its efforts on a study of the neural control of the parotid gland in the rat.

# THE AIMS OF THE PRESENT STUDY

1. To briefly review the literature associated with salivary glands and their control.

2. To examine the topographical anatomy and microscopic structure of the otic ganglion in the rat.

3. To inject tracers (enzymes or fluorochromes) into the substance of the parotid gland and to examine a variety of neural tissues for evidence of retrograde transport of the tracer to neuronal cell perikarya. These studies should provide evidence of neural connections to the parotid gland.

4. The results of the experiments mentioned in (3) above should provide the possible sites of origin of the neuropeptides mentioned previously. These areas will be investigated by means of immunocytochemical techniques for substance P, VIP, CGRP and choline acetyltransferase activity.

5. The evidence obtained from the experiments described in (4) above will be used in conjunction with retrograde tracer experiments to colocalize the tracer and neuropeptide in the same neurones. This should provide direct evidence that such cells may be responsible for the physiological effects described previously.

6. The parotid gland will be examined at the ultrastructural level to identify the different types of nerve endings which might account for the variety of neuropeptides which are currently being implicated in salivary control. Where possible immunoperoxidase cytochemistry and/or immunogold techniques will be used to confirm which type of nerve ending contains which neuropeptide. The results of these experiments should provide further information on the possibility of co-localization of the neuropeptides with one another or with the classical neurotransmitters acetylcholine or noradrenaline.

# CHAPTER TWO

# LITERATURE REVIEW

# LITERATURE REVIEW

## THE EVOLUTION AND EMBRYOLOGY OF THE SALIVARY GLANDS

Salivary glands are not unknown amongst the invertebrates e.g. malaria is transmitted by the saliva of mosquitoes. However it is doubtful if these salivary glands have any genetic relationship with those of vertebrates. In vertebrates the complexity of the salivary glands increases with progress up the evolutionary scale. Salivary glands are absent in fishes which contain only unicellular mucous glands. However, it is generally assumed that the multicellular salivary glands in higher vertebrates have their begining in such unicellular glands. The salivary glands are well developed in reptiles, birds and mammals (Fahrenhalz, 1967,Ihle et al, 1927, Tucker, 1958).

Although mammalian salivary glands are well developed their complexity is determined by other factors such as feeding habits and the immediate environment. For example in aquatic mammals where lubrication of food is not necessary the glands may be absent as in most Cetacea (dolphins and whales) (Fahrenhalz, 1967). In ruminants who ingest very dry food the salivary glands , particularly the parotid gland,are well developed. The volume of saliva secreted by these animals during feeding is so great that it effects the pH and electrolyte concentrations of plasma (Stacy, 1969). As a general rule the parotid gland tends to be more conspicuous amongst herbivores whereas the mandibular gland is more conspicuous among carnivores.

The salivary glands arise as outgrowths from the epithelium of the floor of the mouth. This forms a solid rod of epithelial cells which bud to form lobules. Degeneration of the cells in the centre of the rod converts it to a tube (Chievitz, 1885, Schulte, 1913, Borghese, 1950).

In man the parotid gland can be recognized in an 8mm embryo, the submandibular gland in a 13mm embryo and the sublingual gland in a 20mm embryo. In man primitive acini have formed in a 138mm (4th month) foetus and they are presumed to be mature at birth (de Plessis, 1957).

In the rat no acini are present at birth and full development of the gland awaits maturity of the animal (3-6 months) Screebny, Meyer, Bachem and Weinman, 1955, Leeson, 1959, Leeson and Jacoby, 1959). In the rat, at least, active secretion can take place in the absence of acini.

The early development of the duct system is accompanied by an ingrowth of blood vessels (Flint, 1902-1903). This pattern persists after maturation and the acinar-vascular network appears to be an extension of this ductal system.

In rodents subsequent development of the gland is influenced by hormonal factors. Castration of the male causes atrophy of the tubules (Shafer and Muhler, 1953, Junqueira, 1949) which is corrected by testosterone. The tubules are also affected by thyroid hormones (Grad and Leblond, 1949, Bixler, Webster and Muhler, 1957, Raynaud, 1950). Atrophy takes place after hypophysectomy (Lacassagne and Chamorro, 1940, Screebny, Meyer and Bachem, 1953) which is only partly corrected by thyroxine and testosterone.

# GROSS ANATOMY OF THE SALIVARY GLANDS

## INTRODUCTION

The salivary glands in mammals are generally classified into two types. The small glands situated in the oral mucous membrane whose secretions constantly lubricate and moisten the oral cavity and the larger paired glands which are situated some distance from the oral cavity and whose secretions reach the oral cavity through ducts. These larger glands are usually referred to as salivary glands proper and include the parotid, mandibular and sublingual glands.

## THE PAROTID GLAND

### Rat:

The parotid gland in the rat is a rather diffuse gland with an average weight in a 250g rat of 407 ± 74mg (S.D.). Each gland is situated on the lateral aspect of the neck and extends behind the ear and caudally it extends over the shoulder and the clavicle. Ventrally it lies along the course of the posterior facial vein. Lying over its anterior border is a well-defined extraorbital lacrimal gland with its duct(s) extending upwards towards the orbit. The main parotid duct is formed from the union of three lobar ducts and passes forward over the surface of the anterior superficial masseter muscle where it is accompanied by the mandibular marginal nerve. It passes through a break in the buccinator muscle group and opens into the oral cavity anterior to the 1st molar tooth. Approximately 3 mm before its opening it is joined by another duct which originates from the anterior buccal gland

(Redman,1972). This is a pure mucous gland which lies in a space lateral and mostly superior to the maxillary buccal sulcus beneath the bulge of the masseter muscle near to where the buccal and mandibular marginal branches of the facial nerve cross one another. It is the only salivary gland in the bucccal wall anterior to the third molar tooth. There are other mucous glands in the buccal wall located opposite the third molar tooth which are known as the posterior buccal glands (Redman,1972). They open independently into the buccal cavity.

Human :

The average weight of the parotid gland in adult humans is about 25gms. It has a wedge shape with the base of the wedge facing outwards. It lies below the external auditory meatus between the mandible and the sternomastoid muscle. For descriptive purposes it is divided into three lobes; superficial, deep and glenoid. The superficial lobe extends over the masseter muscle for a variable distance. It has a convex anterior border from which emerge the parotid duct and the branches of the facial nerve. It is related anteriorly to the masseter muscle, the mandible and the medial pterygoid muscle. Posteriorly it is related to the sternomastoid muscle, the mastoid process and the digastric muscle. The deep lobe is the tapered part of the wedge. It lies in contact with the internal jugular vein below the glenoid lobe. The glenoid lobe is the upper part of the parotid gland which reaches the glenoid fossa behind the mandibular joint. It is in contact with both the osseus and cartilaginous parts of the external auditory meatus. The auriculotemporal nerve which carries the parasympathetic fibres from the otic ganglion is in contact with the glenoid lobe.

The parotid gland in adult humans is covered by the parotid fascia which is an upward extension of the deep cervical fascia. Inside the parotid fascia there are lymph nodes lying on the surface and deep within the gland and they are part of the preauricular lymph nodes. The parotid gland also contains other non-glandular structures such as the facial nerve and its plexus, deeper still the retromandibular vein and deepest of all the external carotid artery and branches. The parotid duct is about 5cm in length. It begins by a confluence of two main ducts within the anterior part of the gland. It passes forward over the masseter muscle where it receives the duct(s) of the accessory glands. It then turns around the anterior border of the masseter to pierce the buccinator muscle. It opens on the mucous membrane of the cheek opposite the second upper molar tooth. This point is more anterior to where the duct pierces the buccinator. This acts as a valve preventing inflation of the gland when the intra-oral pressure is raised.

## THE MANDIBULAR AND SUBLINGUAL GLANDS

### Rat

The weight of the mandibular gland in a 250g animal is about 195 ± 33mg (S.D.). It is the most conspicuous structure in the ventral part of the neck. It extends from the level of the hyoid bone to just short of the manubrium almost in contact with the midline. On the anterior pole of the gland there are some lymph nodes which might conceal the major monostomatic sublingual glands which weigh approximately 44.5 ± 10.5mg (S.D.). The major sublingual gland is closely applied to the anterior lateral surface of the mandibular gland and both share a common connective tissue sheath. The sublingual gland is lighter in colour, more variagated and can be completely separated from the mandibular gland without any damage to either.

The main ducts of the two glands leave the anterior pole of each gland (one pair on each side) and share a common connective tissue sheath. They pass deep to the digastric muscle and beneath the mylohyoid muscle to open into the buccal cavity by separate parafrenular openings on the plica sublingualis immediately behind the lower incisor tooth. The orifice of the mandibular duct is constantly located on the ventral surface of small fleshy papilla whilst the sublingual duct, which is more variable in postion, opens immediately lateral and posterior. Separate minor sublingual glands (polystomatic sublingual glands) exist directly beneath the mucous membrane of the floor of the mouth and they open directly into the mouth by several ducts along the lesser sublingual ridge. In some strains of rat a salivary reservoir connected to the main mandibular duct has been described (Butcher, 1972, Schneyer and Flatland, 1975). These reservoirs with volumes of 2-5µL lie behind the two incisors and open into the main duct just prior to its opening. They extend from the mandibular papillae to the base of the tongue and are lined by pseudostratified columnar epithelium and have striated muscle in their walls. They probably function as a readily available source of saliva for the animal.

### Human:

### The Submandibular gland

This gland the size of a walnut, lies in the submandibular fossa between the mylohyoid muscle and the investing layer of deep cervical fascia and partly under cover of the mandible. It extends posteriorly and curves around the posterior border of the mylohyoid muscle and therefore a small part of the gland lies within the floor of the mouth between the mylohyoid and hyoglossus muscle. The facial artery lies in a deep groove in the submandibular gland before it arches upwards around the border of the mandible to reach the face in front of the masseter muscle. The common facial vein grooves the posterior surface of the gland and the mandibular branch of the facial nerve crosses the gland here. Some lymph nodes lie on the surface of the submandibular gland. The submandibular duct is about 5 cms in length. It begins as numerous branches in the superficial part of the gland and runs in the deep part of the gland before coming free. Then it extends between the mylohyoid and hyoglossus muscles and passes between the sublingual glands and the genioglossus to open in the sublingual papilla which is a low elevation at the side of the frenulum of the tongue. On the hyoglossus muscle it lies between the lingual and hypoglossal nerves but at the anterior border of that muscle it is crossed laterally by the lingual nerve.

## The Sublingual gland

This gland has the shape and size of an almond and weighs 3-4 gms. It is situated between the mucous membrane of the floor of the mouth and the mylohyoid muscle. It is related anteriorly with its fellow of the opposite side, posteriorly with the deep part of the submandibular gland, laterally with the mandible and medially with the genioglossus muscle from which it is separated by the lingual nerve and the submandibular duct. The sublingual ducts vary in number from 8-20. Some of the smaller ducts open directly into the floor of the mouth on the summit of the sublingual fold and a few may open into the duct of the submandibular gland. From the anterior part of the gland some of the ducts join to form a major sublingual duct which opens within or near to the mandibular duct.

# BLOOD SUPPLY OF THE SALIVARY GLANDS

In humans the arterial supply to the parotid glands comes from branches of the external carotid artery in or near the gland and its venous drainage is to the retromandibular vein and then to the external jugular vein. The sublingual gland receives its arterial supply from the lingual branch of the external carotid artery and the submental branch of the facial artery. The venous drainage is to the internal jugular vein. The submandibular gland receives its arterial supply from the facial and lingual arteries. The venous drainage is into the common facial vein.

In the rat the arteries to the major sublingual and mandibular glands come from a branch of the external maxillary artery which arises near the angle of the mandible.The parotid gland in the rat is supplied by the posterior auricular artery, a branch of the external carotid artery, which arises near the origin of the sternomastoid muscle and passes dorsally near the ear.

The branches to the major sublingual and mandibular glands accompany the excretory ducts and nerves and so create a sort of hilus. Sometimes there are additional branches from the periphery which supply the interlobular and capsular connective tissue. In the parotid gland there is no such hilus and the blood vessels enter at irregular intervals and in an inconstant fashion.

In general these blood vessels follow the duct system and so each lobule has its own separate blood supply. Three separate layers of blood vessels are found in relation to the duct system: firstly an intramural plexus of capillaries which lie internal to the ducts; secondly an intermediate arteriolar plexus which connects the intrinsic intra mural capillary bed to the third and deepest layer which is composed of two or three arteries and veins.

This pattern continues around intralobular, granular tubules and intercalated ducts. Near the ends of the intralobular ducts ring shaped arterial arcades are formed from which branches come off to supply the secretory endpiece. Embryologically the duct and its blood vessels develop before the secretory endpieces and its vessels. Spanner (1937) was impressed by the prominence of arterio-venous anastomoses throughout the lobule but they are most prominent in the region of the ring vessels and not very prominent in the duct wall.

Although it is agreed that the intralobular duct has a richer blood supply compared to the secretory endpiece, opinions differ on how the capillary beds of both connect, and what the effect is of parasympathetic stimulation on blood flow. Burgen and Seeman, (1958) have stressed that intralobular ducts receive a blood supply running countercurrent to the direction of flow of the salivary secretions. Blair-West et al., (1969) stressed that capillaries of the secretory endpieces join together to make venous sinusoids which form a plexus around the excretory ducts before emptying into wide bore chambers less numerous than the sinusoids which in turn empty into trunk veins which accompany the interlobular ducts. Stimulation of cholinergic nerves to the salivary gland raises both the secretion and blood flow to the gland. This rise in blood flow is attributed to a rise in perfusion of the capillary bed and opening of the arterio-venous anastomoses which were described by Spanner in 1937, Burgen and Seeman (1958), Haggendal and Sivertsson (1967) and Fraser and Smaje(1976) seem to establish that the rise in blood flow represents a true rise in capillary bed perfusion.

Although it is clear that there is a larger rise in blood flow to the intralobular ducts than the secretory endpiece, the results are conflicting. Burgen and Seeman, (1958), Holzlohner and Niessing (1936a,b ) showed a fall of perfusion of the secretory endpiece during activity studies. While Brücke and Zwiauer ( 1938) showed a rise of perfusion of the capillary bed of the secretory endpiece during activity and showed that a decreased flow was seen only if the digastric muscle was contracted and obstructed secretory blood flow. Fraser and Smaje (1976) have shown in the rabbit mandibular gland that cholinergic stimulation causes a rise in capillary blood flow both to ducts and endpieces but the increase to the ducts is more rapid in onset.

The venous drainage of the gland also follows the duct system. These veins which occur especially in the hilar region contain sacculations and were first described by Spanner (1936 - 1937, 1937 ) as Drosselvenen (throttle veins) and they occur especially in the hilar region. Spanner suggested that these veins serve as a reservoir and may also serve to back up the pressure in the capillary circulation and maintain a high outflow resistance in the face of rapid and wide fluctuations in vascular perfusion rate . Spanner (1942) also described the presence of large epitheloid cells in the wall of the arterioles and arterio-venous anastomoses near the excretory duct. These cells have a proposed endocrine function by secreting vaso-active substances which act on the arterio-venous anastomoses (Leeson, 1967).

## LYMPHATIC DRAINAGE OF THE SALIVARY GLANDS

In man the parotid gland lymph drains into the lymph nodes within the parotid fascia. These drain into the deep cervical lymph nodes surrounding the internal jugular vein especially it's anterior superior group. The lymph from the submandibular and sublingual glands in man drain into the submandibular lymph nodes before draining into the deep cervical lymph nodes, especially the jugular-omohyoid group. The lymphatic system in the salivary glands is composed mainly of lymphatic spaces surrounding the secretory endpieces and their intercalated ducts and lymph ducts which accompany the excretory duct system and the blood vessels (Klein, 1882). The lymph spaces communicate with each other. The lymph capillaries which drain the lymph spaces appear first at the level of the intralobular ducts. The lymph ducts do not seem to penetrate between the secretory endpieces (Klein, 1882; Holzlohner and Niessing,1936b; Wenzel, 1967; Sviridova, 1970). The lymph flow from the resting salivary gland is very sparse but when the salivary gland is secreting there is a brisk rise in lymph flow. This rise in lymph flow is induced by parasympathetic nerve stimulation or administration of parasympathomimetic drugs and it can be blocked by atropine. In the absence of a rise in salivary secretion the rise in lymph flow is independant of blood flow. However the rise in lymph flow can be augmented by obstructing venous drainage. Bainbridge (1900-1901) pointed out that salivary gland lymph flow may be influenced by the fact that saliva is hypotonic so the interstitial fluid is probably hypertonic. This would favour the movement of fluid from the intravascular to the extravascular compartment.

# HISTOLOGY OF THE SALIVARY GLANDS

## LIGHT MICROSCOPY

The salivary glands are composed of a glandular component (parenchyma) and a connective tissue framework (stroma). The stroma consists of septa which divide the glandular tissue into lobes and lobules. The large excretory ducts, blood vessels, nerve fibres, occasional ganglion cells, mast cells, and some leucocytes may all be found in the connective tissue septa.

The glandular tissue consists of secretory endpieces and the excretory duct system. The endpieces consist of acini, the shape and staining properties of which differ from one species to another and even from gland to gland in the same species. In man the three major salivary glands are described as tubulo-acinar. The secretory epithelial cells of the acini can be classified as serous and mucous. Accordingly the salivary glands can be classified as pure mucous, pure serous or mixed. The parotid gland in human, dog, cat and rabbit is a pure serous gland. The sublingual and mandibular glands are mixed glands in most mammals but the ratio of mucous and serous components differ from one species to another. In humans the submandibular gland is predominantly serous whilst the sublingual gland is predominantly mucous. The anterior buccal glands in the rat and the palatine glands in humans are examples of pure mucous glands.

The serous cells which produce amylase are pyramidal shaped, with a basal nucleus and an indistinct cell boundary. In haematoxylin and eosin stained sections the cytoplasm appears deep reddish purple and contains in the apical region varying numbers of secretory granules. These granules have been shown to contain peroxidase in many species. The mucous cells in ordinary haematoxylin and eosin stained sections have a light bluish purple appearance. The lumen of the mucous acinus is fairly large and may contain large amounts of mucin. The mucous cells also rest on a basal lamina but , unlike the serous cells, their boundary is quite distinct. The nucleus is oval or spheroidal in shape and the cytoplasm contains variable numbers of secretory droplets which are invariably removed in the fluids used in the preparation of the paraffin wax sections. In acini which contain both serous and mucous secreting cells the serous cells form basophilic crescent-shaped groups (called demilunes) around the outside of the mucous cells.

These acini drain into the first part of the duct system which is the intercalated duct. The intercalated duct is lined by low cuboidal epithelium and its cells contain a large nucleus, little cytoplasm and as a rule few secretory granules. The intercalated ducts drain into the intralobular striated ducts. The striated ducts are lined by simple columnar epithelium whose cells have a centrally placed nucleus. These cells show the characteristic basal striations and in the apical region a variable number of secretory granules may be seen. The striated ducts are best seen in the parotid and submandibular glands and are much less conspicuous in the sublingual glands. In adult rodents the proximal part of the striated duct is greatly developed and may become convoluted. The lining cells of this part are very granular but show little or no basal striations. These ducts are known as granular ducts, are under hormonal influence and are more numerous in the male than in the female. These ducts atrophy following hypophysectomy or castration. The intralobular ducts drain into interlobular and then interlobar ducts. The lining epithelium of these ducts becomes pseudostratified and then stratified as the main duct is approached (Leeson, 1967).

Another type of cell which can be seen in both acini and in some parts of the duct system is the Basket cell (Basel) or the myoepithelial cell. These cells appear as flat cells with long cytoplasmic processes which extend over the outer surface of the acinus in a basket configuration. They are located in the region between the secretory cells and the basal lamina. These cells are best demonstrated by incubating sections for alkaline phosphatase activity. It is assumed that these myoepithelial cells contract and so assist in drainage of the secretory products into the excretory duct.

## ULTRASTRUCTURE

### a) The acinus:

The serous cell is usually squat with a broad base and has a narrow apex. The basal plasma membrane which is usually smooth or slighlty folded is either in contact with the basement membrane or is separated from it by the cytoplasmic processes of the myoepithelial cell. The lateral plasma membrane is plicated and may interdigitate with that of adjacent cells where the lateral intercellular space is narrow. The apical plasma membrane commonly shows irregular microvilli and often extends between the adjacent cells creating an inter cellular secretory canaliculus. Junctional complexes such as zonula occludens and zonula adherens separate the lateral intercellular space from the secretory canaliculus. A number of desmosomes may also be seen between adjacent cells. The nucleus is usually spheroidal or elliptical in shape but irregular forms with deep indentations are not uncommon (Wilborn and Shackleford, 1969) and one or more nucleoli are frequently present. The position of the nucleus in the cell is dependent upon the amount of secretion the cell contains. The more secretory material present the nearer to the base of the cell becomes the nucleus.

The basophilic cytoplasm of the serous cell is attributed to the large amount of rough endoplasmic reticulum and to the large numbers of secretory granules. This is a feature of most protein secreting cells.

The mucous cell has no single ultrastructural feature specific to it. However a common feature of these cells is the presence of large, electron translucent secretory droplets but exceptions do occur and it is wise to identify cells on the basis of histochemical staining.

The mucous cell is more cuboidal in shape (the apical surface is more extensive). Basal and lateral plasma membranes are smooth or only very slightly plicated. There are no microvilli in the apical plasma membrane and the secretory canaliculi are invariably absent in mucous cells. The nucleus is usually situated close to the base of the cell due to its large content of mucous. The rough endoplasmic reticulum consists of closely-packed cisternae in the basal part of the cell.

### b) The duct system

#### Intercalated duct

The lining epithelium is composed of cuboidal cells with a large spheroidal nucleus which occupies most of the cell. The basal and lateral plasma membranes are fairly smooth with no or little plication. The apical plasma membrane shows some irregular microvilli. Extensive junctional complexes are usually present in the form of zonula occludens, zonnula adherens and desmosomes.

The cytoplasm contains the normal complement of organelles. The intercalated duct cells in many species contain in their apical area variable number of spheroidal granules which are usually described as secretory granules (Dorey and Bhoola, 1972, Shackleford and Klapper, 1962, Shackleford and Wilborn, 1968, Shear, 1969, Tandler and MacCallum, 1974). The small number of these secretory granules and lack of morphological evidence of their formation and discharge suggest a very low rate of secretion.

#### Striated ducts

The most striking feature in the histology of the striated ducts (and indeed that is why they are called so) is the basal striation of its simple columnar epithelium. This basal striation bears a great resemblence to the basal striations seen in renal tubular epithelium. Benda in 1903 concluded that these basal striations seen in the renal tubules are due to packing of numerous mitochondria. In 1909 Regaud and Mawas reached a similar conclusion when they studied mammalian salivary glands. Ultrastrutural studies on striated ducts first published by Pease, in 1956 confirmed this conclusion. These basal striations are created by vertically orientated mitochondria within numerous cytoplasmic compartments which are outlined by deep infoldings of the basal plasma membrane. These extensive plasma membrane infoldings or invaginations and an abundance of mitochondria are morphological features of many electrolyte transporting epithelial cells (Berridge and Oschman, 1972).

c) Myoepithelial cells (Basket, Basal cells).

The myoepithelial cells are known to be present in many mammalian exocrine glands such as sweat glands, salivary glands, mammary glands and lacrimal glands. Detailed descriptions of the ultrastructure of the myoepithelial cells in salivary glands come from studies on rat mandibular gland (Tamarin, 1966) and human submandibular (Tandler. 1965) and labial glands (Tandler et al, 1970). The ultrastructure of the myoepithelial cell resembles that of a smooth muscle cell because of the presence of bundles of parallel filaments which occupy large areas of the cytoplasm. Mitochondria and other organelles are sparse and are mainly concentrated within a circumscribed juxta- nuclear area. The myoepithelial cells generally take two forms dependant on whether they are present in the secretory endpiece or in the intercalated ducts. In the secretory endpiece the myoepithelial cells have the shape of an octopus sitting on a rock. There is usually a single myoepithelial cell associated with each acinus. Four to eight processes radiate out from the cell body and each process gives rise to two or more generations of branches. The processes from the acinar cells do not extend to the intercalated ducts. However the myoepithelial cells of the intercalated ducts do extend to the base of the secretory endpiece. In the intercalated ducts the myoepithelial cells are spindle-shaped and their processes seldom subdivide. They mainly lie in a longitudinal direction along the ducts.

## PHYSIOLOGY OF THE SALIVARY GLANDS

### 1. Function of the salivary glands.

Saliva has many important physiological roles some of which are listed below:

a) Lubrication. Saliva which contains a glycoprotein called mucin lubricates the tongue and lips during the acts of swallowing and speaking and also acts as a solvent to stimulate the taste buds. This process of lubrication helps keep the mouth moist and clean and indirectly helps prevent infection. Other factors present in saliva such as thiocyanate ions assist in this protective role. Under normal conditions of pH the saliva is saturated with calcium ions and this prevents the loss of calcium from teeth into the oral fluids.

b) Saliva plays a minor role in the digestion of carbohydrates. The enzyme - amylase attacks the 1:4 linkage in the glycogen molecule.

c) A large number of hormones and pharmacologically active compounds are produced by the salivary glands . Renin, serotonin, glucagon, somatostatin and various growth factors have all been found in the salivary glands of some species (Bing and Farup, 1965, Bing et al., 1967, Cohen et al., 1972,Bhoola, et al., 1973, Gutman et al., 1973). Amongst the growth factors nerve growth factor has received the most attention.

Nerve growth factor (NGF) was isolated from the mandibular gland of adult mice (Levi-Montalcini and Brooker, 1960, Levi-Montalcini and Cohen, 1960, Levi-Montalcini and Angeletti, 1961, Schwab, Stockel and Thoenen, 1976). Cohen,(1960) identified this NGF as a protein with a molecular weight of 44,000 daltons which was non - dialysable, heat labile, destroyed by acid, stable in alkali and its biological activity is destroyed by proteolytic enzymes.

Injection of NGF into adult and newborn mice icreases the size of their sympathetic ganglia (Levi-Montalcini and Booker, 1960).This increase depends upon the age of the animal, and on the amount and purity of the injected protein. In newborn mice the increase in size of the sympathetic ganglia may be 6 fold and is due to both hypertrophy and hyperplasia of the neuronal cells. In mice injected nine or more days after birth, when mitotic activity has ceased, NGF results in hypertrophy only (Levi-Montalcini, 1964). Comparable results were obtained when tissue cultures of foetal human sensory and sympathetic ganglia were incubated in the presence of NGF. In these studies a dense halo of neurites was seen emerging from the ganglia (Levi-Montalcicni and Booker, 1960).

An antiserum to NGF when injected into newborn mice, rats or rabbits has been found to destroy sympathetic nerve cells (Levi-Montalcini and Booker, 1960). NGF is a normal constituent of sympathetic cells and is present in the blood and body fluids of a variety of vertebrates including man (Levi-Montalcini, 1964).

d) Saliva may play a role in thermoregulation especially in fur bearing animals such as the cat and rat. These animals moisten their fur with saliva when they are hot, the saliva evaporates and thus cools the animal (Young and Van Lennep, 1978).

## 2. Composition of saliva.

Generalisations about the composition of saliva are difficult because of the variations in composition amongst species and amongst different glands within the same species. As the mode or intensity of stimulation is changed so the composition of the saliva in the gland changes. In humans the salivary secretion from the three major salivary glands is hypotonic. On the other hand the salivary secretion from the parotid gland of rat and from the sublingual glands of cat and dog is isotonic.

Salivary secretion is a two stage operation and the composition of saliva changes as it passes from one stage to the next. In the first stage the primary secretion produced by the acini contains enzymes in an ionic concentration similar to that of plasma. The second stage takes place while the secretion is passing through the salivary ducts. Two major transport processes occur here to change the composition of saliva considerably. The first transport mechanism involves the active reabsorption of sodium ions and a secretion of potassium ions. This process is under hormonal control. Mineral corticoids increase the reabsorption of sodium ions and chloride ions from the saliva and increase the secretion of potassium ions.

The second mechanism involves the secretion of bicarbonate ions. This reaction is catalysed by the enzyme carbonic anhydrase which is present in the epithelial cells of the ducts. Chloride ions are reabsorbed in exchange for the bicarbonate ions lost. It has been said that reabsorption of bicarbonate ions takes place in the ductal system of the rat parotid and the cat sublingual glands. During maximal salivation this reabsorbtion process along the salivary ducts fails and so the sodium and chloride ion concentrations rise whilst the potassium and bicarbonate ion concentrations fall.

Other inorganic components of saliva include iodide, fluoride, phosphate, calcium, perchloride and thiocyanate. Iodide is concentrated in the salivary glands of many species. Not~~e~~ably in the human parotid and submandibular glands and in the mouse and hamster mandibular glands (Cohen and Myant, 1959). However many glands which produce saliva rich in iodide do not themselves have higher concentrations of iodide than that of plasma. Rats are unable to concentrate iodide in their saliva or selectively accumulate it in their glands (Cohen, Logothetopoulos and Myant, 1955, Logothetopoulos and Myant, 1956). No organic iodine is present in human salivary secretions but dog parotid saliva contains iodo tyrosine

(Davenport, 1978). Thiocyanate is found in the saliva of many species and it appears to be mostly produced endogenously from cyanide. The thiocyanate concentration is raised considerably in saliva from smokers (Burgen and Emmmelin, 1961)

Amongst the organic components of saliva are the following:

1. Mucin - this consists of a whole family of proteins associated with different sugars or sugar derivatives.
2. Amylase - is derived from the acini and the granular cells of the proximal ducts. $\alpha$ - amylase is found in the saliva of many species including man, rat and rabbit. Amylase is absent or in very low concentrations in secretions from the parotid and submaxillary gland of dog, cat horse and sheep. The concentration of amylase in human parotid saliva is independent of the rate of salivary secretion (Ferguson, Krahn and Hildes, 1958). The $\alpha$ - amylase splits the 1:4 glycoside linkage and has no affect on the 1:6 linkage.
3. Lipase is said to be produced in the lingual gland and may be of value in neonates and in other situations where there are inadequate levels of pancreatic enzymes (Sanford, 1982).
4. Soluble blood groups have been identified in saliva of man, horse, rabbit and guinea pig. Among the other organic components described in saliva are lysozymes, killikrinin, some blood coagulating factors, IgA and iodo - proteins. Lysozyme causes lysis of many bacteria including staphylococcus, streptococcus and proteus. The lysozyme acts by hydrolysing a constituent of the bacterial cell wall which appears to be made of a mucoprotein. No organic iodo-proteins are present in human saliva (Ferguson, Krahn and Hildes, 1958) but organic iodine compounds are produced from slices of rat mandibular gland in vitro (Taurog, Potter, Tong and Chaikoff, 1956).

# NERVE SUPPLY AND NEURONAL CONNECTIONS OF THE SALIVARY GLANDS

The salivary glands receive a nerve supply from both divisions of the autonomic nervous system in addition to a sensory supply. The innervation to the salivary glands includes that to the acini, ducts, myoepithelial cells and blood vessels. The antagonistic effect of the sympathetic and parasympathetic nervous systems is only effective at the vascular level. Sympathetic stimulation produces vasoconstriction whereas the parasympathetic nervous system produces vasodilation. Parasympathetic stimulation invariably results in a rise in secretion. The effect of sympathetic stimulation on salivary secretion differs in different species and in different glands. It might cause a vigorous secretion e.g from the cat mandibular gland (Langley, 1878, Langley and Fletcher, 1889) or a moderate secretion e.g. from the rat submandibular gland, a sparse secretion e.g. from dog mandibular gland or no secretion e.g. rabbit mandibular and dog parotid.

When sympathetic secretory fibres exist, stimulation may occur through action on either $\alpha$ or $\beta$ - adrenergic receptors or both. It is said that $\alpha$- receptor stimulation results in secretion of fluid, electrolytes and newly formed proteins. $\beta$ - receptor stimulation results in exocytosis of preformed secretory granules (Young, 1979),

Preganglionic sympathetic fibres to the salivary gland arise from cell bodies in the lateral horn cells in upper thoracic segments of the spinal cord. These fibres ascend in the sympathetic trunk and synapse in the superior cervical ganglion.

Postganglionic sympathetic fibres reach the glands along the connective tissue coats of the arteries. Although little is known about supraspinal pathways, the finding that stimulation of the medulla results in a salivary secretion which is abolished when the cervical sympathetic trunk is sectioned would indicate the existance of such a higher central control (Sanford, 1982).

Preganglionic parasympathetic fibres arise from the superior and inferior salivatory nuclei which are located in the lateral reticular formation of the brain stem. The superior salivatory nucleus is traditionally said to supply the submandibular and sublingual glands while the inferior salivatory nucleus supplies the parotid gland.

Largely on the basis of studies carried out on human cadavers in the 19th century it has been accepted that the parasympathetic supply to the parotid gland travels in the glossopharyngeal nerve and terminates in the otic ganglion. The postganglionic fibres from the neurones in the otic ganglion reach the parotid gland via the auriculotemporal nerve. Similarly the traditional view is that the parasympathetic fibres which supply the submandibular and sublingual glands leave the cranium through the seventh cranial nerve and then travel via the chorda tympani to join the lingual nerve before synapsing in the submandibular ganglion. Preganglionic fibres and a few postganglionc fibres leave the ganglion and travel along the main duct to enter the gland at its hilus. The preganglionic fibres terminate along the duct or in the gland itself. In the rat mandibular gland a ganglion is not visible macroscopically but ganglion cells are present in the connective tissue of the gland and around the main duct.

However this traditional pattern appears to be highly variable among species and is more complex than at first thought. More recent studies on the parotid gland in humans have shown that intracranial sectioning of the glossopharyngeal nerve does no more than decrease reflex parotid secretion (Reichert and Poth, 1933). It seems likely that secretory fibres to the human parotid gland come not only from the glossopharyngeal nerve but also from the facial nerve (Diamant and Wiberg, 1965). Studies in the dog have shown that the bulk of the preganglionic parasympathetic nerves to the parotid gland do travel in the glossopharyngeal nerve although a small percentage travel in the facial nerve (Holmberg, 1971; Holmberg, 1972b). A substantial number of the postganglionic parasympathetic fibres travel not in the auriculotemporal nerve but along the internal maxillary artery and its branch to the parotid artery (Emmelin et al, 1968, Garrett and Holmberg, 1970, Holmberg, 1971, 1972 a,b,).

These variations from the classical view apply to the submandibular and sublingual glands as well . Hellekant and Kasahara (1973a) described secretory fibres in the lingual portion of the trigeminal nerve as well as in the chorda tympani branch of the facial nerve in the rat. Similar findings have been described in the monkey (Hellekant and Kasahara, 1973) and in human (Laage - Hellman and Stromblad, 1960, Wiberg, 1972).

Electron microscopy has shown that the major nerve trunks entering the salivary glands are composed mainly of non-myelinated fibres and a few myelinated fibres which are either afferents or preganglionic fibres. The non-myelinated fibres show periodic varicosities containing vesicles. The vesicles within these terminals are thought to contain acetycholine or noradrenaline. More recently substance P, VIP and other neuropeptides have been identified in the salivary glands (Wharton et al., 1979; Robinson, Schwartz and Costa 1980; Goedert, Nagy and Emson, 1982; Gallacher, 1983;Brodin et al, 1984 ; Sharkey and Templeton, 1984; Ekstrom et al, 1984). Both adrenergic and cholinergic nerves terminals have been observed emerging from the same Schwanncell sheath (Bogart and De Lemos, 1973). The neuro-effector junction consists of regions where varicosities come close enough to the parenchymal cells for the released neurotransmitter to reach them in effective concentrations. Two types of junctions are described - epilemmal and hypolemmal (Young and Van-Lennep, 1978). In an epilemmel junction the axon has a free surface adjacent to parenchymal cell and separated from it by a distance of about 100nm. The basal lamina intervenes between the axon and the paranchymal cell. In a hypolemmel junction the axon penetrates the basement membrane and separates it from the parenchymal cell by a distance of 20nm only. Both types of junction are seen in all species and there seems to be no functional difference between them.

One fibre may innervate several parenchymal cells. However it may not be necessary for all parenchymal cells to be innervated since electrical coupling of epithelial cells does occur (Wiener et al., 1964; Loewenstein and Kanno, 1964; Petersen, 1975 ). In most glands the density of innervation is greatest around the secretory endpiece.

## PHARMACOLOGY.

A large number of drugs can effect salivary secretion by acting on the central nervous system, autonomic ganglia or directly on the acinar cells themselves.

Administration of parasympathomimetic agents such as acetylcholine and methacholine results in a copious secretion of saliva, whereas administration of atropine and other parasympatholytic agents blocks this effect. Sympathomimetic drugs are much less effective in producing salivary secretion. Salivary glands which respond well to sympathetic stimulation such as the cat mandibular gland also respond well to sympathomimetic agents. In man when adrenaline is given through the salivary duct to avoid any systemic effect it produces secretion from the submandibular gland (Emmelin and Stromblad, 1954). Adrenaline imitates the effect of sympathetic stimulation. Hoewever, one striking difference is the appearance of glucose in saliva following adrenaline stimulation. Sympatholytic agents such as ergometrine abolish the secretory effect of both adrenaline and sympathetic stimulation from cat mandibular gland whilst the parasympathetic secretion from the chorda tympani is retained.

In some species neuropeptides have been shown to stimulate salivary secretion. The undecapeptide substance P which has been demonstrated in the salivary gland of some species including the rat (Robinson et al., 1980, Brodin and Nilsson, 1981) when administered systemically produces secretion from rat salivary glands (Leeman and Hammerschlag, 1967; Ekstrom and Wahlestedt, 1982; Gallacher, 1983). The secretory response to substance P is unaffected by blockage of $\alpha$ -adrenergic, $\beta$ -adrenergic and muscarinic receptors. It even persists after degeneration of the parasympathetic and sympathetic nerves (Ekstrom and Wahlestedt, 1982). The vasoactive intestinal peptide (VIP) has also been found to induce salivary flow in some species (Ekstrom, Mansson and Tobin, 1982, Ekstrom, Mansson and Tobin, 1983). Substance P, VIP and possibly other peptides may play an important role in the control of the salivary secretion alongside the well established cholinergic and noradrenergic mechanisms.

The literature review described above is not totally exhaustive but should provide a basis which will be further expanded upon in each of the following Chapters, particularly where information relevant to rat salivary glands is concerned.

# CHAPTER THREE

# THE ANATOMY AND MICROSCOPIC STRUCTURE OF THE RAT OTIC GANGLION

# THE ANATOMY AND MICROSCOPIC STRUCTURE
# OF THE RAT OTIC GANGLION

## INTRODUCTION

In mammals it has been generally accepted that there are two brainstem salivatory centres (Hiura, 1977). These are a superior salivatory nucleus whose preganglionic axons leave the brainstem in the nervus intermedius and form synapses with postganglionic neurons within or close to the submandibular and sublingual glands and an inferior nucleus whose preganglionic axons leave the brainstem with the glossopharyngeal nerve to form synapses with postganglionic parasympathetic neurons in the otic ganglion. The postganglionic axons reach the parotid gland via the auriculotemporal nerve.

In experiments where salivation was evoked by electrical stimulation of the brainstem (Donaldson, Mitchell and Templeton, 1984) or when horseradish peroxidase was used as a retrograde tracer (Contreras, Gomez and Norgren, 1980; Mitchell and Templeton, 1981) the results have suggested that, in the rat at least, the salivatory centres are a continuous column of cells within the lateral reticular formation and are not two distinct groups of nuclei.

The route taken by the preganglionic axons to reach the submandibular and sublingual postganglionic neurons appears to conform to the accepted description. However, the route taken by the preganglionic axons which innervate the otic ganglion in the rat is unknown (Contreras, Gomez and Norgren, 1980). This lack of information is probably due firstly to the relative inaccessibility of the otic ganglion in the rat and secondly , because of the complex dissection required to follow the tortuous route of the preganglionic axons.

The present study has used dissection techniques supported by a light microscopic examination of serial histological sections through the rat head and an ultrastructural examination of the otic ganglion to establish the answer to these questions.

# MATERIALS AND METHODS

Adult male Wistar rats each weighing 250g were injected intraperitoneally with a lethal dose of sodium pentobarbitone (200mg/Kg; Sagatal; May and Baker, Dagenham, Essex) and perfused via the heart with 500ml of physiological saline followed by 500ml of fixative. Ten animals used for dissection purposes were perfused with 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4 (see Appendix 1). Dissections were performed with the aid of a Wild M650 operating microscope (Wild Heerbrugg Ltd,Switzerland) fitted with a camera attachment (Nikon, Japan).

Three animals were perfused with Heidenhain's Susa fixative (see Appendix 2)(Culling, 1963). Following perfusion the head was placed in fresh fixative for one week to allow the skull to decalcify. Each head was then processed into paraffin wax (see Appendix 3 ) and serially sectioned at 20µm thickness with the aid of a sliding microtome (Leitz, Wetzlar, Germany). The sections were stained with haematoxylin and eosin (see Appendix 4 ) and examined with an Olympus BH2 photomicroscope fitted with a PM10 AD attachment for photomicrography (Olympus, Japan).

Three animals were perfused with a modified Karnovsky's (1965) fixative (see Appendix 5) and the otic ganglia were removed and processed into Araldite (see Appendix 6). Sections of 1µm thickness were cut with the aid of a Reichert ultramicrotome, mounted onto glass slides and stained with 1% Toluidine Blue for examination at the light microscpope level. Thin sections 90-100nm thick were mounted onto copper grids and stained with 1% uranyl acetate and 1% lead citrate and were examined in a Philips 300 transmission electronmicroscope.

# RESULTS

## Anatomical Dissection

The otic ganglion is a flattened structure measuring approximately 300um in thickness and with a maximum diameter of 1mm. The dorsal surface of the ganglion is related to the sphenoid bone which separates it from the trigeminal ganglion (Figs 1&2). The ventral surface of the ganglion is apposed to the internal pterygoid muscle (Greene, 1963). Along its lateral edge it is in direct relationship with the pterygopalantine branch of the internal carotid artery (Fig 1). Several nerves can be seen radiating from the otic ganglion (Fig 3).

The largest of these nerves arises from the ventral surface at the rostral end of the ganglion, travels caudally along its ventral surface and proceeds medially behind the tympanic bulla. Midway along its passage behind the bulla it is joined by a branch from the superior cervical ganglion . This fused nerve trunk descends through a vertical groove lying between the basisphenoid bone and the pterygoid plate. As it emerges from this groove it separates into two branches (Fig 4); one of them, which is equivalent to the greater petrosal nerve, travels in a rostral direction and accompanies the internal carotid artery along a groove in the pterygoid plate before entering the foramen lacerum. The other branch passes caudally, enters the facial canal and can be traced to its union with the facial nerve (Fig 6).

The anterior end of the ganglion has two nerve trunks (Fig 3) which pass on either side of the pterygopalatine artery before merging with the auriculotemporal branches of the mandibular nerve en route to the parotid gland. From the caudal end of the ganglion two or more small nerve fascicles enter the surrounding pharyngeal musculature.

Fig. 1 Coronal section through rat head to show the otic ganglion (arrow). A - pterygopalatine branch of the internal carotid artery. V - trigeminal ganglion. Bar = 1mm

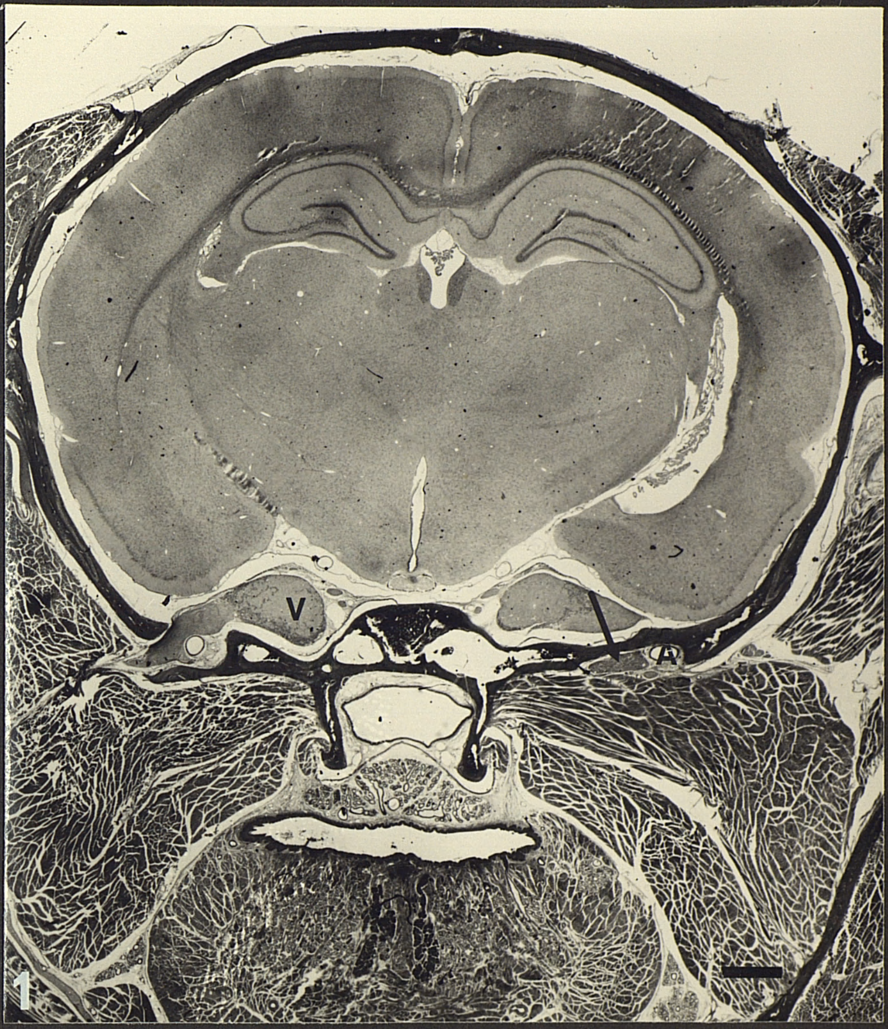

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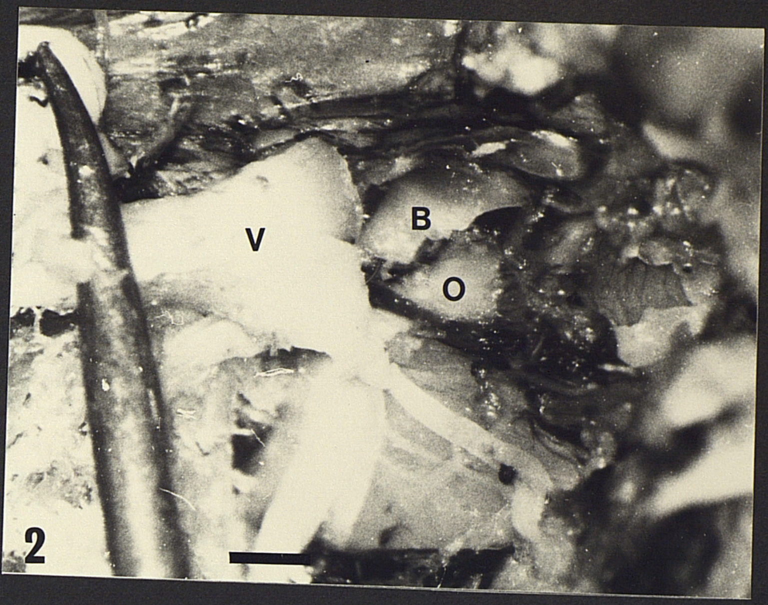
Fig. 2 Dissection with the sphenoid bone (B) partially removed to expose the otic ganglion (O) lying deep to the fifth cranial nerve (V) which has been retracted. Bar = 1mm

Fig. 3a A more complete dissection of the otic ganglion to reveal its neural connections. A piece of black card has been inserted beneath the emerging nerve trunks to aid visualisation. 3b diagram to explain the main features of 3a. AT - auriculotemporal nerve; OTIC - otic ganglion; Bar = 1mm



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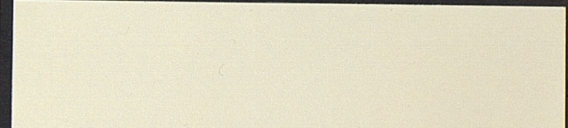

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Fig. 4 Dissection to show the fusion zone (arrow) of the greater petrosal nerve with the branch from the superior cervical ganglion. CT - chorda tympani nerve; GP greater petrosal nerve; TB - tympanic bulla; V branches of the trigeminal nerve. Bar = 1mm.

Fig. 5 A dorsolateral view of a dissection to reveal the anastomosis between the seventh and ninth cranial nerves (arrowheads). AT - auriculotemporal nerve; OG - otic ganglion; VII - facial nerve; IX & X - glossopharyngeal and vagal nerves. Bar = 1mm

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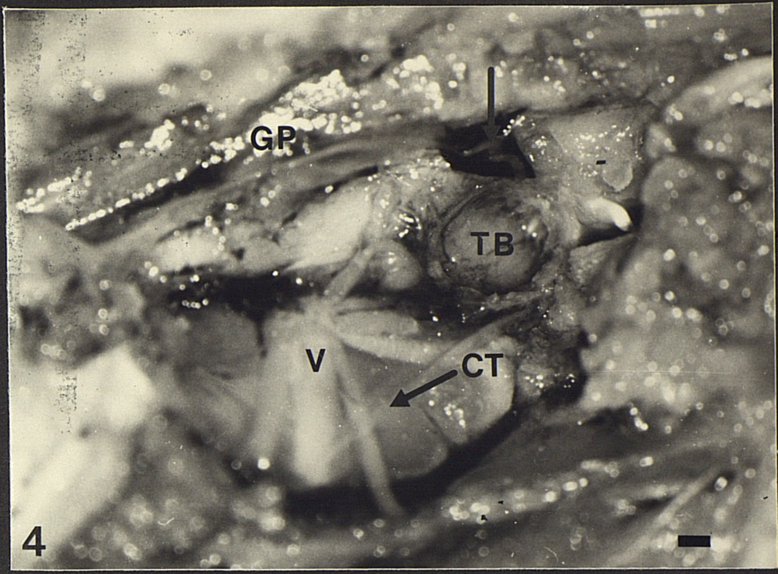

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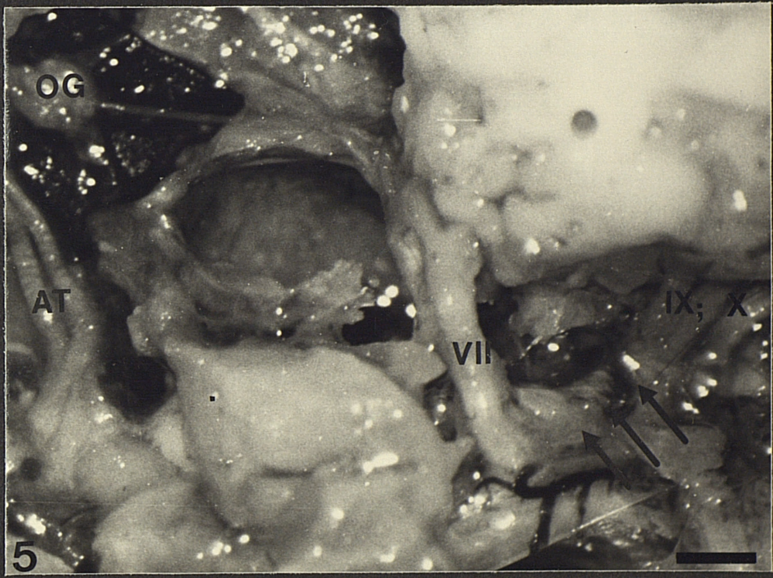
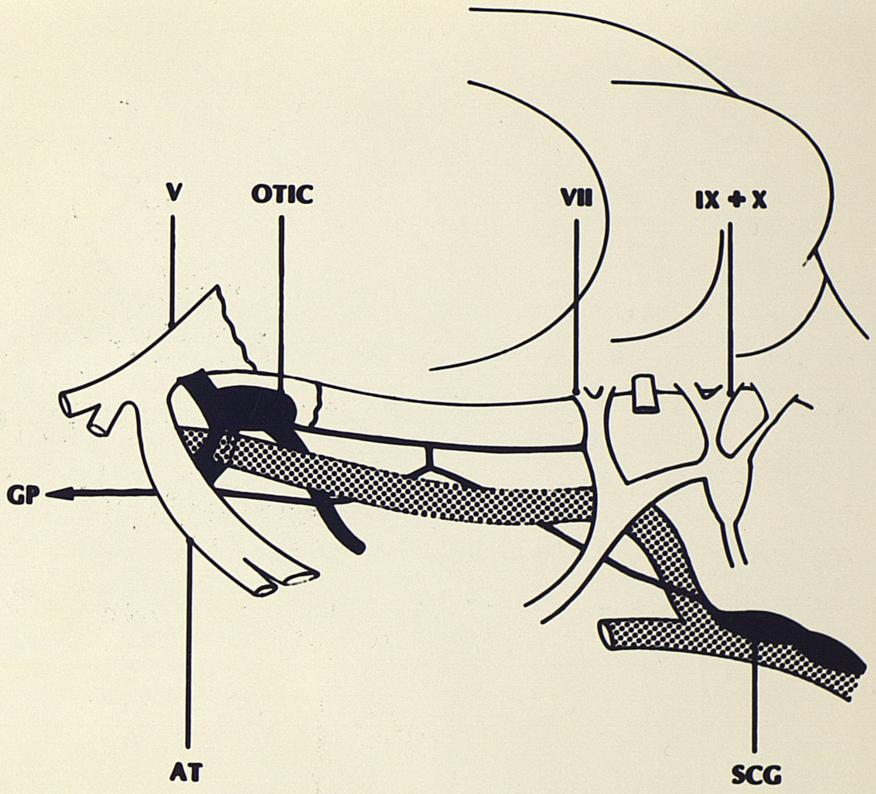


Fig. 6 Diagram to explain the main features of the different neural connections of the otic ganglion. AT - auriculotemporal nerve; GP - greater petrosal nerve; Otic - otic ganglion; SCG - superior cervical ganglion; V, VII, IX & X - respective cranial nerves. NB the chorda tympani branch of the VII nerve has been ommitted to avoid confusion.



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In addition to the small communicating branch of the seventh cranial nerve mentioned above, the chorda tympani also branches off from the rostral edge of the nerve but in a slightly more lateral position. The chorda tympani can be traced into the tympanic bulla where it passes around the head of the malleus before it emerges from the rostral side of the tympanic bulla eventually to fuse with the lingual nerve. From the caudal edge of the facial nerve there is a comparatively large communicating branch which links the facial nerve with the glossopharyngeal-vagal complex (Figs 5,6).

## Light Microscopy of the Otic Ganglion (Figs 7,8)

The otic ganglion like most other autonomic ganglia is surrounded by a thin connective tissue capsule which is composed of fibroblast - like cells. This capsule is continuous with the perineurial covering of the nerves which enter or leave the ganglion and at the light microscope level appears identical to it. This capsular connective tissue penetrates the substance of the ganglion and serves as a conduit for the blood vessels, mainly capillaries, which can be seen throughout the ganglion and its capsule.

The neuronal perikarya are oval or polygonal in shape and are variable in size. There is a tendency for the larger neurones to be centrally placed within the ganglion whilst the smaller ones occupy the periphery . Rather surprisingly these otic ganglion neurones contain few dendrites. The euchromatic nuclei which are variable in position within the cell always contain a prominent nucleolus. The cytoplasm is rich in basophilic Nissl granules and is surrounded by a single layer of supporting cells. Bundles of axons, both myelinated and non-myelinated, are seen both within and emerging from the ganglion.

Fig. 7 Toluidine Blue stained section of the otic ganglion. Bar = 120µm

Fig. 8 Otic ganglion at higher magnification. Bar = 20µm

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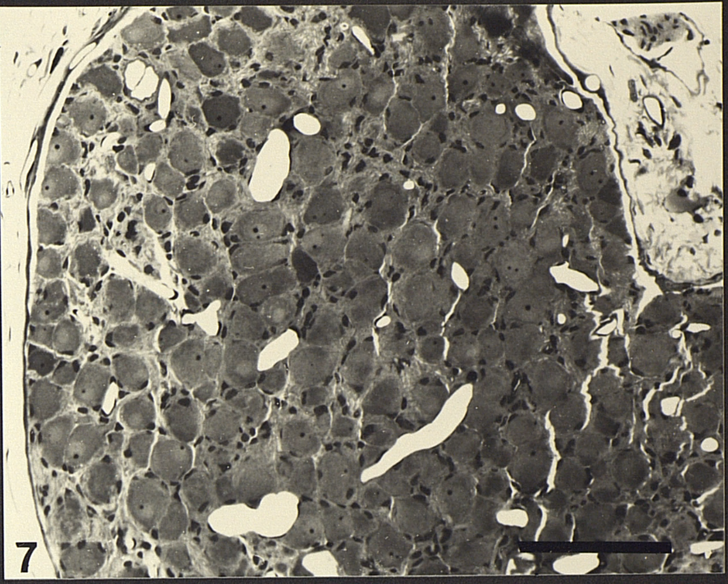

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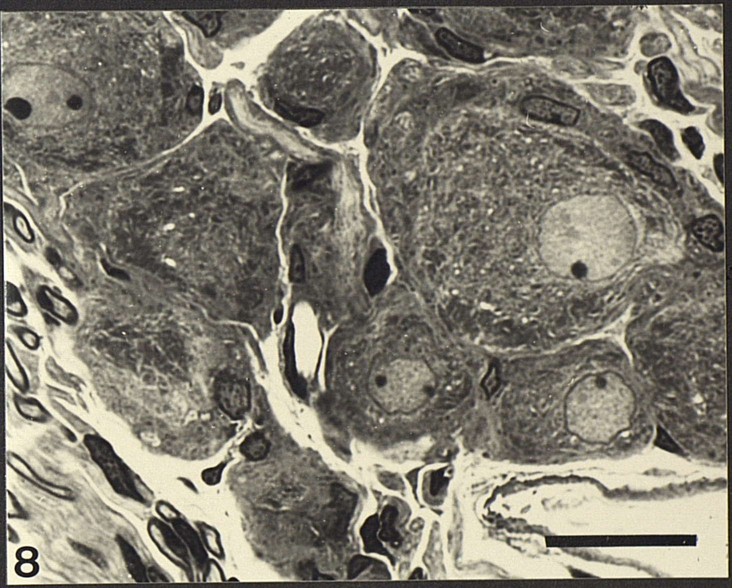


## Ultrastructure of the otic ganglion.

The otic ganglion is surrounded by a connective tissue capsule which is composed of fibroblasts, collagen fibres and blood capillaries.

The multipolar neuronal perikarya are variable in size (the majority fall within the size range of 20-35µm in diameter). There is little or no evidence of substantial structural differences within the population of ganglion neurones. Each cell body contains numerous mitochondria, cisternae of rough endoplasmic reticulum, some free ribosomes, a large Golgi apparatus, lysosomes, microtubules, neurofilaments and vesicles. The nucleus is euchromatic and often contains a prominent nucleolus (Figs 9 & 10).

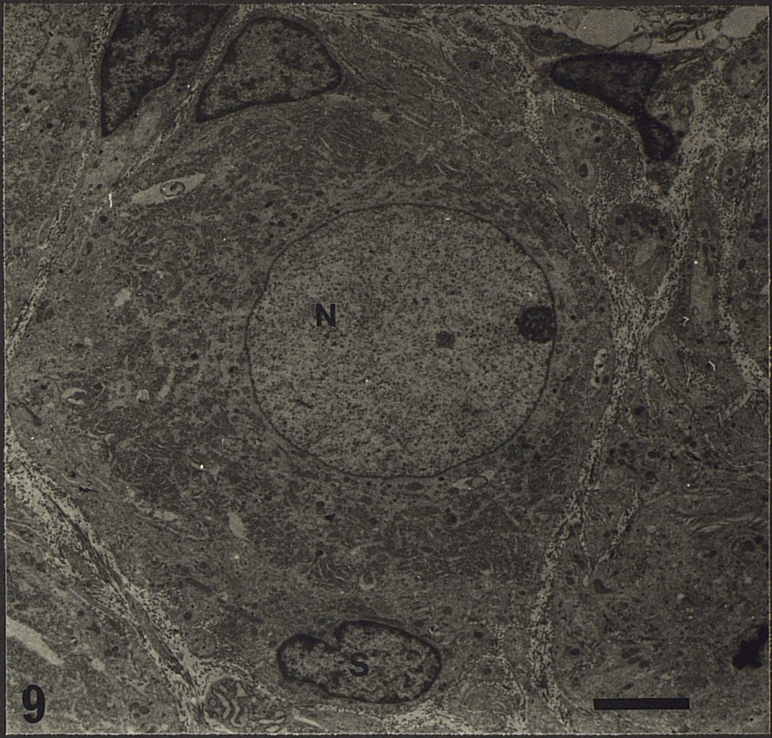
Each ganglion cell is ensheathed by a satellite cell. This glial sheath is continuous over the neuronal soma, although in some areas it is reduced to a thin cytoplasmic process interposed between the neurone on the one side and the basal lamina and connective tissue on the other. The glial sheath also extends over the dendrites. Here, however, there are areas where the neuronal cell membrane lies directly apposed to the basal lamina and connective tissue.

There are abundant nerve endings which synapse on the neuronal cells. The synapses are usually on the dendrites or on dendritic spine-like processes, and only rarely on the cell soma. Most of the intraganglionic nerve endings are packed with small agranular vesicles (50-60nm in diameter) and a few mitochondria, large granular vesicles (110-120nm in diameter) although representing a small proportion of the vesicle population, are also invariably present (Figs 11 & 12).

Both type I and type II synapses (Finn and Geneser, 1986) were found.In type I the post-synaptic densities are very pronounced which results in an asymmetrical appearance at the synapse (Fig 12). In type II synapses the pre and post-synaptic densities are of fairly equal thickness and the synapse appears symmetrical (Fig 11). Type I synapses appear to contain mainly small

Fig. 9 Electronmicrograph of otic ganglion. N - neurone; S - satellite cells. Bar = 2.5µm .

Fig. 10 Electronmicrograph of otic ganglion showing numerous non-myelinated axons and Schwan cells. Bar = 3µm .



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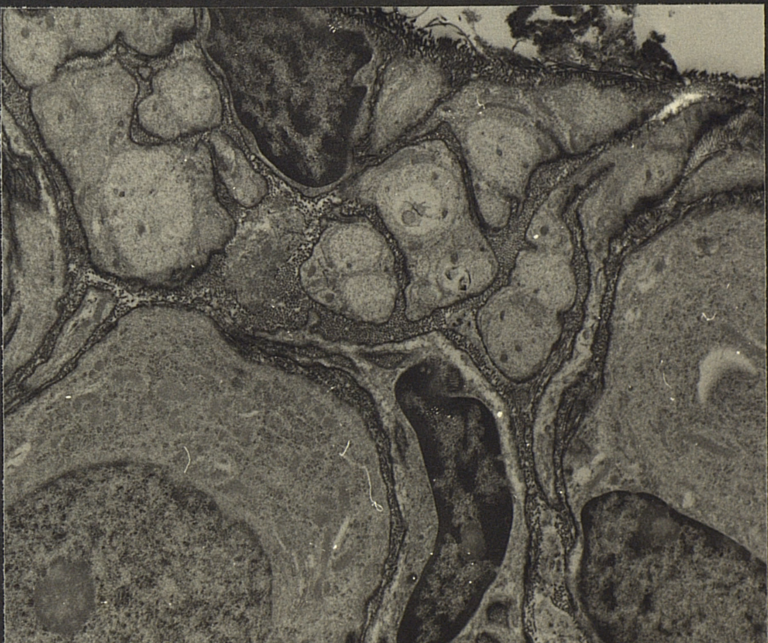

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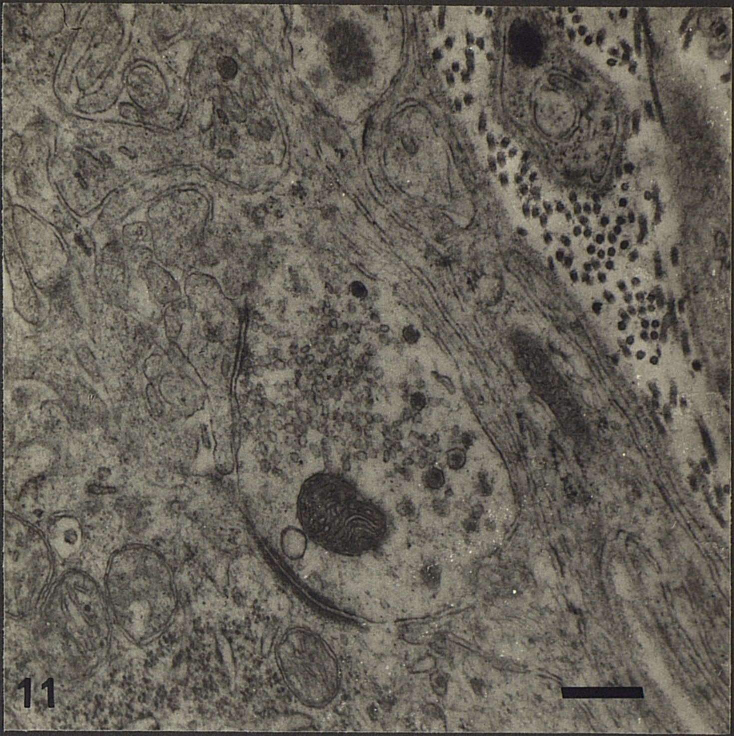
Figs. 11 & 12 Electronmicrographs of synapses within the otic ganglion. NB the symmetric synapse in Fig 11 and the asymmetric synapse in Fig 12. Bar = 250nm .



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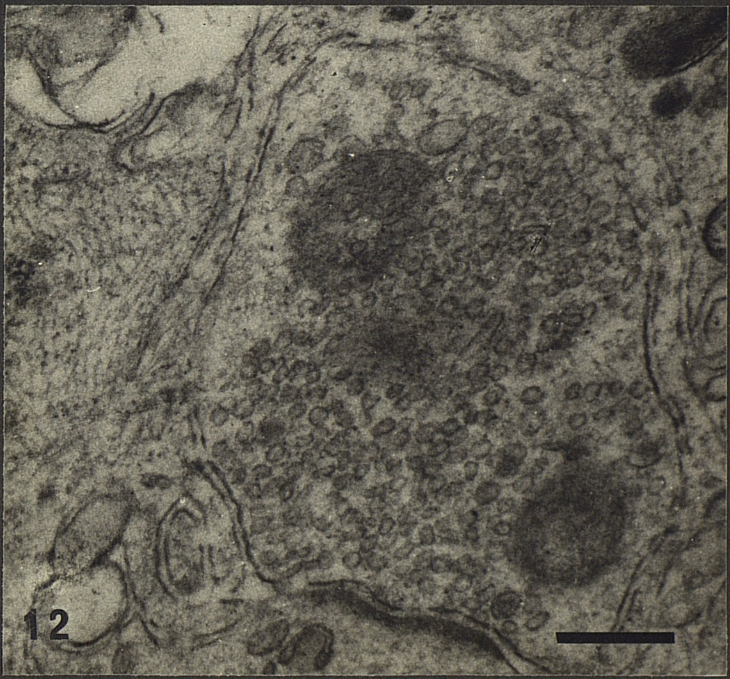

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clear round vesicles and only the occasional large dense-cored vesicle. In the type II synapses the small clear vesicles often appear oval or flattened in shape and the large dense-cored vesicles are more numerous .

Blood vessels, mainly capillaries some of which were fenestrated were found throughout the ganglion and small bundles of axons, both myelinated and non-myelinated, were found towards the periphery of the ganglion.

## DISCUSSION

The results of the present study have established that the location of the otic ganglion in the rat and the topographical anatomy of the nerves that may be important for salivation, in particular for parotid gland secretion, are different from those described for man.

The observations from early anatomical and physiological experiments designed to locate the origin of the preganglionic salivatory neurons can be placed into one of two groups - those which have described small, discrete groups of neurons and those which have reported a diffuse localisation each within the reticular formation of the medulla oblongata. These conflicting results have been obtained from retrograde degeneration studies (Kohnstamm, 1902; Yagita, 1910, Torvik, 1957); histochemical techniques (Shute and Lewis, 1960; Brown and Howlett, 1968) by electrical stimulation studies (Miller, 1913; Magoun and Beaton, 1942; Wang, 1943) and by the use of various animal species.

During the last 20 years there has been increasing evidence from studies employing horseradish peroxidase as a retrograde tracer (Hiura, 1977; Contreras, Gomez & Norgren, 1980; Mitchell and Templeton, 1981) and from more sophisticated electrophysiological methods (Hellekant, 1971; Hellekant and Kasahara, 1973; Donaldson, Mitchell and Templeton, 1984) that the preganglionic parasympathetic salivatory neurons in the rat are located in an uninterrupted column of neurons in the medullary reticular formation. The salivatory neurons innervating the sublingual and submandibular ganglia occupy a rostral location within this column whereas those that supply the otic ganglion lie more caudally and dorsally (Contreras , Gomez & Norgren, 1980).

The axons of the preganglionic parasympathetic salivatory neurons that supply the submandibular and sublingual ganglia leave the brainstem in the nervus intermedius and travel in the chorda tympani through the tympanic bulla to join with the lingual nerve before terminating. This route appears to be common to most mammalian species studied. In species other than the rat the vast majority of preganglionic parasympathetic axons which mediate salivation from the parotid gland travel in the tympanic branch of the glossopharyngeal nerve and terminate in the otic ganglion. However, prior to the present study the route taken by these axons to reach the otic ganglion in the rat was unknown (Contreras, Gomez & Norgren, 1980). By means of dissection and reconstruction from serial histological sections the present study has shown that the only visible connection between the otic ganglion and the cranial nerves is that with the seventh nerve. This very thin branch of the facial nerve passes medially around the outside of the tympanic bulla rather than within the cavity itself. Whether or not the preganglionic salivatory axons actually leave the brainstem with the seventh nerve was not established. The alternative route would be for them to leave the brainstem with the glossopharyngeal nerve and pass along the communicating branch described in this study which connects the glossopharyngeal nerve to the seventh nerve.

This apparently unique situation in the rat deserves further comparison with the petrosal nerves which exist in other species. The greater petrosal nerve in man originates from the seventh cranial nerve and is joined by sympathetic fibres in the deep petrosal nerve to form the nerve of the pterygoid canal.

The branch from the superior cervical ganglion described in the present study could be the equivalent of the deep petrosal nerve. The lesser petrosal nerve in man is formed from the tympanic plexus which consists of the tympanic branch of the glossopharyngeal nerve with branches of the facial nerve (Last, 1978). In the monkey the otic ganglion receives a direct branch from the glossopharyngeal nerve which may represent the lesser petrosal nerve . Such a connection would indicate that a tympanic nerve in the monkey is non-existent . However, a tympanic plexus has been identified which is linked with the facial nerve (Shahid, 1987). In the rat the nerve fascicle observed emerging from the ventral surface of the otic ganglion and which fuses with a branch from the superior cervical ganglion may represent the lesser petrosal nerve. In the rat a tympanic branch of the glossopharyngeal nerve does not exist. The possibility that the communication described in this study linking the glossopharyngeal to the seventh nerve is the rat equivalent of the tympanic nerve cannot be ruled out. These findings are of interest because a similar connection between the glossopharyngeal and geniculate ganglion exists in the early human embryo and gives rise to the lesser petrosal nerve and the tympanic plexus(Gasser, 1966).

In man the parotid gland receives post-ganglionic sympathetic fibres from the superior cervical ganglion via two routes. Firstly, via nerves around blood vessels destined for the gland and secondly through sympathetic fibres picked up by the tympanic plexus from the carotid plexus prior to forming the lesser petrosal nerve (Gray, 1973). In both monkey and rat these sympathetic fibres are probably replaced by the nerves connecting the superior cervical ganglion with the respective cranial nerve (ie glossopharyngeal in the monkey and facial in the rat).

Since Reichert and Poth, (1933) showed that surgical section of the glossopharyngeal nerve in humans did no more than to reduce reflex parotid secretion , the classical description of autonomic control of the parotid gland has been subjected to modification. It seems likely that preganglionic fibres involved in secretion from the human parotid gland come not only from the glossopharyngeal nerve but also via the facial nerve and chorda tympani (Diamant and Wiberg, 1965). Vidic and Young,( 1967) and Vidic ( 1968) describe a nerve in humans which connects the facial nerve complex to the lesser petrosal nerve and which they believe to carry parasympathetic fibres to the parotid gland.

The location of the otic ganglion described in this study also differs from that observed in man and monkey. In man the otic ganglion is 2-3mm in diameter and lies lateral to the tensor palati muscle on the medial side of the mandibular branch of the trigeminal nerve just below the foramen ovale and has no direct relationship with the maxillary artery except in those uncommon cases when the artery passes deep to the mandibular nerve. In the rat the otic ganglion always lies medial to the pterygopalatine branch of the internal carotid artery (equivalent to the maxillary artery in humans) and is separated from the trigeminal ganglion above by the sphenoid bone.

The postganglionic parasympathetic fibres leave the otic ganglion via branches of the auriculotemporal nerve but recent evidence from stimulation experiments in the rat (Al-Hadithi and Breen, 1986) indicate that not all of these branches are crucial for salivation from the parotid gland.

Although the microscopic structure of the otic ganglion is similar to that of other autonomic ganglia the two types of nerve terminal observed within the ganglion are of interest and need further discussion, albeit of a speculative nature.

Numerous attempts have been made to classify nerve terminals according to their morphological structure (Uchizona. 1965; Gray, 1971; Jones, 1975; Geneser, 1986 ) however, great care must be excercised in relating structure to presumed function. The electron density of pre - and postsynaptic thickenings and the shape of synaptic terminals is very much dependent upon fixation protocols and whether or not there is any evidence of degeneration in the tissues being examined. Nonetheless the current view is that asymmetrical synapses are excitatory and symmetrical synapses inhibitory (FitzGerald, 1985). As far as synaptic vesicles are concerned it is generally believed that small round agranular vesicles are excitatory and small elliptical or flattened agranular vesicles may be inhibitory (Uchizona,1965).

The two types of nerve ending found in the otic ganglion would certainly satisfy these criteria. The asymmetrical synapses contained rounded, clear vesicles indicative of excitatory terminals whereas the symmetrical synapses contained flattened vesicles indicative of inhibtory nerve endings. The excitatory terminals are characterisitic of the cholinergic nerve terminals reported by other workers (Richardson, 1966; Johansson and Lundberg, 1981) and may well represent the preganglionic parasympathetic input to the otic ganglion which is known to be cholinergic. The possibility that the inhibitory terminals found in this study contain a neuropeptide awaits further study. Indeed Sharkey and Templeton, (1984) have demonstrated the presence of substance P-containing neurones in the otic ganglion and the possibility cannot be excluded that some of these substance P-containing neurones are intrinsic neurones which synapse within the ganglion.

That neuropeptides appear to have an important role to play in the process of salivation is now well established (Gallacher, 1983; Sharkey and Templeton, 1984). The search for the origin and mechanism of control of these peptide-containing cells necessitated this re-examination of the neuronal connections involved in salivation in the rat; the possibility that the connections observed in the rat may also occur in other species requires further study.

# CHAPTER FOUR

# MICROSCOPY OF THE RAT PAROTID GLAND

# MICROSCOPY OF THE RAT PAROTID GLAND

## INTRODUCTION

Most salivary glands are composed of acinar or tubuloacinar secretory units. The movement of saliva from the secretory units into the duct system is assisted by the contraction of myoepithelial cells. This secretory activity is under the control of the autonomic nervous system. In most cases the postganglionic parasympathetic nerves, which use acetylcholine as their neurotransmitter, innervate the acini and the ducts, whereas the sympathetic fibres which use noradrenaline as their neurotransmitter are believed to innervate the vasculature. Although most of the above-mentioned features are common to all salivary glands there are differences in structure and function within individual salivary glands.

Recent evidence has implicated the importance of the neuropeptides substance P, vasoactive intestinal polypeptide (VIP) and calcitonin gene related peptide (CGRP) in the control of secretion from the rat parotid gland (Uddman et al, 1980; Gallacher, 1983; Thesleff, 1986; Ekman et al., 1986). All three peptides have been identified in the rat parotid gland either by radioimmunoassay or by immunofluorescence techniques (Wharton et al., 1979; Robinson, Schwartz and Costa,1980; Brodin et al., 1983; Goedert, Nagy and Emson,1982; Gallacher, 1983; Sharkey and Templeton, 1984; Ekstrom, et al., 1984; Ekman et al., 1986).

The present study has re-examined the microscopic structure of the rat parotid gland and particular emphasis has been given to the ultrastructural content and location of the nerve terminals within the gland.

## MATERIALS AND METHODS

Seven male Wistar rats each weighing 250g were given an overdose of Sagatal and perfused, via a cannula inserted into the left ventricle. One rat was perfused with 500ml of physiological saline followed by 500ml of 4% glutaraldehyde in 0.1M phosphate buffer pH 7.4. The parotid glands were dissected free of fat and small blocks of tissue approximately 2mm cubed were removed with the aid of a scalpel and placed in fresh fixative overnight. The blocks of tissue were post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer pH 7.2 for 1 hour. After thorough washing in distilled water the tissues were dehydrated through graded alcohols and processed into Araldite. For light microscopy 1 µm sections were stained with 1% Toluidine Blue in 1% Borax. For electron microscopy ultrathin sections 100nm in thickness were mounted onto copper grids and stained with 1% uranyl acetate and 1% lead citrate and examined in a Philips 300 transmission electron microscope.

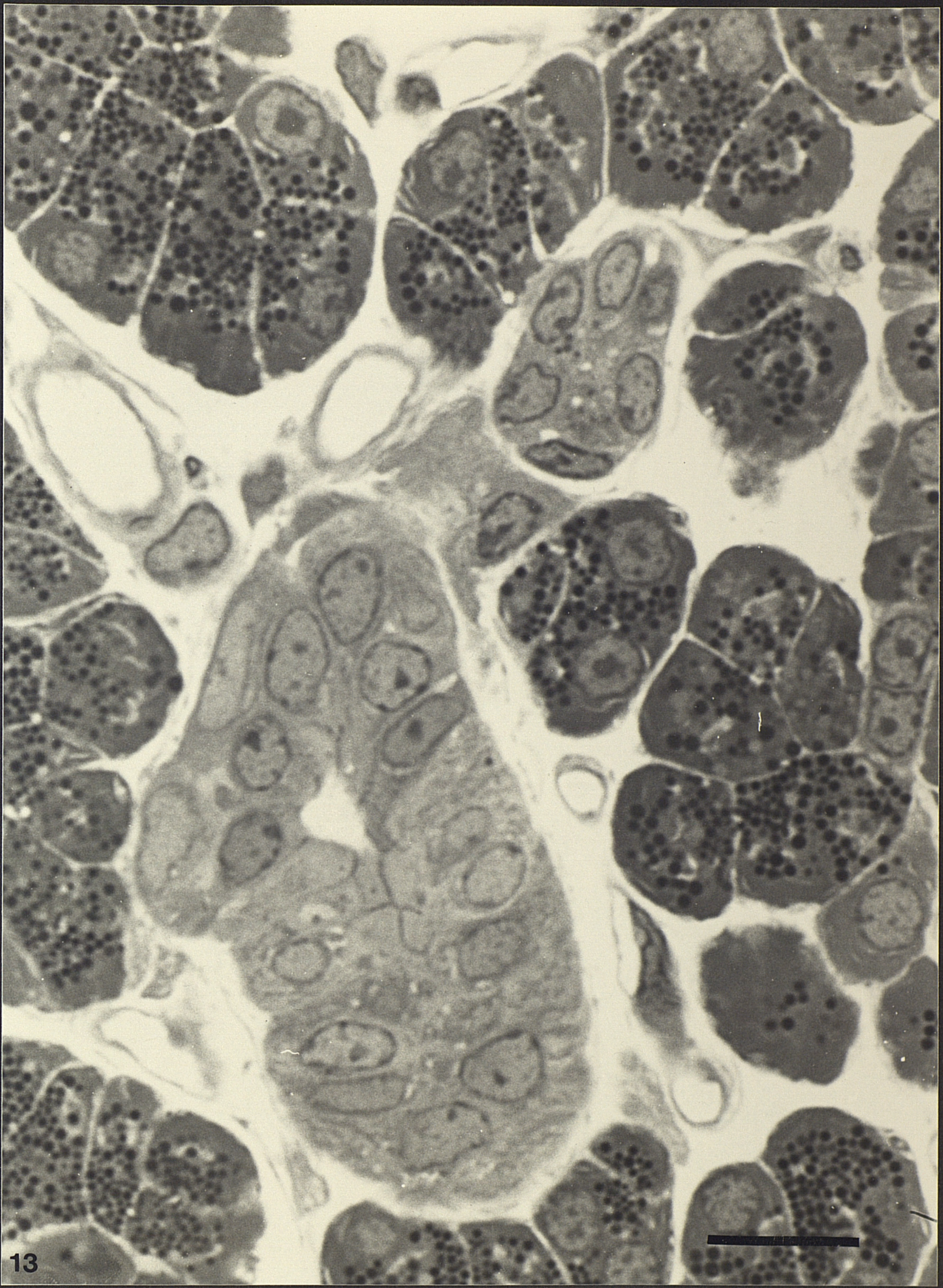
## RESULTS

### LIGHT MICROSCOPY

Typical of most major salivary glands the rat parotid is covered by a connective tissue capsule composed primarily of collagen fibres and fibroblast cells. Strands of connective tissue 'septa' extend into its substance and divide the gland into numerous lobules. Each lobule consists of three secretory components - acini, intercalated ducts and striated ducts.

The acini consist of groups of serous cells with numerous secretory granules (Fig 13 ). Very few cells were found around the outside of the acini that might represent the myoepithelial cells described in other species. Interspersed

Fig. 13 One micron section of parotid gland stained with Toluidine Blue. NB intercalated duct in the centre of photograph. Bar = 10µm



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amongst the acini were numerous cross-sectional profiles of ducts which were lined by cuboidal or columnar epithelium. The larger the duct the taller the epithelial lining. Profiles of 'striated ducts' were occasionally observed . These ducts are so-called because of the noticeable striations in the basal portion of the columnar epithelial cells.

ELECTRON MICROSCOPY:

Acinar cells

The acini consist of groups of cells whose nuclei tend to be situated towards the base of the cell. The apical cytoplasm is filled with secretory granules of varying size and electron density (Fig 14 ). The cytoplasm at the base of the cells contains abundant amounts of rough endoplasmic reticulum (RER). Interspersed amongst the secretory granules and RER the cytoplasm contains a few mitochondria and usually more than one Golgi apparatus. Rarely Were smooth endoplasmic reticulum or polyribosomes seen. The basal membrane of the acinar cells was usually smooth except at sites where nerve endings or the cytoplasmic processes of other cells were present. The lateral and apical plasma membranes were often plicated and numerous cytoplasmic extensions interdigitated with those of adjacent cells. The apical plasma membrane commonly showed irregular microvilli which extend betweeen adjacent cells forming an intercellular secretory canaliculus. Junctional complexes, both zonula occludens and zonula adherens separate the lateral intercellular space from the secretory canaliculus. No myoepithelial cells were seen surrounding the acini.

## Cells lining ducts

The cytoplasmic content of the cells lining both the intercalated and 'striated' ducts was similar. Only the complexity of the infoldings of the basal plasma membrane and the orientation of the mitochondria were different. The nucleus was usually situated towards the base of the cell. Although a few strands of RER were present the bulk of the cytoplasm contained clusters of polyribosomes , numerous mitochondria, a few lysosomes and a prominent Golgi apparatus . The apical cytoplasm contains numerous small vacuoles and occasional small dense granules and the cell surface contains numerous microvilli (Fig 15 ). The basal part of the cell is invaginated by deep folds of the cell membrane, and the resulting cytoplasmic processes interdigitate with similar processes of adjacent cells. These cytoplasmic infoldings contain elongated mitochondria, orientated perpendicular to the base of the cell (Fig 16 ). It is this structural organisation of the cytoplasmic infoldings and orientation of the mitochondria that is more marked in , and accounts for the term 'striated ducts'. Numerous junctional complexes were observed between adjacent cells lining the ducts, these include zonula occludens and zonula adherens. These junctional complexes were most numerous towards the apical region of the cells but some were found at the base of the cell. Myoepithelial cells were not seen around any of the ducts examined.

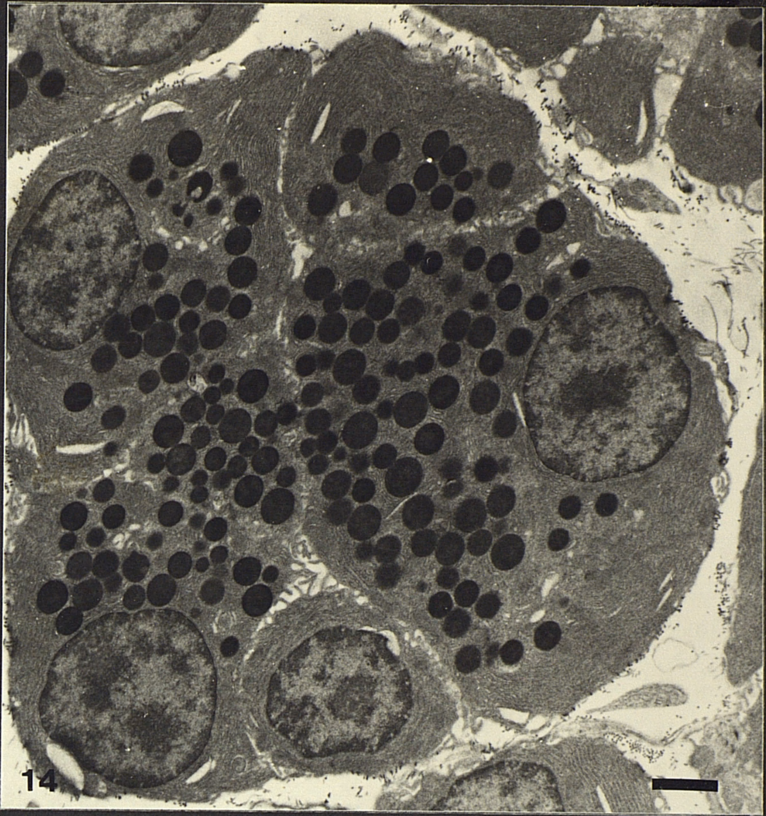
## Nerve terminals and axons.

Bundles of non-myelinated axons were found within the connective tissue septa which separated the gland into lobules , around the periphery of blood vessels and ducts and occasionally amongst acini.

Fig. 14 Electronmicrograph of an acinus showing numerous electron dense secretory granules. Bar = 1μm

Fig. 15 Electronmicrograph of apical region of duct cells. NB microvilli (arrowheads) and junctional complexes between the two duct cells. Bar = 0.5μm

Fig. 16 Electronmicrograph of basal region of duct cells. NB numerous infoldings of the basal membrane and the orientation of the mitochondria. Bar = 0.5μm



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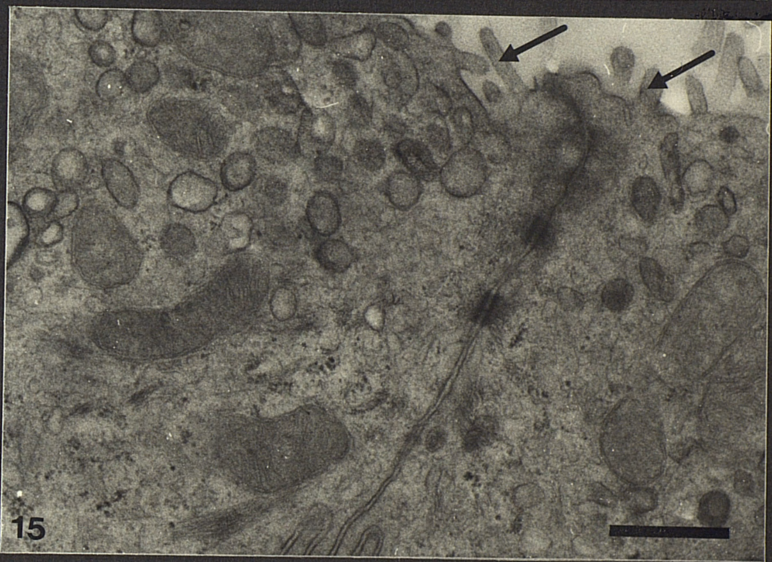

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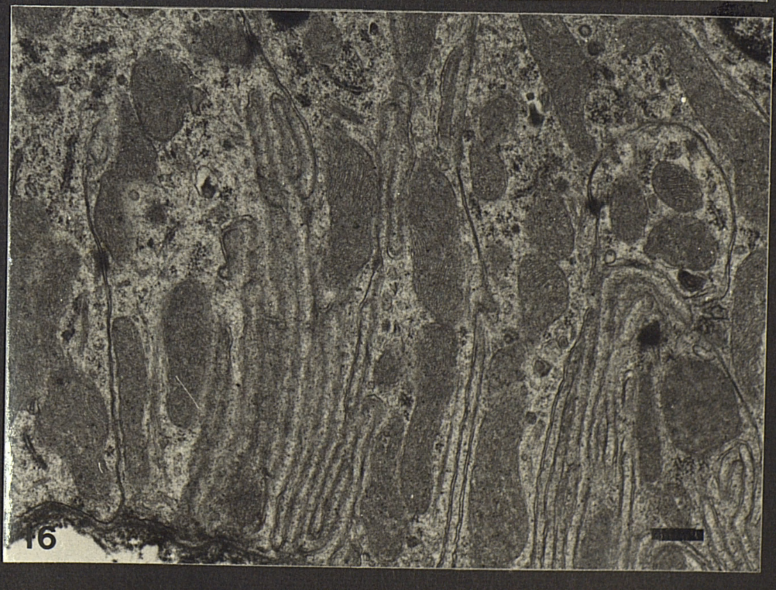

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Nerve terminals associated with acini were found in two sites. They were either located beneath the basement membrane of the epithelium where they were in direct apposition to one or more secretory cells (Fig 17 ) , or outside of the basement membrane where they were invariably surrounded by their Schwan cell cytoplasm (Figs 17, 18 & 19 ). Those nerve terminals beneath the basement membrane were often found some distance from the base of the cell and were lying amongst the cytoplasmic extensions of adjacent cells.

Nerve terminals were also found associated with blood vessels which were frequently fenestrated (Fig 19 ). In this situation, unlike those associated with acini, more than one terminal was apparent. Very few nerve terminals were found associated with the ducts.

In all situations where nerve endings were found their content was similar. The majority of vesicles were small (40-60nm in diameter) spherical or elipsoid and clear. A few larger vesicles (100-140nm in diameter) with an electron dense core were also present. In addition to the vesicles mitochondria were often present.

## DISCUSSION

The contraction of myoepithelial cells around the secretory acinar cells has always been considered to be one of the mechanisms involved in the control of salivary secretion. The apparent lack of such cells in the present study raises the question of their importance in salivary secretion from the rat parotid gland. Scott & Pease( 1959) report a similar absence

Fig. 17 Electronmicrograph showing a hypolemmel nerve terminal lying between the acinar cells and some distance from the basement membrane. Bar = 1µm

Fig. 18 Electronmicrograph of epilemmel nerve terminal containg small spherical agranular vesicles and the occasional large vesicle with an electron dense core. Arrowheads show basement membrane of acinar cells. Bar = 200nm

Fig.19 Electronmicrograph of a Schwanncell containing numerous axon profiles and a nerve terminal. NB the fenestrated capillary. Bar = 200nm

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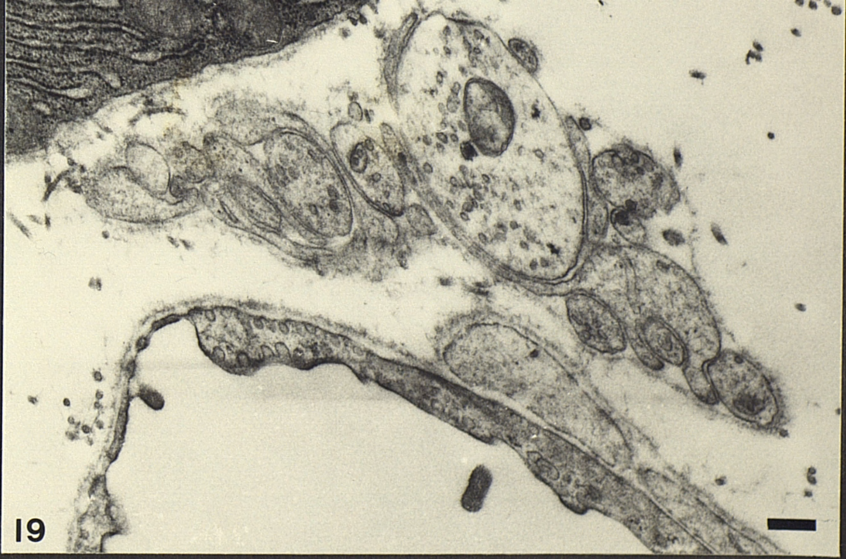


of myoepithelial cells from the rat parotid gland whereas Leeson & Leeson (1971) using freeze-etched preparations are equally positive that myoepithelial cells are present and that they do invest both the acinar and duct secretory cells. Myoepithelial cells are rich in alkaline phosphatase activity (Young and Van Lennep , 1978; Leeson & Leeson, 1981) and this property has been used to identify the cells at the light microscope level. Garret & Parsons (1973) showed that the alkaline phosphatase positive myoepithelial cells in the rat parotid are very atypical, and are confined mainly to the intercalated ducts. Occasionally the processes of the duct myoepithelial cells may extend to the base of some acinar cells. However it is questionable if they play any major role in the mechanism of salivary secretion in the rat parotid gland.

The presence of nerve endings in the two sites described in this study have been reported previously. Those located beneath the basement membrane have been called hypolemmel terminals and those found outside of the basement membrane as epilemmel ( Garret, 1975 ). The close contact of the nerve terminals with the acinar cells has been observed in both the parotid and sublingual glands of the rat but not in the mandibular gland (Scott & Pease , 1959; Parks, 1961; Tamarin, 1966; Bogart, 1970; Hand, 1970).

The rat parotid gland contains both cholinergic and adrenergic nerve terminals. Terminals with small dense-cored vesicles measuring 40-60nm in diameter have usually been described as adrenergic, whereas those containing small, agranular vesicles are usually considered to be cholinergic. In the present study no terminals were seen which contained small vesicles with a dense core. However this is probably due to the use of glutaraldehyde and osmium tetroxide fixation protocols which are not the ideal fixatives for the demonstration of the electron dense cores (Richardson, 1966; Tranzer and Thoenen, 1967). Histochemical techniques for acetylcholinesterase and the uptake of tritiated noradrenaline have also been used to identify cholinergic and adrenergic terminals, respectively in the rat parotid gland (Bogart, 1971; Bogart and Delemos, 1973).

Both cholinergic and adrenergic terminals have been reported to contact the same cell (Hand, 1972). Young and Van Lennep, (1978) report that most hypolemmel terminals in the parotid gland are cholinergic. However both cholinergic and adrenergic terminals have been observed outside of the acinar cell basement membrane. Based on morphological evidence alone it has been suggested by Hand (1972) that about 2/3 of nerve terminals in the rat parotid gland are adrenergic and the remaining are cholinergic.

More recently other neuropeptides such as substance P, VIP and CGRP have been identified in the rat parotid gland either by radioimmunoassay or by immunofluorescence techniques (Wharton et al., 1979, Uddman et al., 1980, Ekman et al., 1986). The ultrastructural morphology of the nerve terminals observed in the present study give no indication as to which terminals contain the neuropeptides. The lack of any obvious difference between the nerve terminals may indicate that the neuropeptides are co-localized with one another and / or with the classical neurotransmitters acetylcholine and noradrenaline. Such co-localization would not be unusual. VIP and acetylcholine have been reported to co-exist in nerves of exocrine glands of the cat (Lundberg, 1981) and substance P and CGRP are known to co-exist in many sensory neurons such as those in the trigeminal ganglion (Lee et al., 1985a,b)

Further work is needed not only to localize the neuropeptide-containing neurones whose axons project to the parotid gland but also to positively identify the neuropeptides at the ultrastructural level within the the nerve terminals.

# CHAPTER FIVE

# NEURAL CONNECTIONS TO THE PAROTID GLAND USING THREE DIFFERENT RETROGRADE TRACERS

# NEURAL CONNECTIONS TO THE PAROTID GLAND: A COMPARISON WITH THREE DIFFERENT RETROGRADE TRACERS.

## INTRODUCTION

Classical knowledge about the autonomic connections involved in the control of secretion from the salivary glands was based on dissection or retrograde degeneration studies carried out in the early part of the 20th century (Kohnstamm, 1902; Yagita, 1910; Miller, 1913; Magoun and Beaton, 1942 and Wang, 1943). More recent studies (Garrett and Holmberg, 1970, 1972) and the use of more sophisticated electrophysiological (Hellekant and Kasahara, 1983; Donaldson, Mitchell and Templeton, 1984) enzyme histochemical (Contreras, Gomez and Norgren, 1980; Mitchell and Templeton, 1981) and dissection techniques (Al-Hadithi and Mitchell, 1987) have, in the rat at least, produced new evidence about the origin and the anatomical course that some of the preganglionic parasympathetic neurones take to reach their destination.

In addition to the involvement of the classical neurotransmitters noradrenaline and acetylcholine recent evidence has implicated the importance of the neuropeptides substance P (SP), vasoactive intestinal polypeptide (VIP) and calcitonin gene related peptide (CGRP) in the control of secretion from the salivary glands (Uddman et al, 1980; Gallacher, 1983; Thesleff, 1984; Ekstrom et al, 1986). Each of the above-mentioned neuropeptides have been demonstrated in the rat parotid gland either by immunocytochemistry and/or radioimmunoassay.

Although there is some evidence that substance P in the rat parotid gland may originate from two sources (Sharkey and Templeton, 1984). The origin of the VIP and CGRP is uncertain. The present study has used three different retrograde tracers to establish the neural connections to the parotid gland. The results provide further evidence for the possible origin of the neuropeptide-containing cells which control salivation from the rat parotid gland but they also suggest caution in interpretation of results obtained with the different tracers.

## MATERIALS AND METHODS

Adult male Wistar rats each weighing 250g body weight were used for this study. Each animal was anaethetised with an intraperitoneal injection of Sagatal (40mg/Kg body weight: May and Baker, Dagenham). A unilateral incision was made to expose the parotid gland. With a 10µL Hamilton syringe multiple injections (usually three) of the tracer were made into the substance of the parotid gland. Following each injection the needle was left in situ for 2 minutes to allow maximum absorption of the tracer and care was taken to minimise leakage on withdrawl.The total volume injected of either of the two fluorochromes True Blue 1%(n=14) or FastBlue 1%(n = 13)(DrIlling GmbH & Co. KG, Germany) varied between 1-9µL. Animals (n = 6) injected with horseradish peroxidase conjugated to wheatgerm agglutinin (HRP/WGA; Sigma Chemical Co.Ltd., Dorset, England) received a total volume of 0.5 or 1µL of a 5% solution.(n= 6).

Each animal received an overdose of Sagatal and was perfused via the heart. Animals which had been injected with a fluorochrome were killed 7 - 10 days after injection and those which had received an injection of HRP/WGA were killed after 48 hours. Each animal was perfused with 500ml of physiological saline followed by 500ml of fixative; for the fluorochrome injected animals the fixative was 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 and for the HRP injected animals the fixative was 1.25% glutaraldehyde, 1% paraformaldehyde in 0.1M phosphate buffer pH7.2. This was followed by 300ml of 7.5% sucrose in 0.1M phosphate buffer pH 7.2.

The following tissues were removed from each animal - the otic, trigeminal, geniculate and superior cervical ganglia from both sides; the spinal cord with its attached dorsal root ganglia ; and 5mm slice of brainstem cut in the coronal plane to include the salivatory nuclei. The rostral cut was made through the inferior colliculi and the caudal cut just distal to the paraflocculus.

Frozen sections were cut at 20μm thickness and were either reacted for peroxidase activity according to the method of Mesulam,( 1978) or mounted onto albumenised slides and examined in an Olympus fluorescence microscope fitted with a UG-1 exciter filter, a U ( DM-400 + L-420) dichroic mirror and an L435 barrier filter.

As a control for the leakage of dye from the injection site, the parotid gland was exposed on one side and 9uL of either fluorochrome or 1uL of HRP/WGA was allowed to drip onto the surface of the gland. The incision was sutured and the animals left to survive for 7 days in the case of those which had been exposed to a fluorochrome (1 animal for each tracer) and 48 hours for the animal which had been injected with HRP/WGA.

## RESULTS

Irrespective of the tracer used heavily labelled neuronal perikarya were observed in the ipsilateral otic, superior cervical and trigeminal ganglia (Figs 20, 21, 22 & 23 ). Interestingly even with the largest injection sites the number of cells retrogradely labelled in the otic ganglion never exceeded 60%. No labelled neurones were found in the geniculate ganglion or in the contralateral otic trigeminal or superior cervical ganglia.

With each tracer sections taken from the brainstem pontomedullary junctional area revealed heavy retrograde labelling of neurones in the facial and suprafacial nuclei on the ipsilateral side (Figs 24, & 25 ). However an additional group of neurones were observed in the ipsilateral dorsolateral reticular formation when HRP/WGA was used as the tracer.

When Fast Blue was used as the tracer labelled neurones were also found bilaterally in the dorsal root ganglia associated with spinal cord segments C4 through to T3 and in the intermediolateral column of spinal cord segments C8 through to T3 (Figs 26, & 27). No equivalent labelling was found when either True Blue or HRP/WGA was used as the tracer.

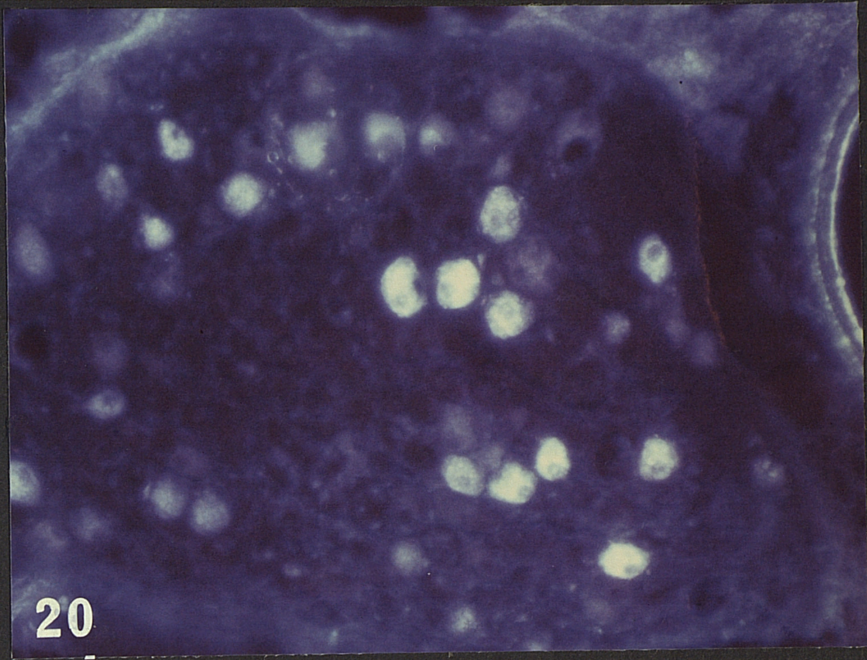
Examination of the tissues from the control animals, where the fluorochrome dye or HRP/WGA was applied to the surface of the parotid gland, revealed no retrograde labelling of neuronal perikarya.

## DISCUSSION

The use of tracers, which are transported axonally either in a retrograde or anterograde direction, as tools to map neural

Fig. 20 Neurones in the otic ganglion which have been retrogradely - labelled with Fast Blue. x 250 magnification .

Fig. 21 Otic ganglion neurones which have been retrogradely-labelled with True Blue.x 350 magnification .



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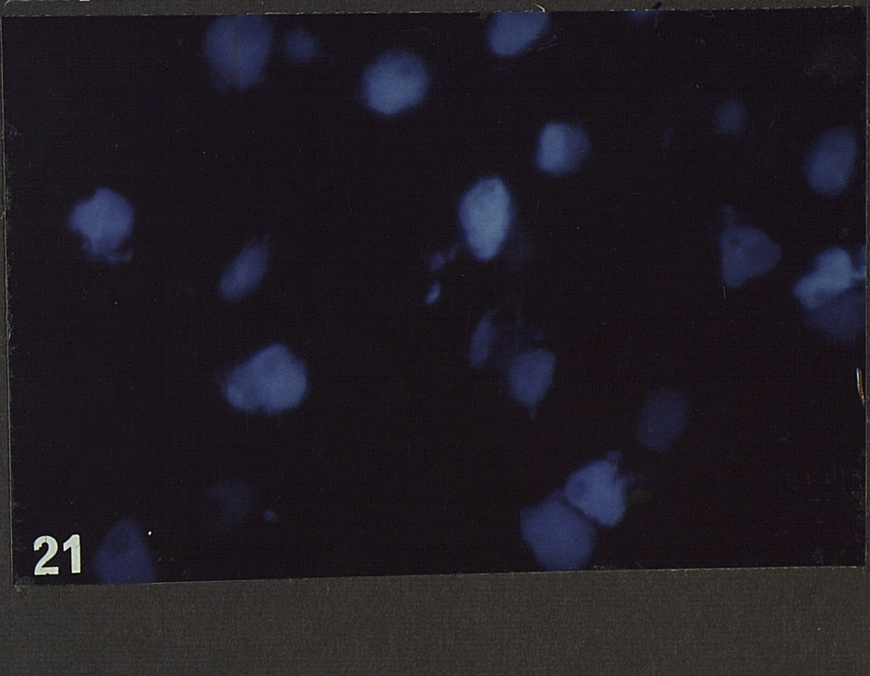

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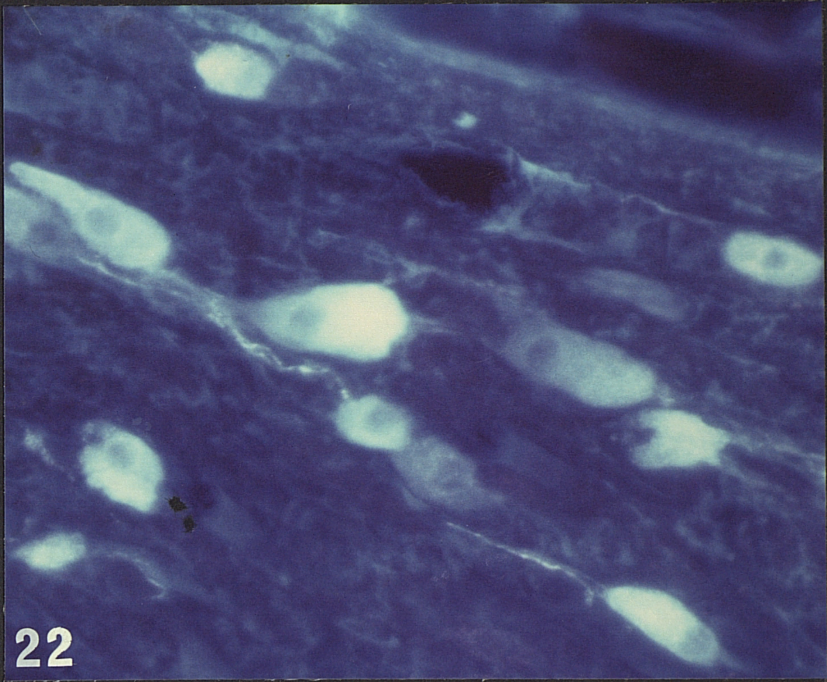
Fig. 22 Neurones in the superior cervical ganglion which have been retrogradely-labelled with Trus Blue. Magnification x 350.

Fig. 23 Neurones in the trigeminal ganglion which have been retrogradely-labelled with Fast Blue . x 350 magnification .



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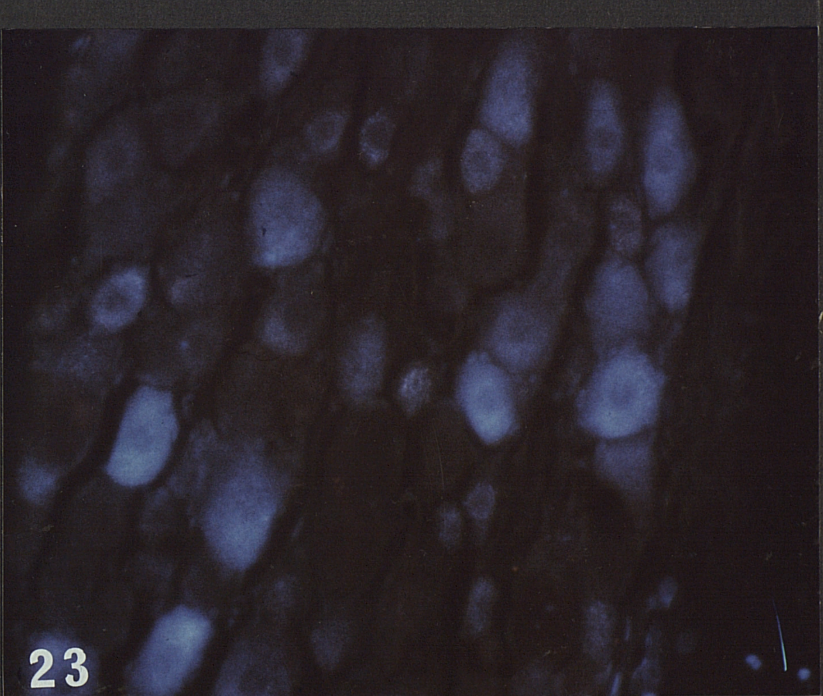

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Fig. 24 Diagram of coronal sections through the rat brain to illustrate neurones retrogradely-labelled with HRP/WGA following injection into the parotid gland. CER - cerebellum; F - facial motor nucleus; P - pyramids; SO - superior olivary nucleus; V - trigeminal sensory nucleus.

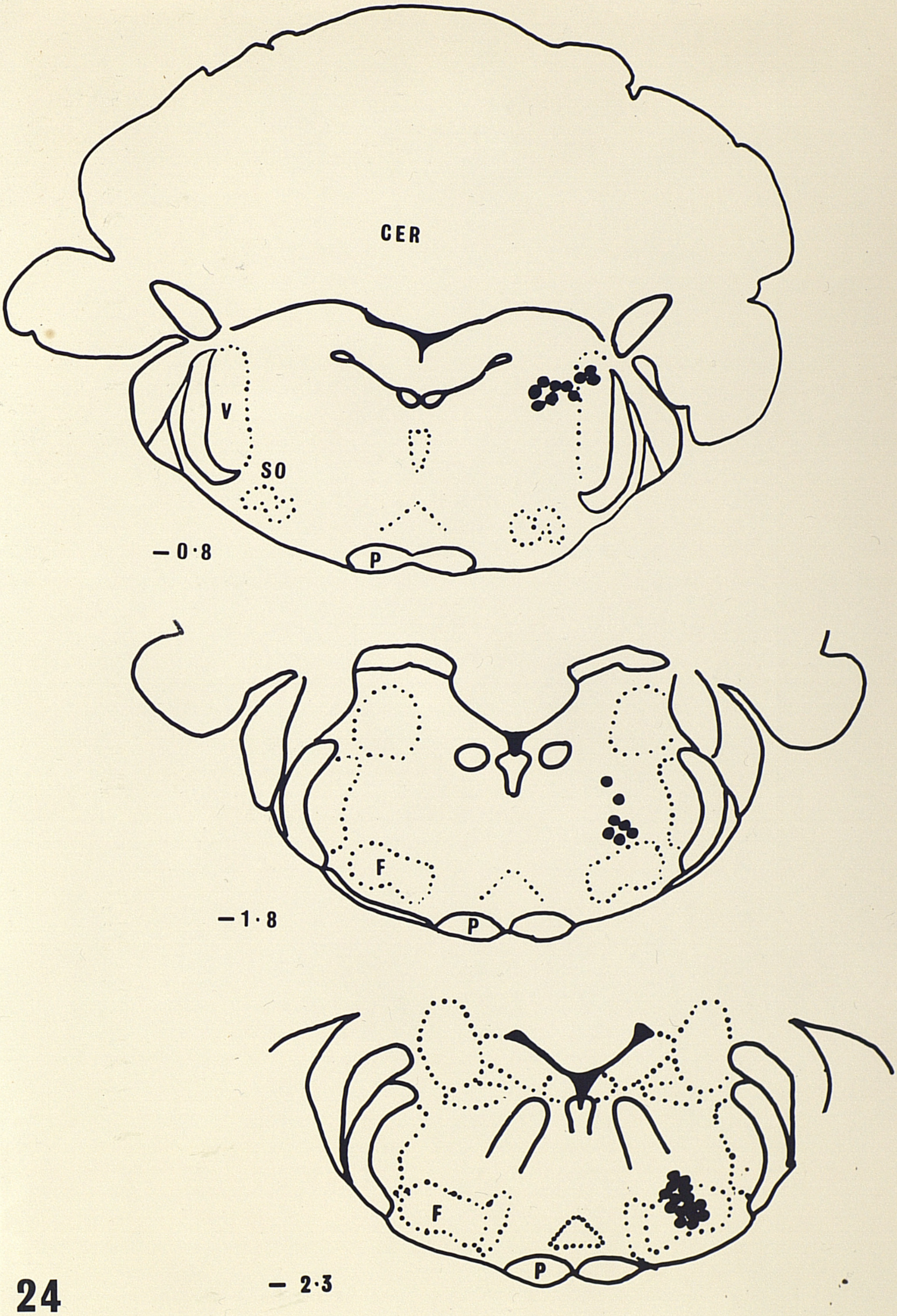

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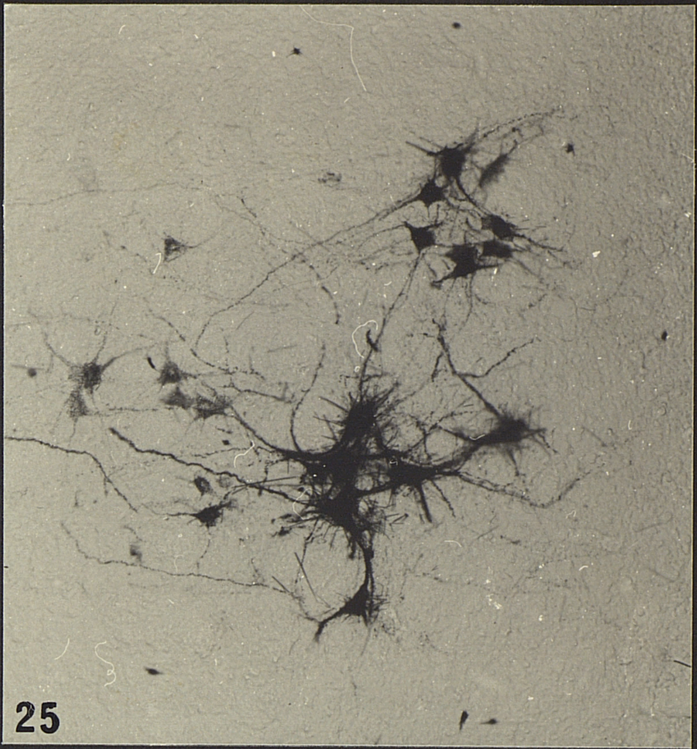
Fig. 25 Neurones in the facial and suprafacial motor nuclei which have been retrogradely-labelled with HRP/WGA x 250 magnification .

Figs. 26 & 27 Neurones in the intermediolateral column of the spinal cord (26) and dorsal root ganglion (27) which have been retrogradely-labelled with Fast Blue. x 350 magnification.



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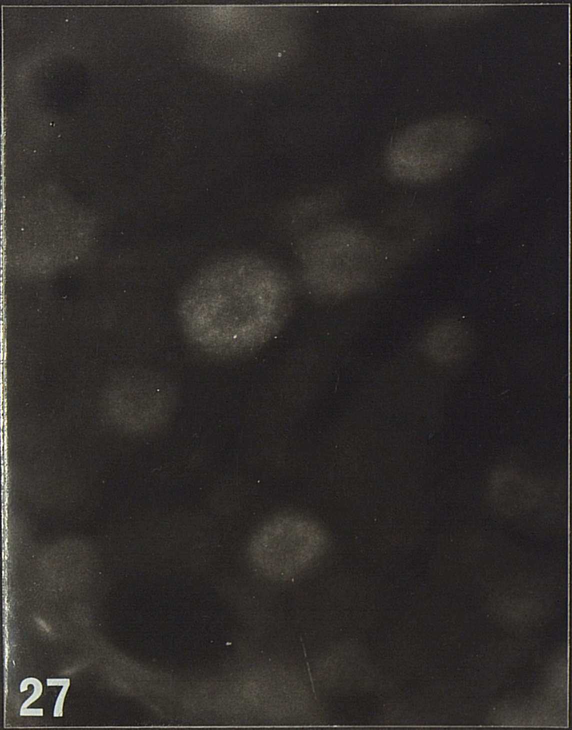

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pathways is well established. A wide variety of compounds have been tested as potential tracers each with its own advantages and/or disadvantages. The most useful include fluorescent dyes (Bentivoglio et al, 1979) horseradish peroxidase (HRP) (Kristensson and Olsson, 1971; LaVail and LaVail,, 1972) and HRP conjugated to wheatgerm agglutinin HRP/WGA (Lechan, Nestler and Jacobson, 1981).

Of the fluorescent dyes, True Blue and Fast Blue have been used most extensively primarily because they can be left in situ for 7 days or more without any apparent leakage from the retrogradely labelled neuronal perikarya and can therefore be used to study long-tract pathways. In previous studies where fluorochromes have been compared with HRP as potential retrograde tracers similar results were obtained irrespective of whether True Blue, Fast Blue or HRP was used as a retrograde tracer (Aschoff and Hollander, 1982, Illert et al, 1982).

In preliminary experiments Fast Blue was chosen as the tracer to investigate the afferent connections to the parotid gland. The retrograde labelling of neurones in the ipsilateral otic, superior cervical and trigeminal ganglia can be explained on the basis that these sites are the established origins of the postganglionic parasympathetic, sympathetic and sensory projections respectively, to the parotid gland. The possibility that one or more of these ganglia contain the neuropeptides that have been implicated in the control of secretion from the rat parotid gland is supported by other evidence in the literature.

When the auriculotemporal nerve is sectioned the levels of SP and VIP fall by 95% (Brodin et al., 1984; Ekstrom et al., 1984) which would suggest that the otic and/or trigeminal ganglion rather than the superior cervical ganglion are the main source of these two neuropeptides in the parotid gland. Sharkey and Templeton (1984) showed that following capsaicin treatment nerve fibres around the vasculature within the parotid gland disappeared whereas those around the secretory acini remained. These authors therefore suggested that the SP in the rat parotid gland originates from two different sources, and that the innervation of the vasculature is from the trigeminal ganglion whereas that to the secretory acini is from neurones in the otic ganglion.

CGRP and SP have been co-localized in trigeminal ganglion cells (Lee et al., 1985) and CGRP like SP, has been shown to have a vasodilatatory effect (Edvinsson and Uddman, 1982; Fischer et al., 1983). CGRP has also been shown to enhance the effect of SP (Ekman et al., 1986) although alone it has no effect on secretion. However, the origin of the CGRP in the parotid gland is much more difficult to establish because when the auriculotemporal nerve is sectioned the levels of CGRP drop by only 20% which would suggest that the otic and trigeminal ganglia are not the main source of supply. The possibility that the superior cervical ganglion contains CGRP neurones can also be eliminated because following sympathectomy, levels of CGRP increase by 150% (Ekman et al., 1986).

Although the otic ganglion contains the postganglionic parasympathetic neurones it was interesting to note that even when large volumes of fluorochrome were injected into the salivary gland no more than 60% of the cells in the otic ganglion were labelled. The possibility that many of the otic ganglion neurones are intrinsic neurones cannot be ruled out.

Retrograde labelling of neurones in the ipsilateral facial and suprafacial brainstem nuclei may be explained on the basis of uptake of the dye into axons of the facial motor nuclei which branch extensively within the substance of the parotid gland (Greene, 1963;Sawchenko and Swanson, 1981). Although care was taken to avoid leakage from the injection site the possibility that the dye was taken up into intact nerve endings within the facial musculature cannot be excluded. However when the fluorochrome dye was dripped onto the surface of the parotid gland no retrograde labelling of the facial neurones was observed. A more likely explanation for this retrograde labelling would be uptake into axons which were inevitably damaged during the injection procedure especially as multiple injections were given. A similar retrograde labelling of facial motor neurones following salivary gland injections has been reported previously (Mitchell and Templeton, 1981) when HRP was used as the tracer.

The bilateral labelling of neurones in the dorsal root ganglia and in the intermediolateral columns of the spinal cord is more difficult to explain. The most likely explanation for the bilateral labelling would be uptake of the Fast Blue dye via leakage from the vasculature. Such labelling of neurones in the dorsal root ganglia and intermediolateral columns of the spinal cord has been reported previously following either direct injection into or via absorption from the circulation (Van Der Krans and Hoogland, 1983, Sterner, Fox and Powley, 1985). Why only these spinal cord neurones should so readily transport the Fast Blue dye from the vasculature is uncertain.

In the experiments where True Blue and HRP/WGA were used as the tracer no labelling of dorsal root ganglia or spinal cord neurones was observed. Such results would imply that leakage into the vasculature is not common to all fluorochromes or indeed to HRP/WGA. When HRP was injected intravenously it was shown by Jacobs, Macfarlane & Cavanagh (1976) that rat dorsal root ganglion neurones will pinocytose any of the protein which has leaked from the vasculature. Howewver, such protein uptake was minimal and its removal by macrophages occurred very rapidly.

The retrograde labelling of neurones in the ipsilateral, dorsolateral reticular formation, corresponds to an area described by Szentagothai (1952) as a 'lacrimosalivary column' and also to an area which when directly stimulated causes secretion from the parotid gland in the rat (Donaldson, Mitchell and Templeton, 1984). Whether or not these retrogradely labelled neurones are the preganglionic salivatory neurones cannot be ascertained without further study. However, their position in the lateral reticular formation is further rostral than has been reported previously for salivatory neurones (Contreras et al., 1980, Mitchell and Templeton, 1981; Donaldson et al., 1984). The possibility that these neurones have been labelled by transynaptic transport rather than by direct retrograde transport from the parotid gland cannot be excluded. Such transynaptic transport has been reported for HRP (Harrison, et al., 1984, Jankowska and Skoog, 1986).

In summary, the present study has demonstrated that the otic, trigeminal and superior cervical ganglia each contain neurones whose axons innervate the parotid gland and which may therefore contain the neuropeptides which have been implicated in the control of salivation from this gland. The results have also shown that when the fluorochrome Fast Blue is used as a retrograde tracer, care should be taken in interpretation of the results as it appears to be readily absorbed into the circulation.

# CHAPTER SIX

# THE ORIGIN OF NEUROPEPTIDES IN THE RAT PAROTID GLAND
## A COMBINED IMMUNOFLUORESCENCE AND RETROGRADE TRANSPORT STUDY

# THE ORIGIN OF THE NEUROPEPTIDES IN THE RAT PAROTID GLAND
# A COMBINED IMMUNOFLUORESCENCE AND RETROGRADE TRANSPORT STUDY

## INTRODUCTION

The salivary glands are innervated by nerve fibres from both the sympathetic and parasympathetic nervous systems. Classically this would imply the use of the neurotransmitters noradrenaline and acetylcholine respectively. However the results of recent work have suggested that the control of secretion from the salivary glands may involve other potential neurotransmitters , the neuropeptides substance P (SP), vasoactive intestinal polypeptide (VIP), and calcitonin gene-related peptide (CGRP) (Wharton et al., 1979, Uddman et al., 1980, Gallacher, 1983, Ekman et al., 1986, and Thesleff, 1986). Each of these neuropeptides has been demonstrated in the rat salivary glands ( Wharton et al, 1979, Uddman et al., 1980, Brodin and Nilsson, 1981, Ekstrom et al., 1984, Sharkey and Templeton, 1984, Ekman et al., 1986).

When the auriculotemporal nerve was sectioned in rats SP, VIP and choline acetyltransferase levels fell by 95% and CGRP levels by 20%. These results would suggest that the neuropeptides SP , VIP and some of the CGRP might originate in the trigeminal and or otic ganglia. Sharkey and Templeton, (1984) have suggested a dual origin for SP fibres in the rat parotid gland but the origin of the VIP, and CGRP fibres is unknown.

In the previous chapter it was shown by using fluorochrome dyes as retrograde tracers that the otic, trigeminal and superior cervical ganglia each had projections to the rat parotid gland. The present study will investigate the origins of the neuropeptides in the parotid gland by combining retrograde transport studies with immunocytochemical techniques to localalise SP, VIP, CGRP and choline acetyltransferase. In addition sections from the otic ganglion will be incubated for acetyl cholinesterase activity.

Evidence for the existence of neuropeptides with classical neurotransmitters or with other neuropeptides is now abundant (Lundberg et al., 1980, Johanson and Lundberg, 1981, Wiesenfeld - Hallin et al., 1984, Lee et al., 1985). The possibility that one or more of the neuropeptides present in the rat parotid gland might co-exist in the same neurone will also be investigated.

## MATERIALS AND METHODS

### I IMMUNOCYTOCHEMICAL LOCALISATION OF SP, VIP AND CGRP

Seven adult Wistar rats each weighing 250g body weight were used for this part of the study. The animals were heavily anaesthetised with an intraperitoneal injection of Sagatal (60mg/Kg) and perfused ,via a cannula inserted into the left ventricle. The perfusates were 500ml of 0.85% physiological saline followed by 500ml of 4% paraformaldehyde in 0.1M phosphate buffer at a pH of 7.4.

Both parotid glands,, otic, trigeminal and superior cervical ganglia were removed from each animal and placed in fresh fixative for a further 4 hours and then transferred overnight into 0.1M phosphate buffer (pH 7.4) which contained 10% sucrose. The next day the tissues were placed in a solution of 30% sucrose in 0.1M phosphate buffer (pH7.4).

Frozen sections with an optimum thickness of 10$\mu$m were taken serially from each block of tissue and thaw-mounted onto clean glass microscope slides which had been previously coated with poly-L-lysine (Huang et al., 1983). All sections were processed for immunocytochemistry using an indirect immunofluorescence technique to demonstrate SP, VIP or CGRP (see below for details of the technique used). Sections were mounted on to the slides as illustrated in Figure 28. All sections were examined in an Olympus BH RFL-W fluorescence microscope fitted with a HBO mercury vapour lamp. A dichroic mirror block BH-DMBG containing a 20BG-W and an EY455 filter was used for excitation and an O-530 filter was used as a barrier.
The optimum conditions for the immunofluorescence technique were established by a series of experiments. The method detailed below is the one which was found to be most reliable for the peptides under investigation.

## The Immunofluorescence Method Used To Demonstrate SP, VIP And GGRP In The Rat Parotid Gland, Otic, Trigeminal and Superior Cervical Ganglia.

1. Control sections: sections from dorsal root ganglia and spinal cord were used as positive controls for SP-like immunoreactivity. As negative controls for each neuropeptide the incubation of the sections in the primary antibody was ommitted and replaced by incubation in the presence of phosphate-buffered-saline.

2. The serial sections mounted onto poly-L-lysine-coated slides were allowed to dry for 1 hour before incubating in the primary antibody (see below for source and dilution etc.) for 24 hours, at room temperature in the case of SP and at $4^{\circ}$C for CGRP and VIP.

3. The primary antibody:

a) Two sources of Substance P antibody were used one was obtained from Cambridge Research Biochemicals Ltd, Cambridge, Batch No. VG 18/312, the other was a gift from Dr Keith Sharkey, MRC Secretory Control Group, Physiological Laboratory,University of Liverpool. These C-terminal specific antibodies were reconstituted with distilled water to give a stock dilution of 1:10. This stock was further diluted with solution A listed below to give a final dilution of 1:500.

| | | |
|---|---|---|
| Solution A: | Phosphate buffered saline pH 7.4 | 100ml |
| | Triton-X (Sigma Chem.Co. Ltd) | 100µl |
| | Thyroglobulin (bovine; Sigma ) | 5mg |
| | Sodium azide (Sigma) | 250mg |
| | Ethylenediaminetetraacetic acid (EDTA: Sigma) | 40mg |
| | Bovine serum albumin, fraction V (Sigma) | 100mg |

b) The VIP antibody was a gift from Professor Julia Polak, The Royal Postgraduate Medical School, Hammersmith Hospital, London. The optimum dilution was found to be 1:2000.

c) The CGRP antibody was also a gift from Professor Polak. The optimum dilution for this antibody was found to be 1:800.

4. Wash in three changes (each of 10 minutes duration) of phosphate-buffered-saline.

5. Incubate the slides in the Fluoroscein Isothiocyanate (FITC)- conjugated goat anti-rabbit immunoglobulin (Sera-Lab Ltd, Crawley Down, Sussex, England) for 1 hour at room temperature (22°C). A dilution of 1:50 with phosphate-buffered-saline was found to be optimal.

6. Wash thoroughly in three changes (10 minutes each) of phosphate-buffered-saline.

7. Mount sections in 'Citifluor' (Citifluor Ltd, London, England) and examine in a fluorescence microscope.

## II IMMUNOCYTOCHEMICAL LOCALISATION OF CHOLINE ACETYLTRANSFERASE AND THE HISTOCHEMICAL LOCALISATION OF ACETYLCHOLINESTERASE

Four adult rats each weighing 250g body weight were heavily anaesthetised with an intraperitoneal injection of Sagatal (60mg/Kg) and perfused via the heart as described above but with 500ml of physiological saline followed by 500ml of 4% paraformaldehyde containing 0.1% glutaraldehyde in 0.1M phosphate buffer. Throughout the perfusion procedure the animal was chilled by immersion in an ice bath.

Following fixation 300ml of ice-cold 10% sucrose in 0.1M phosphate buffer was perfused through the vasculature. The otic ganglia and small blocks of forebrain (to include the nucleus basalis) were removed and placed in 30% sucrose in 0.1M phosphate buffer.

Frozen sections 20um thick were cut from each block of tissue. Alternate sections were collected serially and placed in 0.1M phosphate buffered saline (PBS) and incubated for choline acetyltransferase activity (see below for details).

Adjacent sections were mounted onto clean glass microscope slides and reacted for acetylcholinesterase activity using the method of Karnovsky and Roots ( 1964 ).(See Appendix 7 for details).

## Method Used For Choline Acetyltransferase (ChAT).

1. Incubate the free-floating sections with gentle agitation in 0.4% Triton X-100 in PBS at room temperature for 30 minutes.
2. Wash in two changes of cold PBS. 5 minutes each.
3. Incubate with 0.2% Triton X-100 and 1% normal rabbit serum in PBS at room temperature for 1 hour.
4. Wash in two changes of cold PBS. 5 minutes each.
5. Incubate in ChAT antibody (2 sources of antibody were used
   - (i) From RIA (UK)Ltd. Washington. Lot No8352023. This antibody was known to demonstrate the enzyme in cholinergic neurones of the rat forebrain. Sections from rat forebrain were used as a positive control. The optimum dilution for this antibody was found to be 1:50.
   - (ii) From Boehringer Mannheim, Lewes, Sussex. This rat monoclonal antibody was claimed by the manufacturer to demonstrate choline acetyltransferase activity in the peripheral nervous system. Sections of otic ganglia and rat forebrain were incubated in doubling dilutions over a range of 1:10 to 1:1280.

   Sections were incubated overnight at 4°C.
6. Wash in three changes of cold PBS. 10 minutes each.
7. Incubate in rabbit anti-rat IgG (Sera Labs) diluted to 1:200 with PBS containing 0.2% Triton X-100 and 1% normal rabbit serum.

8. Wash in three changes of cold PBS. 10 minutes each.
9. Incubate sections in rat peroxidase anti-peroxidase (PAP , Sera Labs) diluted 1:100 with PBS containing 0.2% Triton X-100 and 1% normal rabbit serum. Incubate for 90 minutes at room temperature.
10. Wash in three changes of PBS. 10 minutes each.
11. Incubate sections in 3, 3 diaminobenzidine tetrahydrochloride (DAB, Sigma. 5mg/10ml in 0.5M Tris buffer pH 7.6) for 10 minutes at room temperature.
12. Incubate in DAB solution containing 0.01% hydrogen peroxide (BDH Ltd.) for 10 minutes at room temperature.
13. Wash in 5 changes of cold PBS. 5 minutes each.
14. Mount sections in sequence onto clean glass microscope slides.
15. Dehydrate in graded alcohols, clear in xylene (BDH) and mount in a synthetic resin.

Controls: Incubate some sections in normal rat IgG at at a concentration of 0.1mg/ml in place of the choline acetyltransferase antibody.

## III RETROGRADE TRANSPORT OF FLUOROCHROME TRACERS

Adult Wistar rats received bilateral injections of a either a 1% suspension of Fast Blue (15 rats) or a 1% suspension of True Blue (14 rats) into the parotid gland. Details of the procedure are described in Chapter 5 (page 60). Serial cryostat sections (optimum thickness 10μm) were taken from the otic, trigeminal and superior cervical ganglia and thaw-mounted onto clean glass microscope slides previously coated with poly-L-

Fig. 28 Diagram illustrating how serial, sections were mounted on slides for incubation in different antisera.

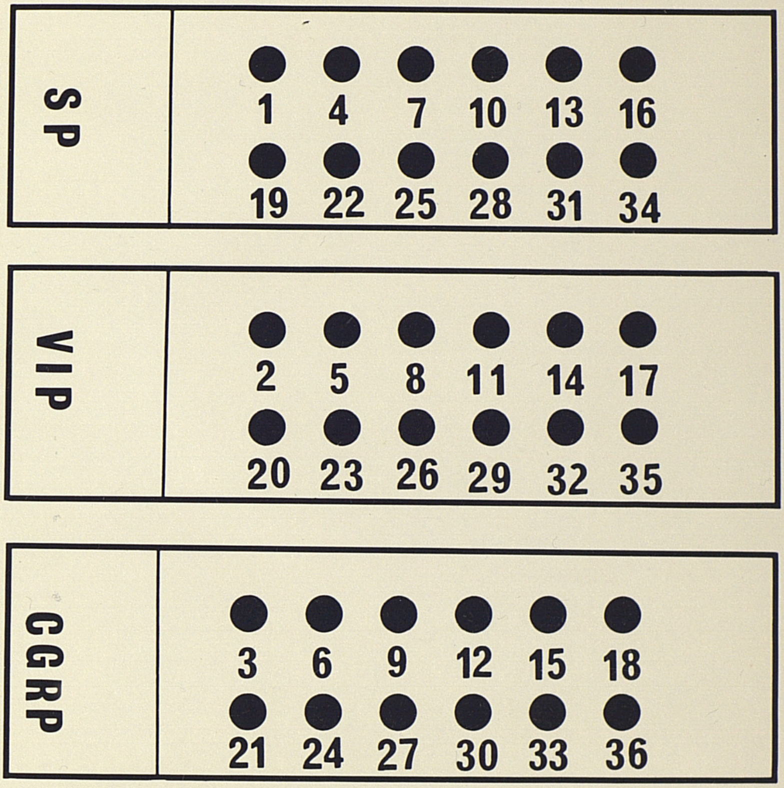

SP
1 4 7 10 13 16
19 22 25 28 31 34
VIP
2 5 8 11 14 17
20 23 26 29 32 35
CGRP
3 6 9 12 15 18
21 24 27 30 33 36

Fig 28

lysine. The sections were arranged on the slides as illustrated in Figure 28 .

Two additional animals (1 for each fluorochrome dye) were given an intraperitoneal injection of colchicine (Sigma Chemical Co. Ltd, Poole, Dorset: 2mg/Kg), 24 hours prior to sacrifice. This was an attempt to increase the amount of neuropeptide within the neuronal cell perikarya by blocking axoplasmic transport.

## IV COLOCALISATION OF THE FLUOROCHROME WITH THE NEUROPEPTIDES

Adjacent slides containing sections of neurones which had been retrogradely-labelled with a fluorochrome were incubated for SP, VIP and CGRP immunoreactivity according to the method described above.

## V COLOCALISATION OF NEUROPEPTIDES IN THE SAME NEURONE

Adjacent sections from each of the ganglia which had been incubated for immunocytochemical localisation of different peptides were carefully examined for possible co-localisation of neuropeptides.

## VI QUANTITATION OF NEUROPEPTIDE-CONTAINING NEURONES.

The total number of neurones immunoreactive for each peptide was obtained from serial sections taken through a single otic, trigeminal and superior cervical ganglion.

# RESULTS

## I IMMUNOCYTOCHEMISTRY AND HISTOCHEMISTRY

### a) CONTROL SECTIONS

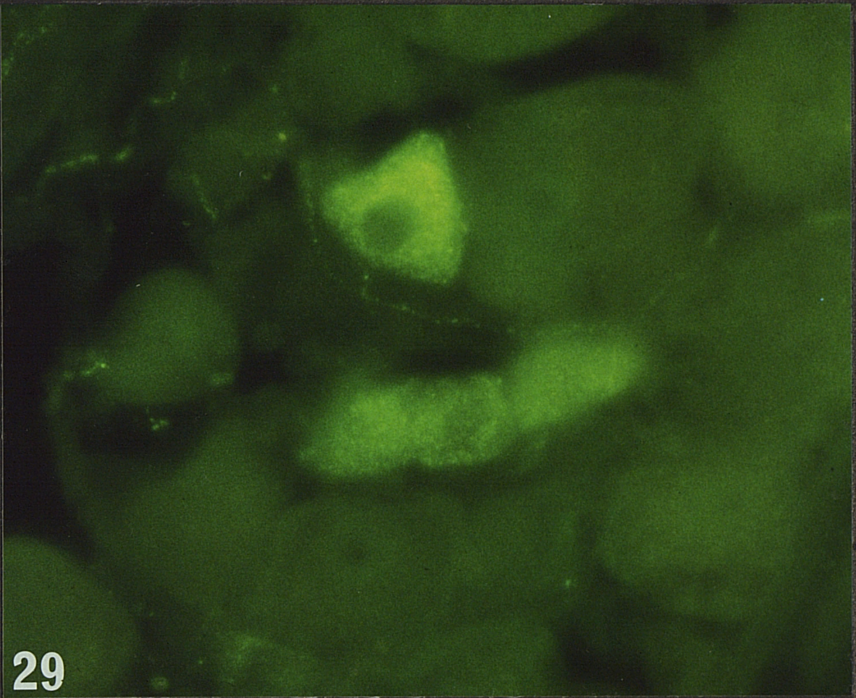
Sections of dorsal root ganglia and spinal cord which had been incubated for SP immunoreactivity because they were known to contain the neuropeptide revealed numerous neuronal perikarya and nerve fibres in the dorsal root ganglion which were strongly postive for SP immunoreactivity. In the occasional cell it was possible to see the axon emerging from its cell body before splitting into its peripheral and central components (Fig 29 ). In the spinal cord intensely fluorescent SP immunoreactivity was observed in the nerve terminals of the substantia gelatinosa layer (Fig 30 ).

Control sections incubated in the absence of a primary antibody contained no fluorescent neuronal perikarya in the otic, trigeminal or superior cervical ganglia. However, in sections of the parotid gland numerous fluorescent cells were observed within the connective tissue lying between the acini (Fig 31). These cells were found irrespective of whether the sections had been incubated in the presence or absence of an antibody. No immunofluorescent nerve fibres were observed in sections which had been incubated in the absence of a primary antibody but a diffuse green fluorescence was observed in the connective tissue of sections taken from each of the ganglia under investigation. However, as the different antibodies were diluted out to increase specificity the diffuse fluorescence gradually disappeared and was considered to be non-specific.

Control sections of rat forebrain incubated for choline acetyltransferase activity using the RIA antibody revealed

Fig 29 SP-like immunoreactivity in dorsal root ganglion cells. x 750 magnification.

Fig 30 SP-like immunoreactivity in nerve terminals of the substantia gelatinosa of the spinal cord. x 750 magnification.



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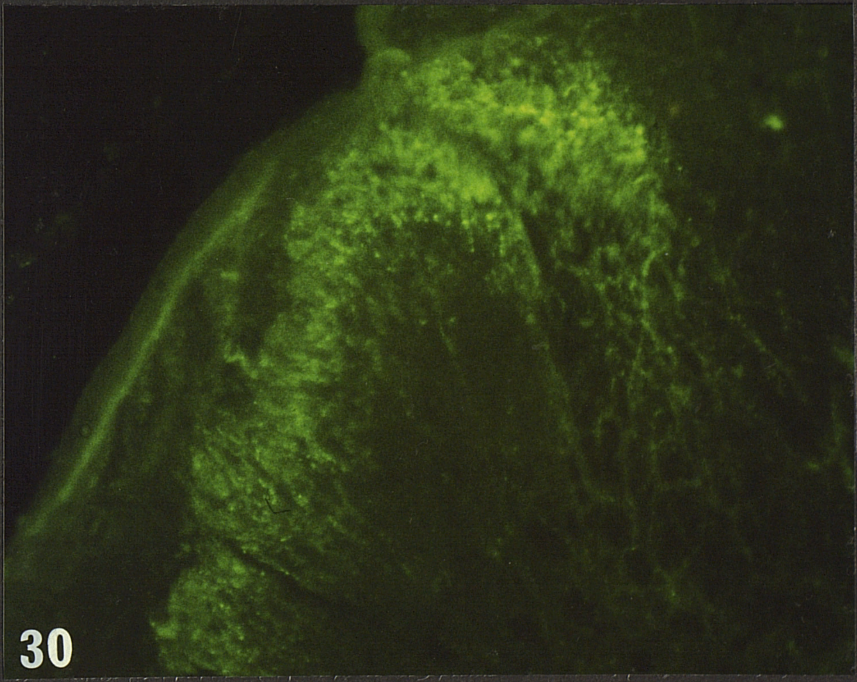

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numerous neurones in the nucleus basalis which were immunoreactive for this enzyme but no immunoreactivity was observed in sections of the otic ganglion. Using the Boehringer antibody no immunoreactivity was observed in neurones of either the nucleus basalis or of the otic ganglion. Sections incubated in the presence of normal rabbit IgG in place of the primary antibody revealed no immunoreactivity.

## b) SUBSTANCE P-LIKE IMMUNOREACTIVITY

### The Otic Ganglion:

Approximately 60% of the neuronal perikarya in the otic ganglion were positive for SP-like immunoreactivity (Fig 39). Numerous fluorescent nerve fibres indicative of SP-like immunoreactivity were scattered throughout the ganglion and of particular interest were nerve fibres which appeared to encircle some neuronal perikarya (Fig 32). Occasionally a neurone was seen in which the immunoreactive nerve fibres completely covered the surface of the neurone as if it were enclosed within a meshwork.

### The Trigeminal Ganglion:

Approximately 20% of the neuronal perikarya in the trigeminal ganglion exhibited an apple-green fluorescence indicative of SP-like immunoreactivity (Fig 33). Immunofluorescent axons were observed scattered throughout the ganglion but were much more abundant in areas where they had collected together to form a bundle .

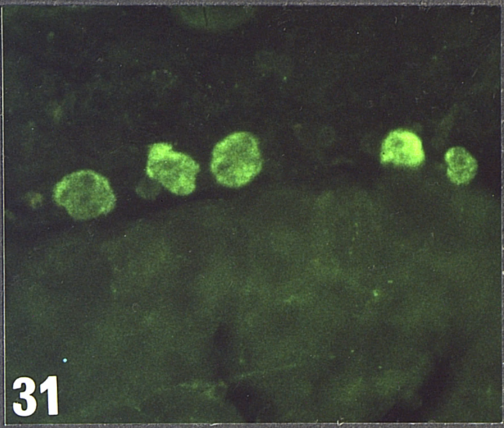
### The Superior Cervical Ganglion:

Although serial sections , which had been incubated for SP-like immunoreactivity, were examined in several superior

Fig 31 Non-specific fluorescence in connective tissue cells of the parotid gland. x 350 magnification.

Fig 32 SP-like immunoreactivity in nerve terminals surrounding neurones in the otic ganglion. x 350 magnification.

Fig 33 SP-like immunoreactivity in neurones of the trigeminal ganglion. x 250 magnification.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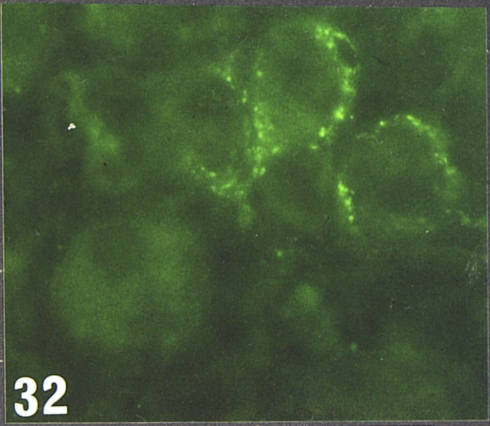
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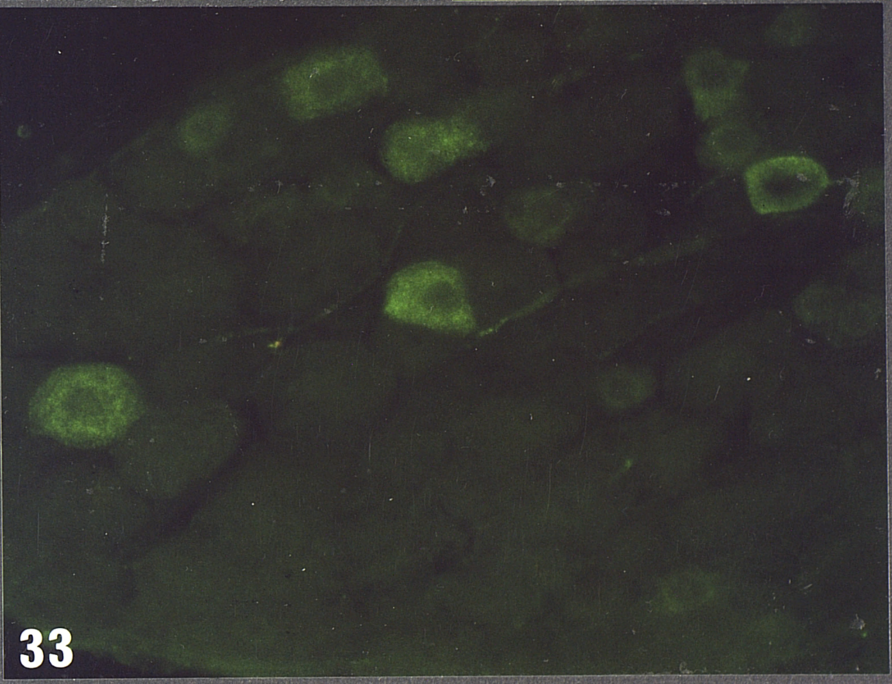
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cervical ganglia only one cell was found which was clearly immunoreactive for this peptide. However numerous fluorescent fibres indicative of SP-like immunoreactivity were found distributed throughout the ganglion amongst the neuronal perikarya.

The Parotid Gland:

Numerous fluorescent beaded fibres indicative of SP-like immunoreactivty were found lying between and appearing to terminate on the acinar secretory units (Figure 34). Very rarely a fluorescent fibre was seen at the periphery of the cells lining a duct.

## c) VIP-LIKE IMMUNOREACTIVITY

The Otic Ganglion:

Between 10 and 20% of neuronal perikarya within the otic ganglion revealed an apple-green immunofluorescence indicative of VIP-like immunoreactivity (Fig 35). Fluorescent nerve fibres were also seen amongst the neurones but were much more apparent when the axons were collected together in a bundle as they entered or left the ganglion.

The Trigeminal and Superior Cervical Ganglia:

No neuronal perikarya or axons exhibited apple-green fluorescence indicative of VIP-like immunoreactivity in either of these two ganglia.

The Parotid Gland:

Nerve fibres and terminals immunoreactive for VIP-like activity were distributed amongst the secretory accini in the gland (Fig 36). No evidence for VIP-like activity was seen around the ducts.

## d) CALCITONIN-GENE-RELATED PEPTIDE IMMUNOREACTIVITY

In the otic and superior cervical ganglia no neuronal perikarya, axons or terminals contained an apple-green fluorescence indicative of CGRP-like immunoreactivity.

The Trigeminal Ganglion:

Approximately 30% of neurones contained CGRP-like immunoreactivity (Fig 37). Numerous beaded fluorescent axons and terminals were observed permeating amongst the trigeminal ganglion cells.

The Parotid Gland:

Very few sections revealed any CGRP-like immunoreactivity. When present the fluorescence was restricted to axons or terminals situated very close to the ducts ( Fig 38 ). No immunofluorescent cell bodies , axons or their terminals were found associated with the secretory accini.

## e) CHOLINE ACETYLTRANSFERASE IMMUNOREACTIVITY

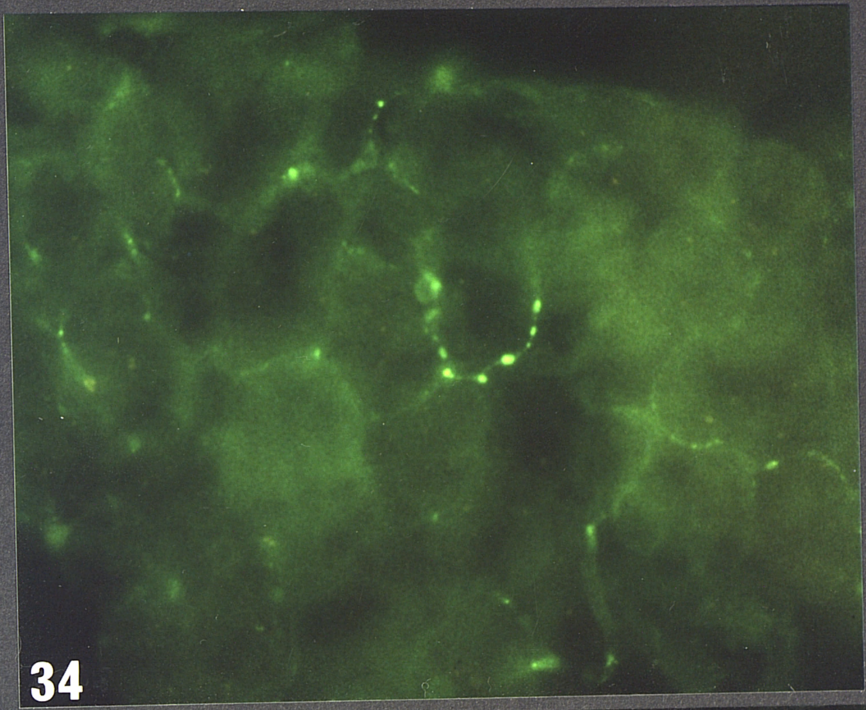
No neurones in the otic ganglion were found which were imunoreactive for this enzyme irrespective of the source of the primary antibody. However as reported above choline acetyltransferase immunoreactive neurones were found in the nucleus basalis of the rat forebrain.

## f) ACETYLCHOLINESTERASE ACTIVITY

All neurones in the otic ganglion exhibited acetylcholinesterase activity of varying levels of intensity.

Fig 34 SP-like immunoreactivity in nerve terminals around acini in the parotid gland x 250 magnification.

Fig 35 VIP-like immunoreactivity in otic ganglion neurones. x 700 magnification.



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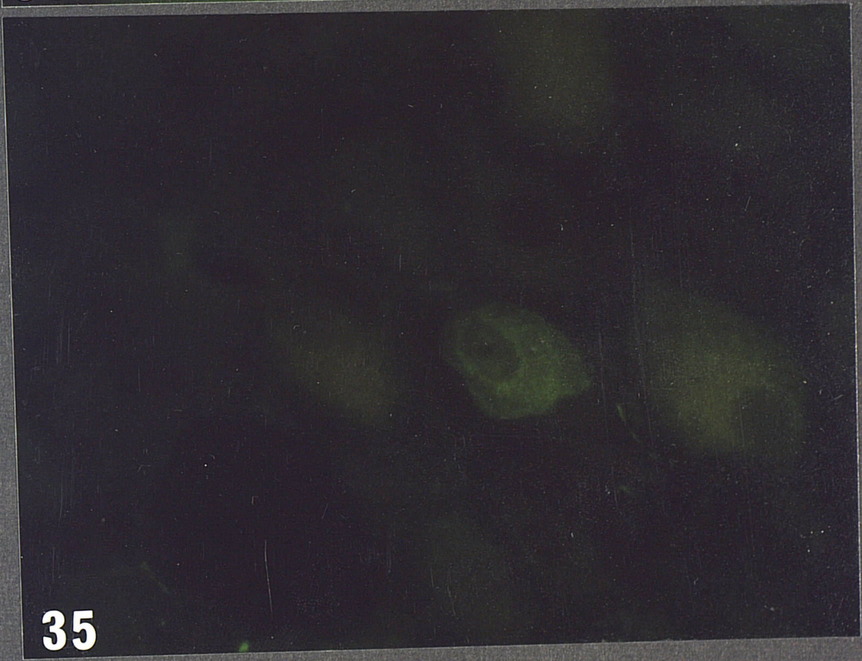

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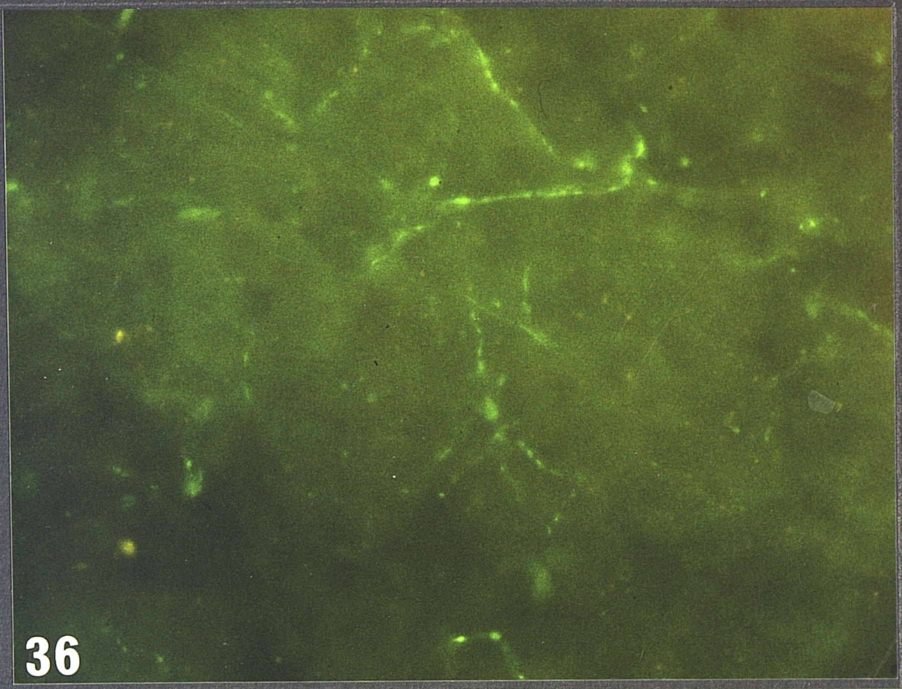
Fig 36 VIP-like immunoreactive nerve terminals in the rat parotid gland x 250 magnification.

Fig 37 CGRP-like immunoreactivity in a neurone of the trigeminal ganglion x 700 magnification.

Fig 38 CGRP-like immunoreactivity in nerve terminals around a striated duct in the rat parotid gland x 350 magnification.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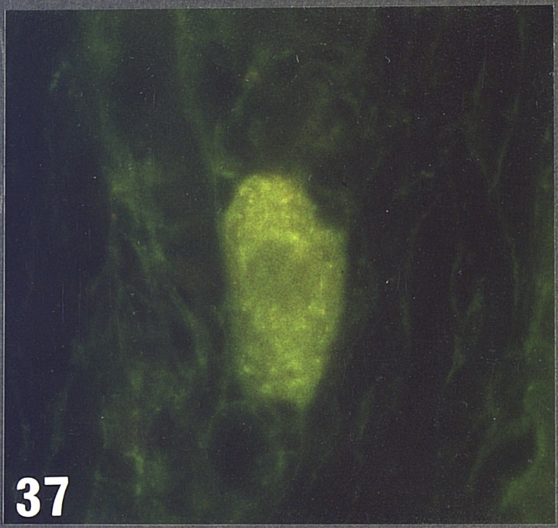
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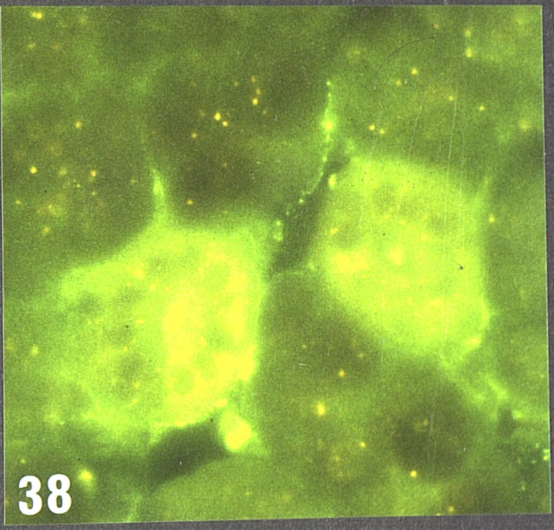
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## II THE CO-LOCALISATION OF NEURONES CONTAINING NEUROPEPTIDES WITH THE RETROGRADELY-TRANSPORTED FLUOROCHROMES

The Otic Ganglion:

Numerous neuronal perikarya were observed in the otic ganglion which contained both a retrograde tracer and a neuropeptide. As there were many more neurones immunoreactive for SP than for VIP it was easier to find SP-containing neurones which also contained either True Blue or Fast Blue (Figs 39,40) than it was to find VIP-containing neurones with a fluorochrome. Nonetheless, neurones which were immunoreactive for VIP and which contained a retrograde tracer were found (Figs 41,42).

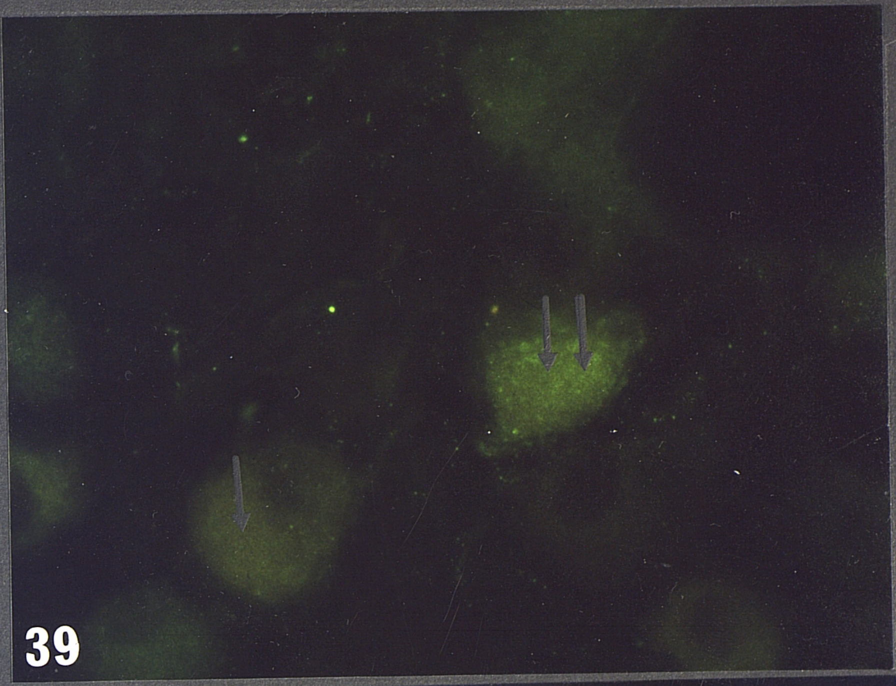
One very interesting feature observed in sections incubated for SP-like immunoreactivity was the presence of a meshwork of SP-terminals around, either other neurones which were SP-positive or around neurones which contained a retrograde tracer but which were not themselves positive for SP-like immunoreactivity (Figs 43,44). Because of technical difficulties it was difficult to record photographically more than one or two neurones per section which contained both a neuropeptide and a retrograde tracer . This difficulty was due to the fact that the fluorochrome dyes faded very rapidly on exposure to ultra violet light and therefore as the exposure times were often in terms of minutes rather than fractions of a second by the time one photograph had been exposed the fluorochrome in neurones other than the one being photographed had already faded.

The Trigeminal Ganglion:

Many neurones in the trigeminal ganglion which were retrogradely-labelled with a fluorochrome were also positive

Fig 39 SP-like immunoreactive otic ganglion neurones. x 750 magnification.

Fig 40 Same section as illustrated in Fig 39 illustrating otic ganglion neurones which have been retrogradely-labelled with Fast Blue. NB the three neurones (arrowed) which are also positive for SP-like immunoreactivity. x 750 magnification.



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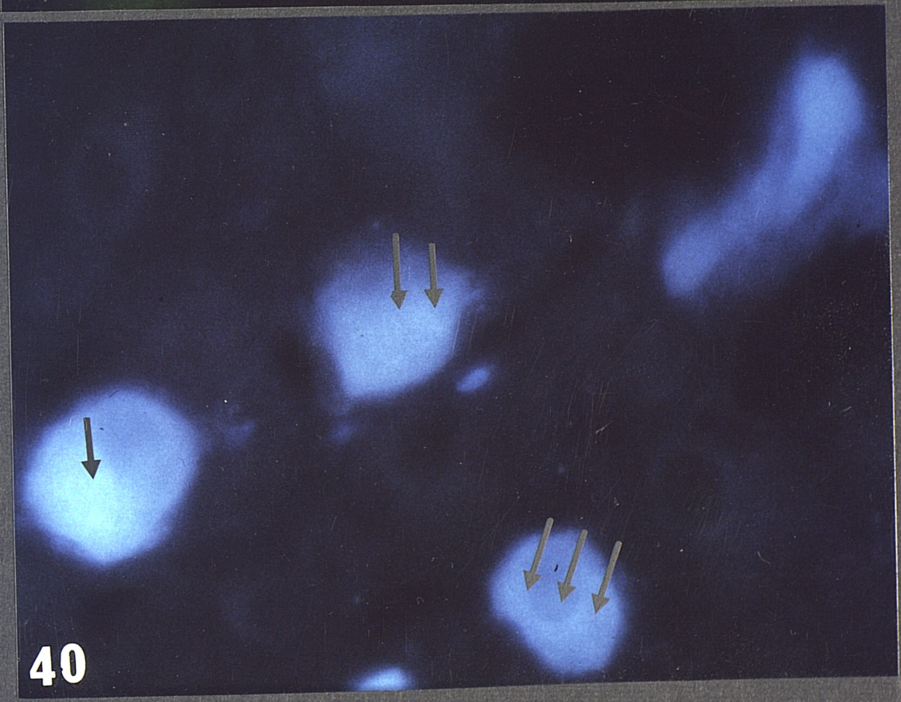

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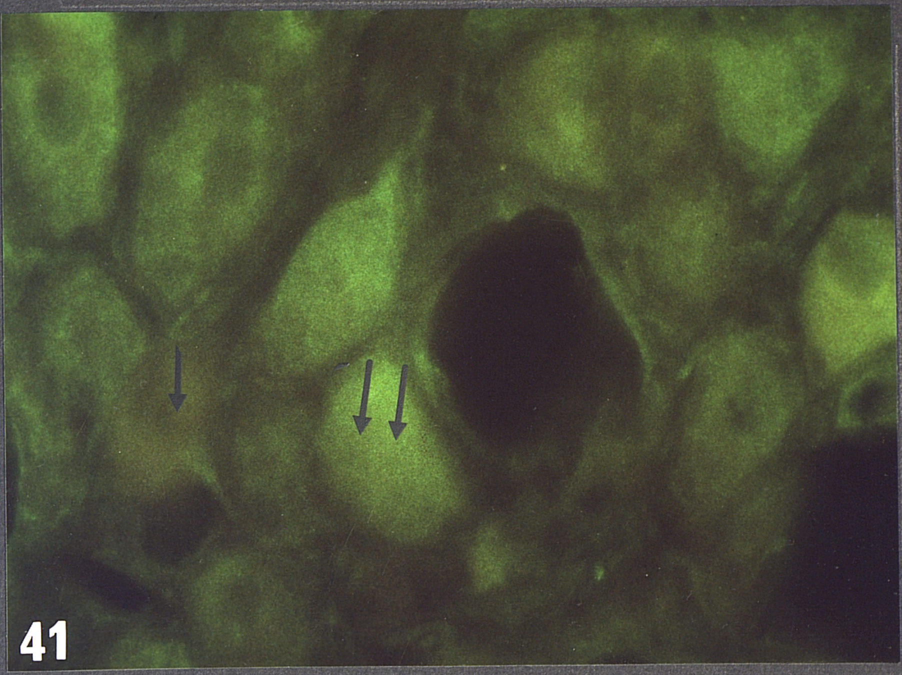
Fig 41 VIP-like immunoreactive neurones in the otic ganglion. x 750 magnification.

Fig 42 Same section as in Fig 41. NB VIP-like immunoreactive cell (double arrows) which is also retrogradely labelled with true blue and the true blue labelled cell which is negative for VIP-like immunoreactivity. x 750 magnification.



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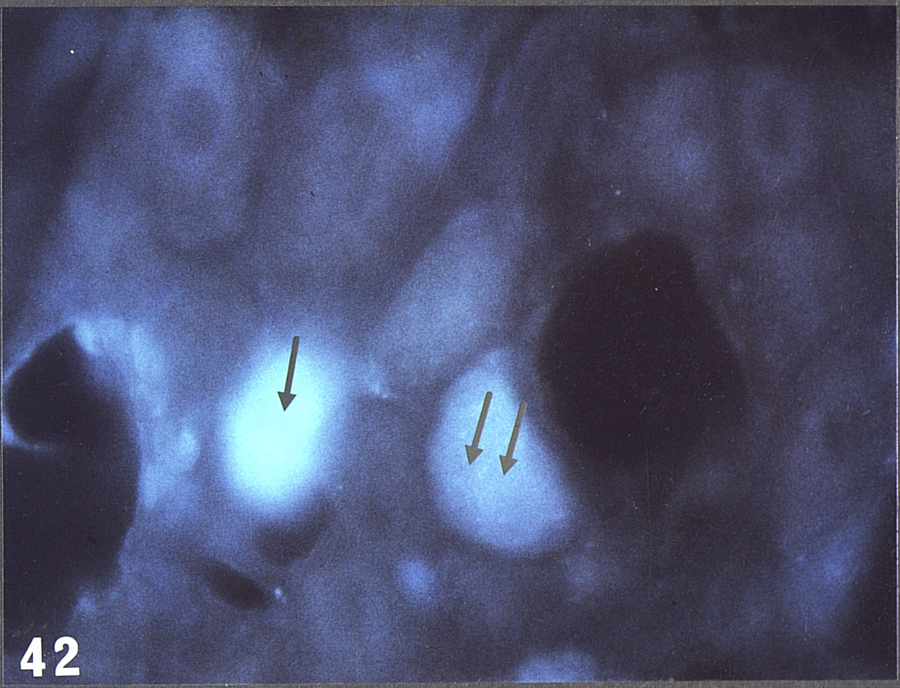

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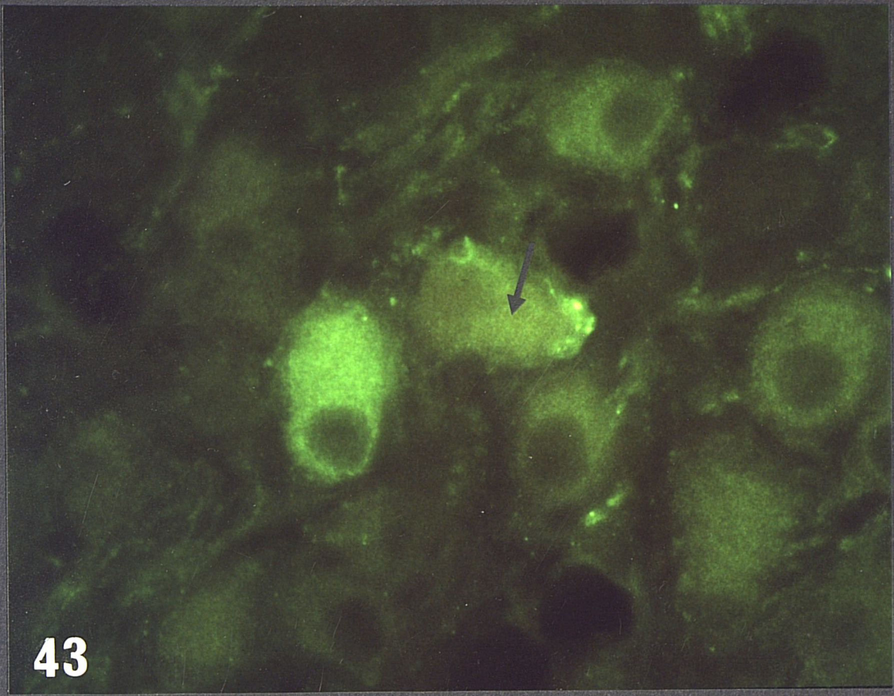
Fig 43 SP-like immunoreactive neurones in the otic ganglion. x 750 magnification.

Fig 44 Same section as in Fig 43. NB cell in centre (arrowed) which is retrogradely labelled with fast blue and is surrounded by SP-like immunoreactive nerve terminals, x 750 magnification.



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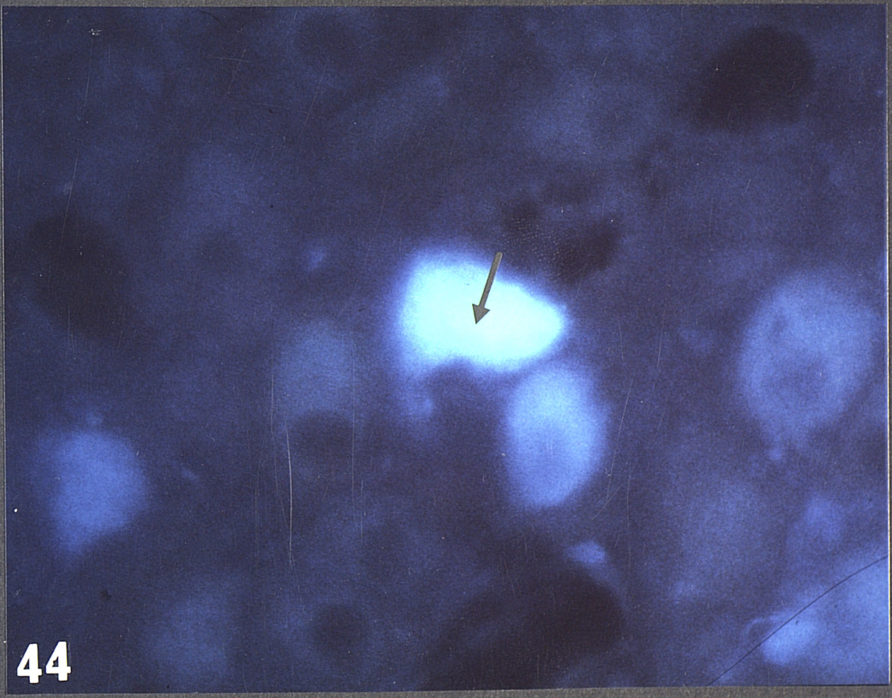

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for either SP-like or CGRP-like immunoreactivity .Unlike in the otic ganglion, neurones in the trigeminal which were immunoreactive for a neuropeptide were never observed to innervate a retogradely-labelled neurone which was negative for that peptide.

The Superior Cervical Ganglion:

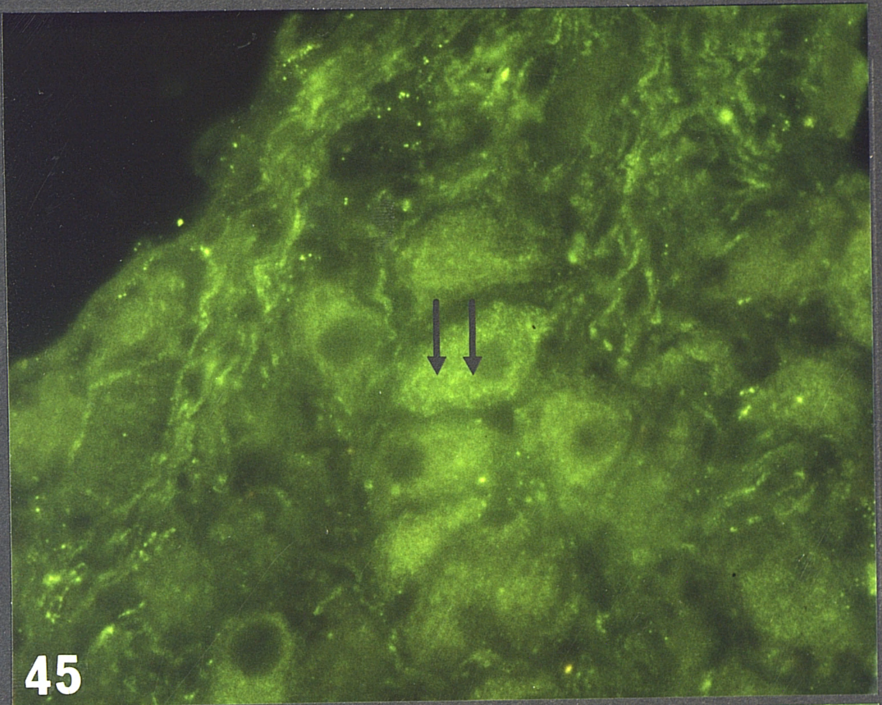
No evidence for co-localisation of a neuropeptide with a retrograde tracer was seen in the superior cervical ganglion. Although numerous retrogradely labelled neurones were found in this ganglion .

## III CO-LOCALISATION OF NEUROPEPTIDES IN THE SAME CELL

The Otic Ganglion: the careful examination of adjacent serial sections taken from the otic ganglion which had been incubated in antisera directed against different neuropeptides revealed neuronal perikarya which were positive for both SP and VIP-like immunoreactivity (Figs 45,46). The number of neurones which contained both peptides was difficult to assess accurately . However between 2 and 5 cells per section were positively identified as containing both neuropeptides but this is probably an underestimate (see Discussion for explanation).

The Trigeminal Ganglion: examination of serial sections showed that all trigeminal perikarya that were positive for SP-like immunoreactivity were also positive for CGRP-like immunoreactivity. However the reverse situation was not so i.e. many CGRP-immunoreactive neurones were negative for SP-like immunoreactivity.

Figs 45 & 46 Adjacent sections incubated for SP (Fig 45) and VIP (Fig 46) immunoreactivity. NB cell arrowed which is immunoreactive for both peptides. x 750 magnification.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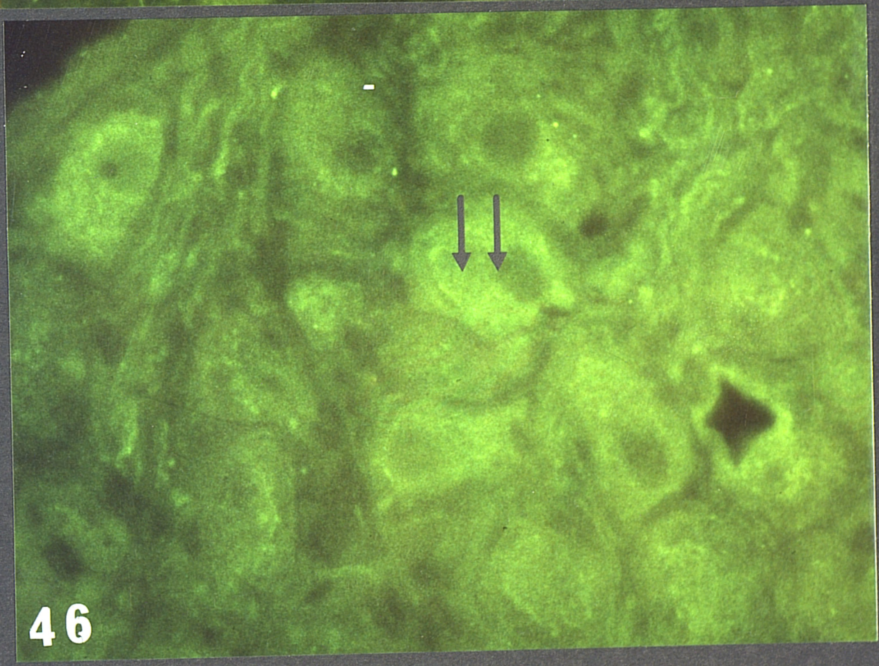
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# DISCUSSION

Previous work using retrograde tracers (Chapter Five) showed that the otic, trigeminal and superior cervical ganglia each contained neurones which projected to the parotid gland and could therefore be the source of neuropeptides which have been implicated in control of salivation from this gland in the rat. When the auriculotemporal nerve was sectioned SP and VIP levels fell by 95% and CGRP levels by 20% (Brodin et al., 1983, Ekström et al., 1984, Ekman et al., 1986) which would suggest that neurones in the otic or trigeminal ganglia rather than in the superior cervical ganglion contain the neuropeptides.

The present study has demonstrated the presence of SP and/or CGRP-like immunoreactivity within the trigeminal ganglion neurones and SP and/or VIP-like immunoreactivity within the otic ganglion neurones.

Sharkey and Templeton (1984) suggested a dual origin for SP in the rat parotid gland. These authors showed that following capsaicin treatment the SP-immunoreactive fibres around the vasculature disappear whereas those around the secretory acini remain. Such results would suggest that the SP-like immunoreactivity around the blood vessels is sensory and probably originates from neurones in the trigeminal ganglion whereas that around the secretory acini originates from postganglionic parasympathetic secretomotor neurones in the otic ganglion. Interestingly both SP and CGRP have both been shown to have vasodilatory effects (Edvinsson and Uddman, 1982, Fischer et al., 1983).

Co-localisation of SP with CGRP-like immunoreactivity has been reported in both trigeminal ganglion neurones and also in other sensory ganglia ( Lee et al., 1985a,1985b. Gibbins et al., 1985).

VIP-like immunoreactive neurones have been described in the submandibular ganglion of the cat and in some exocrine glands it has been co-localised with acetylcholinesterase (Lundberg et al., 1980). In the present study VIP was co-localised with SP-like immunoreactivity in neurones of the otic ganglion at least one of which contained fluorochrome indicating that it projected to the parotid gland.

The inability to retrogradely label more than 60% of the neurones in the otic ganglion following injections of large volumes of the fluorochrome into the parotid gland is surprising. Such results would suggest that either the fluorochrome is unable to reach all of the secretomotor nerve endings in the gland or that many of the otic ganglion neurones project to sites other than the parotid gland or that they are intrinsic to the otic ganglion. In support of the latter possibility is the presence of numerous SP-immunoreactive nerve endings around many of the otic ganglion neurones some of which contain fluorochrome and therefore project to the parotid gland and are probably secretomotor in function. SP-immunoreactive fibres surrounding parasympathetic neurones have been described in the cardiac ganglion (Wharton et al., 1981). Recent work suggests that SP acts by producing a slow synaptic excitation of the cardiac parasympathetic ganglion cells (Konishi, Okamoto and Otsuka, 1985).

The possibility that neurones in the otic ganglion which projected to the parotid gland also contained choline acetyltransferase as well as the two neuropeptides SP and VIP was investigated with a monoclonal antiserum to choline acetyltransferase but for technical reasons this antiserum appeared to be unsuitable for demonstrating the enzyme in the peripheral nervous system. Such difficulties have been encountered by other research workers and a suitable antiserum is eagerly awaited. However all of the cells when incubated for acetylcholinesterase activity were positive to varying degrees and it would seem very likely that many of the otic ganglion cells which project to the parotid gland do contain one or more neuropeptides and acetyl choline as well.

The co-existence of a neuropeptide with a classical neurotransmitter or another peptide is not unusual (Burnstock, 1976, Lundberg et al., 1979, Wiesenfeld-Hallin et al., 1984, Hökfelt et al., 1984, Lee et al, 1985, Lundberg and Hökfelt, 1986, Hökfelt et al., 1987) and indeed is becoming a common phenomenon in the autonomic nervous system. However the functional significance of the presence of SP and VIP with a classical neurotransmitter, acetylcholine, is far from clear. It is a well known fact that acetylcholine induces both salivary secretion and an increase in blood flow and these effects are both atropine sensitive (Lundberg, 1982). SP is a potent sialogogic agent (Leeman and Hammerschlagg, 1967, Ekstrom and Wahlestedt, 1982, Ekstrom, Mansson and Tobin, 1983) and is also known to have a vasodilatory effect (Edvinsson and Uddman, 1982,). VIP, in addition to its vasodilatory action when given intravenously it alters the flow of protein rich saliva (Ekstrom et al., 1983a, 1984) and potentiates the salivary secretion caused by acetylcholine (Lundberg, 1982). It has been suggested that VIP and SP are responsible for the atropine-resistant secretion of the parotid gland when the auriculotemporal nerve is stimulated (Ekström et al., 1983).

One functional significance of co-localisation of CGRP and SP is that CGRP is a potent inhibitor of SP endopeptidase (Le Greves et al., 1986) and so inhibits degradation of SP and prolongs its action.

The significance of co-existence of the peptides with classical neurotransmitters is not well understood. However it is possible that these neurotransmitters can produce selective and differential responses. This can be achieved through presynaptic or postsynaptic selectivity (Hökfelt et al., 1987). With presynaptic selectivity the classical neurotransmitter (acetylcholine) and the peptides can be released differentially depending on the frequency of the action potentials (Lundberg et al., 1982, Edward et al., 1982). According to this theory a low impulse frequency selectively releases acetylcholine whereas higher frequencies or bursts of impulses release in addition SP and VIP. With postsynaptic selectivity the neurone releases all types of messengers under any condition and the distribution and type of receptor provides for selectivity and specificity.

The possible co-localisation of the two neuropeptides SP and VIP in terminals of the rat parotid gland needs further investigation to confirm their co-existence. An attempt at identifying the neuropeptides by means of immunogold-labelling will also be investigated.

# CHAPTER SEVEN

# THE ULTRASTRUCTURAL LOCALISATION OF SUBSTANCE P AND VIP IN NERVE TERMINALS OF THE RAT PAROTID GLAND USING THE IMMUNOGOLD AND IMMUNOPEROXIDASE TECHNIQUES

# THE ULTRASTRUCTURAL LOCALIZATION OF SP AND VIP IN NERVE TERMINALS OF THE RAT PAROTID GLAND USING THE IMMUNOGOLD AND IMMUNOPEROXIDASE TECHNIQUES

## INTRODUCTION

Particles of gold in water carry a net negative charge and their stability is maintained by the repulsion produced by this electrostatic force. However if strong electrolytes are added this will change the environment and lead to aggregation and flocculation of the gold particles (Weiser, 1949). This aggregation can be prevented by adding protective colloids such as proteins. The proteins are adsorbed onto the gold particles and are stabilised against flocculation by electrolytes (Jirgensons and Straumanis, 1964). Faulk and Taylor (1971) stabilised the colloidal gold particles with an antibody and as colloidal gold is electrondense (Van Furth, Hirsch & Fedorko 1970), they suggested the possible value of this immunogold for the detection of various antigens at the ultrastructural level. Faulk and Taylor (1971) adsorbed rabbit anti-salmonella serum to particles of colloidal gold and the immunocolloid was exposed to salmonellae. These authors produced electronmicrographs which showed the colloidal gold particles coating the bacteria.

Subsequently colloidal gold has been linked succesfully to a variety of substances such as enzymes (Horisberger and Rosset, 1977), staphylococcal protein A (Romano and Romano, 1977; Roth , Bendayan and Orci, 1978), lectins (Horisberger and Rosset, 1977;

Roth and Binder, 1978) and immunoglobulins (Geoghegan and Ackerman, 1977; De Mey et al., 1980, 1981) to detect both surface and intracellular antigens. Various modifications have led to the development of an immunostaining technique which can be used at both light and electron microscopical levels ( Romano, Stolinski and Hughes-Jones, 1974; Geoghegan and Ackerman, 1977; Horisberger and Rosset, 1977; DeMay et al., 1981).

These modifications have been successfully used to demonstrate regulatory peptides and catecholamine converting enzymes (Polak et al., 1981; Probert et al., 1981; Varndell et al., 1982a,b). Another advantage of the immunogold technique is the possible simultaneous ultrastructural localisation of more than one substance by the use of different sized gold particles (Tapia et al., 1983; Gulbenkian et al., 1986).

Recent evidence has implicated the importance of the neuropeptides substance P (SP), vasoactive intestinal polypeptide (VIP) and calcitonin gene related peptide (CGRP) in the control of secretion from the salivary glands (Uddman et al., 1980; Gallacher, 1983; Thesleff. 1986; Ekström, Månsson and Tobin, 1982, 1983; Ekman et al., 1986). Previous work has shown by the use of immunofluorescence techniques at the light microscope level (Chapter 6 ) that SP and VIP may be contained within the same otic ganglion neurones.

The present study uses the relatively new immunogold technique described above along with the more established immunoperoxidase method of Sternberger, ( 1979) to confirm at the ultrastructural level the co-localization of SP and VIP in the nerve terminals of the rat parotid gland.

## MATERIALS AND METHODS

### Tissue Preparation (General)

Ten adult male Wistar rats each weighing 250g body weight were deeply anaethetised with Sagatal (60mg/Kg body weight). Each animal was perfused via a cannula inserted into the left ventricle with 500ml of 0.1M phosphate buffered saline (PBS) at a pH of 7.2 followed by 500ml of fixative (see below for details).

The parotid glands were removed , dissected free of fat, cut into small pieces approximately 2mm cubed and immersed in fresh fixative for 4 hours.

### Immunoperoxidase method

The fixative used was 4% paraformaldehyde in 0.1M phosphate buffer pH 7.2. The parotid gland blocks from 6 animals were cut with the aid of a Sorvall Tissue Chopper into 100μm slices. These were then incubated for SP or VIP immunoreactivity using the unlabelled peroxidase antiperoxidase (PAP) technique of Sternberger (1979). Details of the immunoperoxidase method are given below. The optimum dilution for the anti-substance P antiserum (Cambridge Research Biochemicals) was found to be 1:500 whilst that for the VIP antiserum (a gift from Professor Julia Polak, London) was 1:4000. After incubation the tissue slices were thoroughly washed and processed into araldite according to the schedule in Appendix 6. Ultrathin sections (100nm) were cut using a Reichert OM-U3 ultramicrotome (Reichert, Austria), mounted onto copper grids (50μm, Taab

Labs. Ltd., Berkshire, England) and examined without further staining in a Philips 300 transmission electronmicroscope.

Control slices were incubated without specific antiserum but with the preimmune serum or the immunoreactivity was blocked by incubation with the specific antiserum to which had been added the neat peptide (5µl of 1mM solution added to 100µL of undiluted antiserum).

## Immunogold method

The fixative used contained 1% glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer pH 7.2. The parotid gland blocks of tissue from four animals were rinsed in 0.1M PBS pH 7.2 and immersed for 1 minute in 1% Azar II in 70% ethanol in order to stain the tissue for later identification. The tissues were passed through a series of graded alcohols, cleared in propylene oxide and infiltrated with Araldite epoxy resin (see Appendix 6 for details). Ultrathin sections (100nm in thickness) were cut with the aid of a Reichert-Jung ultratome and collected on 200-mesh Nickel grids (EMscope Laboratories Ltd., London, U.K).

## Antisera

A rat monoclonal antiserum to substance P (Sera Lab. Ltd. Crawley Down, Sussex) was used at varying dilutions (optimum dilution was found to be 1:500). A polyclonal antiserum to VIP (a gift from Professor Julia Polak, London) was used at varying dilutions (1:4000 was found to be the optimum dilution which produced minimal background levels).

## Electron-immunocytochemistry

For the localization of SP-like and VIP-like immunoreactivity at the ultrastructural level an on-grid immunogold staining procedure was used (see below for details). The nickel grids containing the sections were floated onto the surface of the various solutions which were contained in microwells (Falcon 96-Multiwell Plate;Becton Dickenson UK Ltd., Oxford). Each well contained 25μL of solution . The nickel grids were continually agitated in the various solutions by placing the Microwell Plate on top of a magnetic stirrer (Chemlab U.K; Model SS3). Handling of the nickel grids was found to be extremely difficult because of their high electrostatic charge and it was found necessary to magnetise the forceps used for handling the grids when transferring from one solution to the next (see Fig 47).

In brief, antiserum to SP and/or VIP raised in different species (rat and rabbit respectively) was applied alone or simultaneously to the ultrathin sections.

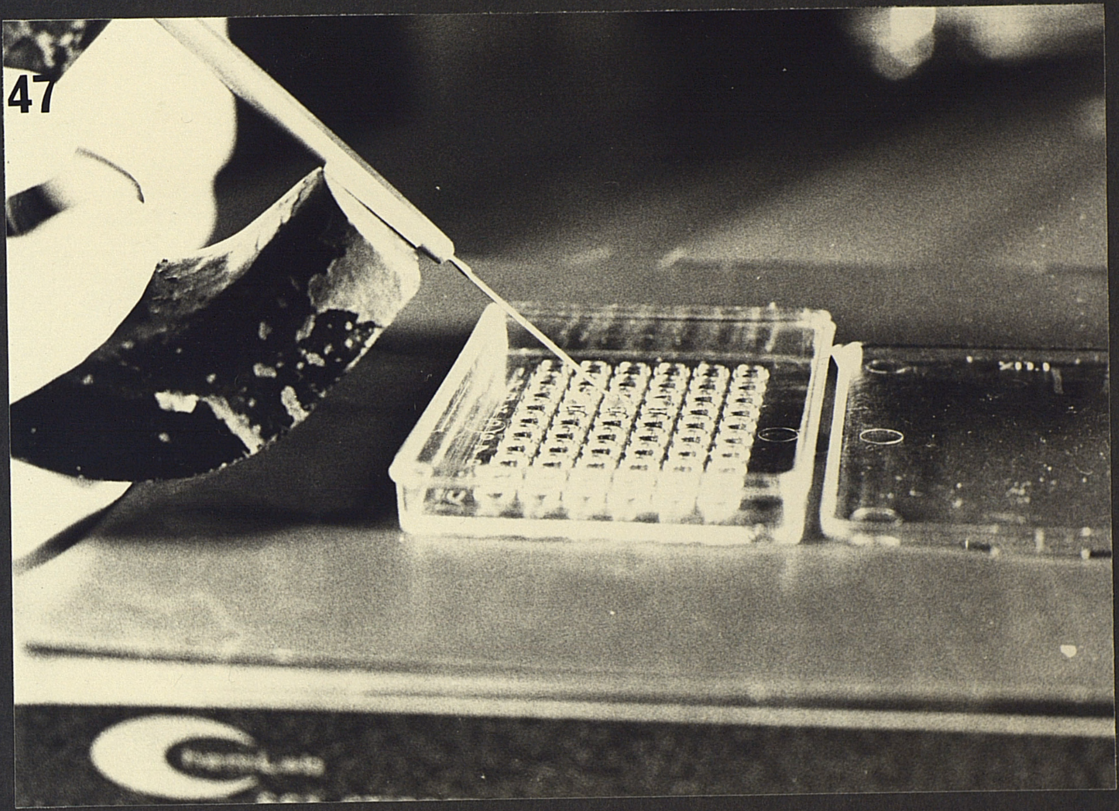
The two peptides were localized by using two species specific antisera labelled with gold particles of different sizes. For localization of SP, goat anti-rat IgG was adsorbed to gold particles 15nm in diameter and for VIP , goat ant-rabbit IgG was adsorbed to gold particles 10nm in diameter. The gold-labelled antisera were obtained from Janssen Pharmaceutical Ltd., Wantage, Oxfordshire, U.K.

Control sections were incubated in non-immune serum for the first layer instead of the primary antisera to SP and VIP.

Following immunogold staining sections were stained with 1% uranyl acetate and 1% lead citrate and examined in a Philips 300 transmission electron microscope.

Fig 47 Shows the technique used for handling nickel grids for immunogold staining.

47



# THE IMMUNOGOLD TECHNIQUE

1. Etch ultrathin sections in 10% hydrogen peroxide... 10mins.

2. Wash in distilled water. Keep agitated............. 2x2mins

3. Normal goat serum diluted 1:20.Agitate...............30mins

4. Incubate in primary antiserum.Agitate at 4°C......overnight

5. Wash in 0.05M Tris buffer 0.1% Tween 20. pH 7.2-7.4.
with constant agitation........................... 4 x 5mins

6. Wash in 0.05M Tris buffer 0.2% bovine serum albumin
0.1% Tween 20. pH 7.2-7.4 with agitation......... 4 x 5mins

7. Wash in 0.05M Tris buffer pH8.2 1% bovine serum albumin
............................................... 10 - 15mins

8. Incubate in gold-labelled IgG suspended in 0.05M Tris
buffer pH 8.2 1% bovine serum albumin....... 1hr at 18°C

9. Wash with constant agitation in 0.05M Tris buffer pH 7.2-
7.4 0.2% bovine serum albumin................. 4 x 5mins

10. Wash with constant agitation in 0.05M Tris buffer,
pH 7.2-4......................................... 4 x 5mins

11. Wash in distilled water with constant agitation.. 2 x 5mins

12. Stain in 1% uranyl acetate...........................2mins

13. Wash briefly with methanol...........................1min

14. Stain in 1% lead citrate............................ 2mins

15. Wash in distilled water . Air dry and examine.

## The Immunoperoxidase Method Used For The Ultrasructural Localisation Of SP AND VIP.

1. Tissue slices 100um thick were washed in several changes of phosphate-buffered saline pH7.4 (PBS).........30mins.
2. Incubate in normal swine serum diluted 1:10 with PBS..30mins
3. Thorough washing in PBS..................................30mins
4. Incubate in primary antiserum at 4 C..............overnight
5. Thorough washing with PBS..........................30mins
6. Incubate in goat anti-rabbit serum diluted 1:50 with PBS.... 60mins
7. Thorough washing with PBS...........................30mins
8. Incubate in rabbit Peroxidase anti-Peroxidase (PAP) diluted 1:50 with PBS..........................................50mins
9. Thorough washing in PBS..................................20mins
10. Tris-buffered saline (TBS) 0.05M pH7.6...............10mins
11. Diaminobenzidine (DAB) 0.06% in TBS.................10mins
12. DAB 0.01% hydrogen peroxide (freshly prepared)......5mins
13. TBS..........................................................10mins
14. PBS......................................................overnight
15. Fix in 1% osmium tetroxide in distilled water.........1 hour
16. After thorough washing process into Araldite.(see Appendix 6)

# RESULTS

## Immunoperoxidase staining of nerve terminals
## (Figs. 48, 49, 50 & 51)

The localization of the electrondense immunoreactive peroxidase reaction product indicative of peptide activity within nerve terminals was similar for each peptide. In varicosities where the larger dense-cored synaptic vesicles were present the electrondense reaction product covered the whole of the vesicle. In addition immunoreactivity was observed around the outside of the smaller agranular vesicles, mitochondria and to a lesser extent the plasma membrane.

Nerve terminals which were immunoreactive for either SP or VIP were located both beneath and outside the acinar cell basement membrane (Figs. 48, 49 & 50). Some immunoreactive nerve terminals were also found between adjacent acinar cells some distance from the basement membrane.

Occasional SP or VIP immunoreactive varicosities were found associated with blood vessels (Fig. 51). Some nerve terminals remained unstained after incubation for SP and VIP.

There was a complete absence of electrondense reaction product in sections taken from control slices which had been incubated in the absence of a primary antiserum.

## Immunogold staining of SP-like immunoreactive nerve terminals (Figs. 52 - 57)

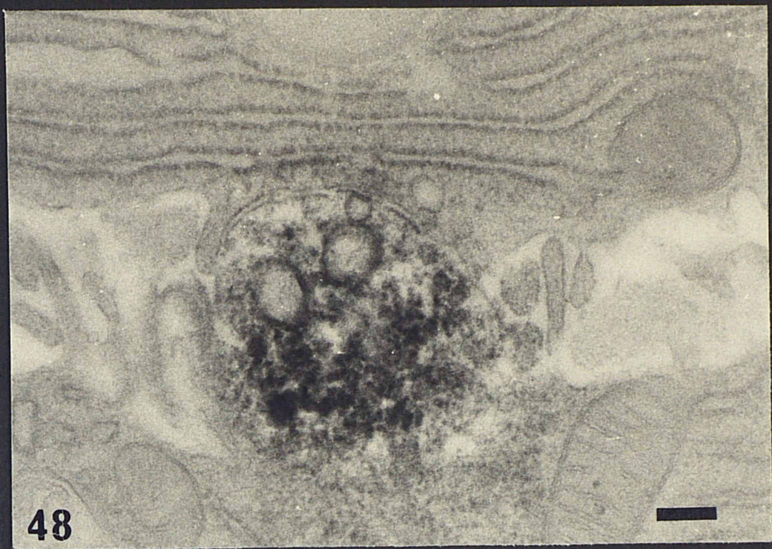
Substance P-like immunoreactivity was located at the ultrastructural level mainly in the larger synaptic

Figs 48 - 51 Unstained electronmicrographs showing immunoreactive peroxidase reaction product in nerve terminals of the rat parotid gland.

SP-like immunoreactivity in a hypolemmel (Fig 48) and an epilemmel (Fig 49) nerve terminal.

VIP-like immunoreactivity in an epilemmel terminal (Fig 50) and in a terminal associated with a blood vessel (Fig 51).

Bar = 200nm.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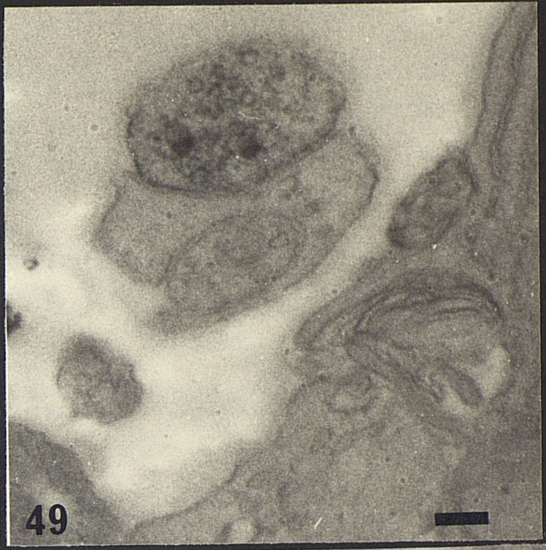
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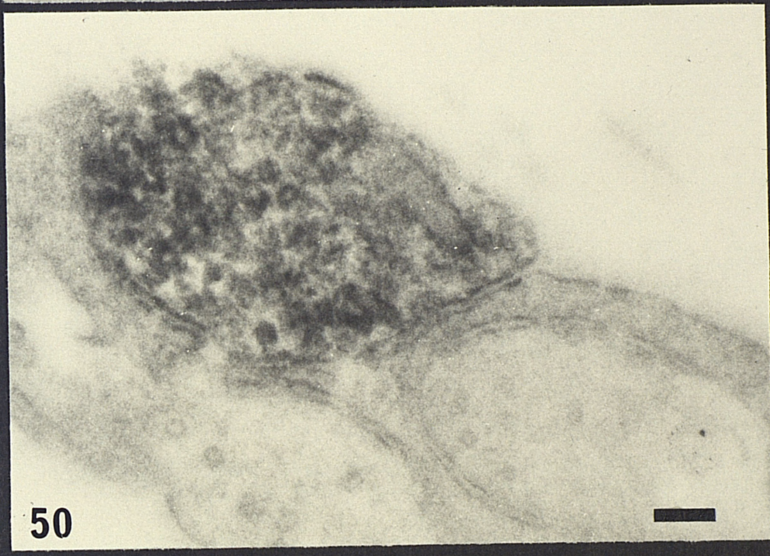
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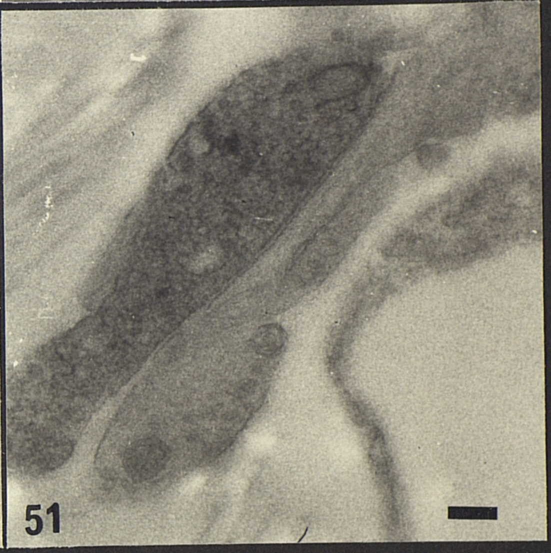
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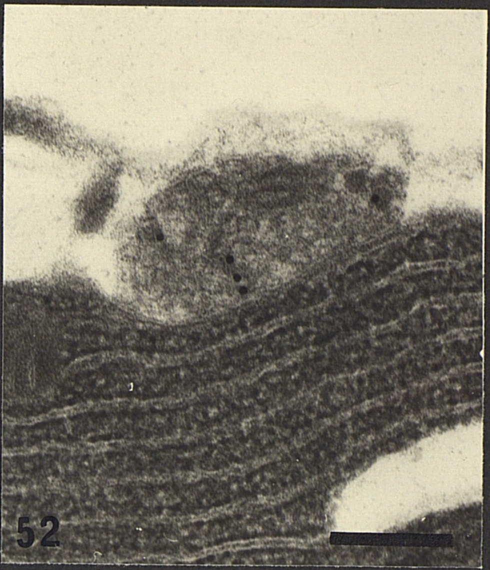
vesicles which measured between 100-140nm and probably contained an electrondense core (see page 44). The gold particles were clearly concentrated on or around these larger vesicles (Figs 52, 53 ) and the background activity was very low in the remainder of the tissue. The smaller much more numerous agranular vesicles seen in osmicated tissues were not readily seen in the immunogold preparations. However gold particles indicative of substance P-like immunoreactivity were rarely seen overlying the colourless spaces occupied by these smaller vesicles.

Immunogold-labelled dense cored synaptic vesicles indicative of substance P - like immunoreactivity were found in nerve terminals situated both below and outside of the acinar basement membrane and in those associated with blood vessels. Not all of the large synaptic vesicles within a terminal were positively labelled with gold particles (Figs.52, 53 ) . Several terminals were observed which showed no immunogold labelling indicative of SP-IR.

## Immunogold-labelling of VIP-like immunoreactive nerve terminals (Figs. 54 - 57)

Vasoactive intestinal polypeptide - like immunoreactivity (VIP-IR) with the immunogold technique was much more difficult to localise accurately and there was always a certain background level of gold labelling. Gold particles were certainly found in or on the surface of the larger dense-cored synaptic vesicles but single gold particles were also seen lying free within the terminal and not apparently

Figs 52-57 Immunogold labelling of nerve terminals in rat parotid gland. SP-like immunogold labelling ( Figs 52 & 53). VIP-like immunogold labelling (Figs 54 & 55). SP + VIP -like immunogold labelling (Figs 56 & 57)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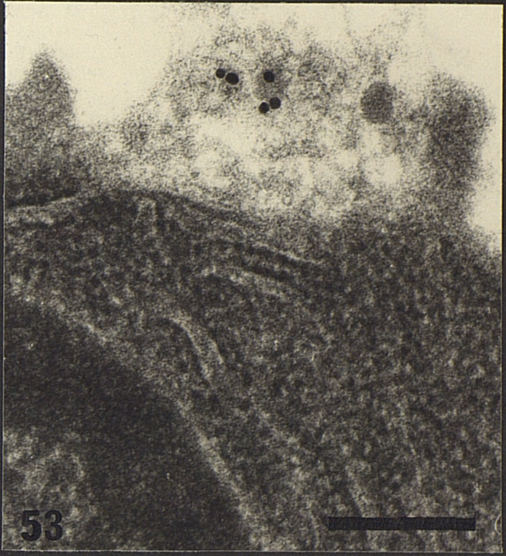
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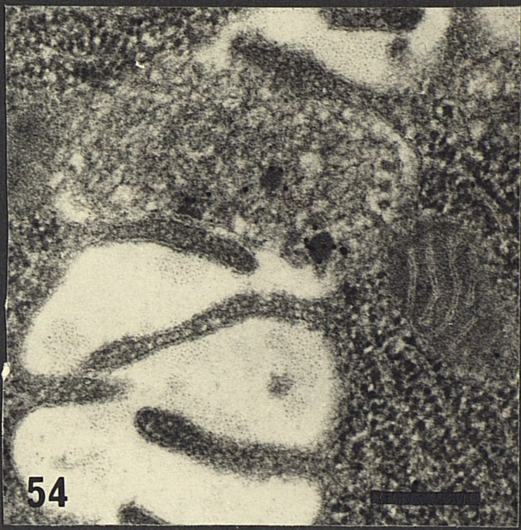
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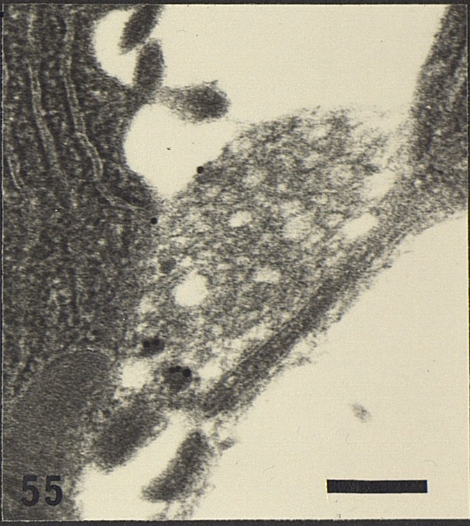
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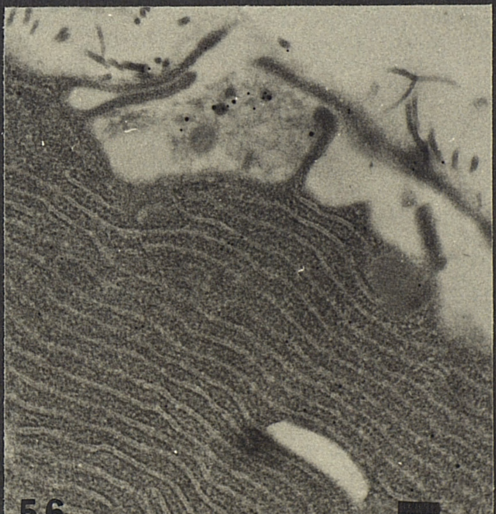
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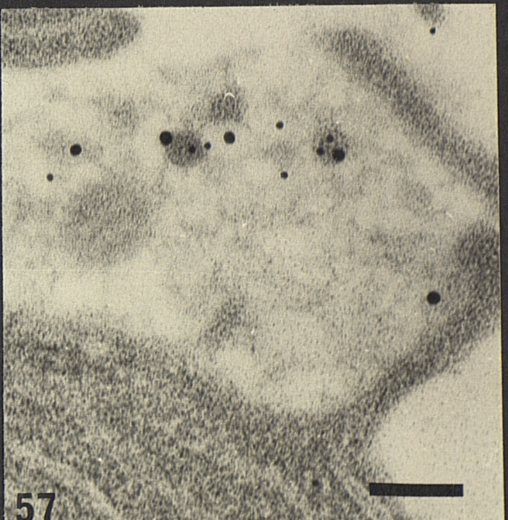
57

associated with any particular vesicle. However, because of the lack of staining of the small agranular vesicles it was possible that these isolated gold particles were lying above the small vesicles.

Terminals which contained synaptic vesicles indicative of VIP-IR were found both beneath and outside of the basal lamina of the secretory acinar cells and occasionally around blood vessels. A few nerve terminals were found which did not contain any gold particles indicative of VIP - IR.

### Co-localization of both SP and VIP in the same terminal
### (Figs. 56,57)

When sections were incubated with antisera to both SP and VIP the majority of the labelled nerve terminals associated with the acinar cells contained dense-cored synaptic vesicles which were clearly labelled with gold particles of different sizes (Figs 56, 57) indicative of co-localization of these two neuropeptides.

Worthy of note was the observation that some nerve terminals associated with the acinar cells did not contain any gold particles .

An extensive search of terminals associated with blood vessels failed to find any which contained synaptic vesicles labelled with gold particles of two different diameters although some terminals were found which contained large synaptic vesicles which were labelled with gold particles of one size only. Only two nerve terminals were found associated with ducts and neither were labelled with gold particles.

Sections incubated in the presence of non-immune serum instead of the primary antiserum revealed a complete absence of gold particles over nerve terminals.

## DISCUSSION

Previous work using retrograde tracers alone (see Chapter 5) or in combination with immunofluorescence techniques (see Chapter 6) has shown that the SP and VIP in the rat parotid gland probably originates from the otic and/or trigeminal ganglion. In addition the immunofluorescence studies provided strong evidence, at least at the light microscope level, that the two peptides may well co-exist within the same otic ganglion neuronal perikarya. The present study has examined the parotid gland tissue at the ultrastructural level to try and confirm the co-localization of these two neuropeptides within the same nerve terminals.

The presence of the peroxidase immunoprecipitate, indicative of the presence of SP or VIP-like immunoreactivity within the larger synaptic vesicles and attached to the outer surface membrane of the smaller synaptic vesicles is similar to that which has been reported in other tissues (Johansson and Lundberg, 1981, Loesch and Burnstock, 1985). The lack of immunoprecipitate within the smaller vesicles may be explained in several ways (see Johansson and Lundberg, 1981 for discussion) but the two most likely explanations would be that either the peptides have diffused away from their original storage sites within the smaller vesicles or that the small vesicles contain only acetylcholine. In support of the latter statement, subcellular fractionation studies of cat submandibular gland nerve terminals have revealed that acetylcholine content was high in fractions containing small synaptic vesicles whereas VIP levels were high in heavier fractions corresponding to large vesicles (Lundberg,1981). The localization of the peroxidase reaction product within the larger dense-cored synaptic vesicles but not within the smaller agranular vesicles would lend support to this hypothesis.

Although the localization of the peroxidase immunoreactive reaction product for SP and VIP within individual nerve terminals was very similar it was not possible from this technique alone to confirm or deny the co-localization of the two neuropeptides within the same nerve terminal.

The results of the immunogold study at the ultrastructural level do however confirm the hypothesis, based on the immunofluorescence study at the light microscope level, that SP and VIP co-exist in the same nerve terminal. In addition strong evidence is provided that the two peptides may well be present within the same synaptic vesicles. The advantages of the immunogold technique over the immunoperoxidase technique are that the antigenic sites are not obscured and conventional counterstaining with lead citrate and uranyl acetate is still possible. The immunogold technique has been used by other workers to show that more than one substance can exist in the same secretory granule. Polak et al., in 1981 confirmed the suggestion made by Ravazzola and Orci (1980) that glucagon and glicentin (proglucagon) are topologically segregated within the alpha granules of human pancreatic cells. Tapia et al., (1983) used a double immunogold technique similar to the one used in the present study to show that insulin and pro-insulin can be localized in the same pancreatic cell granule.

Perhaps of more significance and worthy of discussion is the observation that the ultrastructural features of the nerve terminals in the rat parotid gland (see Chapter four), which have been shown in this study to contain both SP and VIP , are typical of cholinergic nerve terminals. The terminals were characterized by an abundance of small agranular vesicles and some large dense-cored vesicles in a ratio of about 9:1 .Such terminals are believed to be cholinergic (Richardson, 1966; Hökfelt and Nilsson, 1965; Johansson and Lundberg, 1981). However, one must bare in mind the fact that the fixation protocol used in the present study does not allow differentiation between cholinergic and adrenergic terminals.

Adrenergic nerve terminals can be identified after potassium permanganate or 5-hydroxydopamine treatment by the presence of large numbers of small granular vesicles 50nm in diameter and some larger (100nm diameter) dense-cored vesicles (Richardson 1966; Tranzer and Thoenen, 1967). As adrenergic nerve terminals are known to be present in the rat parotid gland it cannot be excluded that the terminals described in the present study which contained SP and VIP-like immunoreactivity were not adrenergic terminals. However, the terminals which were negative for both neuropeptides may equally represent the adrenergic terminals.

The co-existence of neuropeptides with a classical neurotransmitter or with another peptide is not unusual (Lundberg, et al, 1979; Lee et al., 1985;) and indeed is becoming a common phenomenon in the autonomic nervous system. However the functional significance of the presence of two neuropeptides and a classical neurotransmitter in presumed secretomotor neurones is far from clear.

SP is not only a potent sialogogic agent (Ekström and Wahlestedt, 1982; Ekström, Månsson & Tobin, 1983) but it has also been shown to have a vasodilatatory effect (Edvinsson and Uddman, 1982 ). Sharkey and Templeton (1984) showed that following capsaicin treatment the SP-IR fibres around the vas culature disappeared whereas that around the secretory acini remained. Such evidence would support their hypothesis that the SP within the rat parotid gland originates from two different sources, and that the innervation of the vasculature is from the trigeminal ganglion whereas that to the secretory acini comes from the otic ganglion. This is of interest because CGRP and SP have been co-localized in trigeminal ganglion cells (Lee et al., 1985; see Chapter 6) and CGRP like SP, has been shown to have a vasodilatatory effect (Edvinsson and Uddman, 1982; Fischer et al., 1983). CGRP has also been shown to enhance the effect of SP (Ekman et al., 1986) although alone it has no effect on secretion.

When VIP is given intravenously it alters not only the flow of protein rich saliva (Ekström et al, 1983, 1984) but also potentiates the salivary secretion caused by release of acetylcholine (Lundberg, et al, 1982). Lundberg , (1981) has suggested that the VIP and acetylcholine release around vascular nerves may be different from that around secretory acini.

Clearly these different physiological effects of neuropeptides and / or classical neurotransmitters on secretion of saliva , blood flow etc are probably due to the local release of these substances from terminals around blood vessels and ducts as well as from those terminals around the secretory acini. However, the present study has clearly shown not only the co-localization of two neuropeptides with a presumed classical neurotransmitter in the same nerve terminal but also co-localization of SP and VIP-IR within the same vesicles.

There is some evidence that classical transmitters and neuropeptides can be released differentially from the same nerve terminal. The release being dependent upon the frequency of action potentials (Lundberg et al., 1982; Edwards et al.,1982). Such a presynaptic mechanism could operate from the terminals described in the rat parotid gland. However one can only imagine that the SP and VIP contained within the same vesicle must be released at the same time. The possibility exists of course that the two peptides may act on different receptors on the postsynaptic cell to produce their effect, thus producing some sort of selectivity and specificity as suggested by Hökfelt et al., (1987).

In conclusion this study has shown that nerve endings in the rat parotid gland with an appearance similar to that of cholinergic terminals contain two peptides within their large dense-cored vesicles. Therefore it is possible that upon stimulation of the parasympathetic secretomotor innervation of the parotid gland these peptides are released and may potentiate the effect of acetylcholine or indeed of each other.

# CHAPTER EIGHT

# GENERAL DISCUSSION

# GENERAL DISCUSSION

On consideration of which neurotransmitter(s) contribute to the atropine-resistant protein rich salivary secretion evoked by parasympathetic stimulation of the rat parotid gland, SP and VIP seem to be of particular interest (Ekström et al, 1983, Ekström et al., 1984).

Immunoreactive fibres for both SP and VIP have been described in the parotid gland of the rat (Wharton et al., 1979, Uddman et al., 1980, and Sharkey and Templeton, 1984) and their presence has been confirmed in the present study.

SP when given inravenously is certainly a very potent sialogogic agent in the rat parotid gland (Ekström and Wahlestedt, 1982, Ekström et al., 1983). VIP on the other hand , when administred intravenously, produces only a small flow of protein rich saliva from the gland (Ekström et al., 1982, Ekström et al., 1983).

Generally speaking it is considered to be the parasympathetic innervation of the rat parotid gland which contains these two peptides SP and VIP. These considerations are based upon the results of experiments where the activities in the parotid gland of the acetylcholine forming enzyme, choline acetyltransferase, and of the two neuropeptides SP and VIP are reduced to about 5% of their control values following section of the auriculotemporal nerve (Ekström et al., 1984).

More recently it has been demonstrated that continuous stimulation of the parasympathetic nerves to the rat parotid gland causes a reduction in the amount of SP and VIP in the gland . This reduction in the neuropeptide levels was associated with a marked reduction in the secretory response to auriculotemporal nerve stimulation (Ekström et al., 1985). Thus both SP and VIP have been presumed to be released from nerve endings and to act on specific acinar receptors in the rat parotid gland (Gallacher, 1983, Inoue et al., 1985).)

However, the origin of SP and VIP-immunoreactive fibres in the rat parotid gland was far from clear. The results of experiments presented in this thesis have not only identified the neuronal projections to the rat parotid gland but have also established that at least some of the neurones which contain the SP and VIP originate in the otic ganglion. Furthermore, the light and electronmicroscopical observations provide strong evidence for the co-localisation of SP and VIP in "cholinergic" terminals of the rat parotid gland.

Co-localisation of neuropeptides with a classical neurotransmitter is becoming a familiar phenomenon in both the central and peripheral nervous systems (Hökfelt et al., 1987). Acetylcholine has been found to co-localise with SP in the rat pons (Vincent et al., 1983) and with VIP in the rat cerebral cortex (Eckenstein et al., 1984). Co-localisation of two or more peptides with a classical neurotransmitter has been described in the neurones of the gastrointestinal tract (Schultzberg, 1980, Furness et al., 1984). Recently SP and VIP immunoreactivity has been co-localised in nerve fibres supplying vascular and bronchial smooth muscles as well as bronchial glands in the cat lung (Dey et al., 1988). The significance of this co-localisation is not very well understood.

A similar co-localisation of neuropeptides with a classical neurotransmitter has been suggested in the salivary glands of other species. Acetylcholine and VIP co-exist in the parasympathetic nerves around the secretory elements and blood vessels of the cat submandibular gland (Lundberg et al., 1980, Johansson and Lundberg, 1981, Lundberg, 1981).

The significance of co-localisation of neuropeptides with classical neurotransmitters is poorly understood (Hökfelt, et al.,1987). However it has been suggested that they may play a complementary role in regulating salivary function. Such a complementary role seems to be complex and varies in different species.

In the cat submandibular gland acetylcholine infusion produces salivary secretion and vasodilation and both effects are atropine sensitive (Lundberg, Anggard and Fahrenkrug, 1982). VIP infusion produces atropine resistant vasodilation but no salivary secretion (Bloom and Edwards, 1980, Lundberg et al., 1980,Lundberg, Anggard and Fahrenkrug, 1982). It is surprising that in spite of a dense VIP innervation of acini and ducts (Johansson and Lundberg, 1981) that VIP has no secretory effect when injected alone. Combined infusion of acetylcholine and VIP has an additive effect on vasodilation but potentiates the secretory effect of acetylcholine (Lundberg, Ånggård and Fahrenkrug, 1982). Acetylcholine infusion alone does not produce a maximal secretory response when compared to that produced by parasympathetic stimulation. The composition of the saliva produced by muscarinic stimulation also differs from that produced by parasympathetic stimulation ( Baxter, 1932 ). Furthermore, when VIP antiserum was used both the salivatory response and the increased blood flow following parasympathetic stimulation was reduced (Lundberg,et al., 1981). Such results may suggest that VIP has a complementary role with acetylcholine in regulating both salivary secretion and salivary blood flow in the cat submandibular gland. Substance P in the cat submandibular gland seems less likely to be involved in the regulation of secretion since substance P infusion has no secretory effect even when given in very high doses (Lundberg, Ånggård and Fahrenkrug, 1982).

In the rat parotid gland intravenous infusion of substance P produces a rapid onset, profuse salivary secretion, whilst VIP produces a slow onset , small flow secretion of protein rich saliva (Ekström and Wahlestedt, 1982, Ekström et al., 1983). In 1986, Ekstrom and Olgart showed that exogenously applied substance P acts with some agent or agents that are released upon auriculotemporal nerve stimulation. Such an agent or agents are not exerting their effects through muscarinic, or adrenergic receptors. The evidence that one of these agents may be VIP is very strong. Firstly, both nerve stimulation and VIP infusion produced a high amylase output and their responses were affected by substance P injection in the same way. Secondly, after long term continuous stimulation the enhanced response to repeated substance P injection became attenuated. This may be due to VIP depletion in the rat parotid gland. A similar depletion of VIP occurs when the auriculotemporal nerve is subjected to a similar course of stimulation (Ekström et al., 1985).

However, VIP cannot be the only agent responsible for the atropine resistant secretion. The onset of secretion was much delayed and the fluid volume was much less in the VIP induced secretion when compared to that observed following parasympathetic stimulation. The other agent acting with VIP might be substance P. When substance P and VIP were used together the secretory response was augmented and the fluid volume and amylase output was similar to that which occurs during nerve stimulation (Ekström and Olgart, 1986). Salivary secretion from the rat parotid gland also seems to be enhanced when acetylcholine and VIP are injected together. VIP from the rat parotid gland seems to be released on low frequency stimulation of the parasympathetic nerve supply.

The atropine resistant vasodilation produced by predominantly high frequency stimulation of the parasympathetic nerves to the cat submandibular gland is thought to be due to VIP (Bloom and Edwards, 1980, Lundberg, Änggård and Fahrenkrug, 1981). Of interest is the fact that atropine resistant vasodilation is seen in the rat parotid gland but on low frequency stimulation of the auriculotemoral nerve. Such results could indicate that VIP is released at low frequencies.

The new information presented in this thesis taken together with information already reported in the literature would indicate that the two neuropeptides SP and VIP are found not only within the same nerve terminal but possibly within the same synaptic vesicles. These peptides are always found alongside synaptic vesicles which morphologically, resemble those which are believed to contain acetylcholine. The terminals which contain these peptides originate from postganglionic parasympathetic neurones in the otic ganglion. There is no doubt that these two peptides have an effect on salivary secretion from the rat parotid gland although the significance of such effects remains far from clear.

# CHAPTER NINE

# SUMMARY AND CONCLUSIONS

# SUMMARY AND CONCLUSIONS

As a result of experiments reported in this thesis the precise location of the otic ganglion in the rat has been described. In addition the probable route by which the preganglionic axons from the brainstem salivatory neurones take to reach the postganglionic parasympathetic neurones in the otic ganglion has been described for the first time.

The results of the retrograde tracer experiments in which fluorochrome dyes were injected into the substance of the parotid gland and from which several ganglia were examined for retrogradely labelled cells clearly showed that projections to the parotid gland of the rat were restricted to the superior cervical ganglion (sympathetic supply), trigeminal ganglion (sensory supply) and the otic ganglion (postganglionic parasympathetic supply).

The immunofluorescence studies demonstrated that neurones which contained SP were to be found not only in the trigeminal ganglion but also in the otic ganglion. VIP on the other hand was restricted to otic ganglion neurones. CGRP was only found in the trigeminal ganglion neurones. All three neuropeptides were found as fibres of passage or as terminals within the parotid gland itself.

The combination of the immunofluorescence and the retrograde transport studies showed that at least some of the neurones in the otic ganglion which contained SP and VIP did project to the parotid gland.

The use of the relatively new immunogold technique provided conclusive evidence for the co-localisation of SP and VIP in dense-cored vesicles which were contained , at least on a morphological basis, in cholinergic nerve terminals.

The possible significance of this co-localisation of the two neuropeptides SP and VIP in cholinergic nerve terminals is discussed on the basis of their known effects on secretion of saliva from the parotid gland.

# APPENDICES

# APPENDIX 1

## PHYSIOLOGICAL SALINE

| | |
|---|---|
| Sodium chloride (B.D.H. Ltd., Poole, Dorset, U.K.) | 8.5g |
| Distilled Water | 1 litre |

---

## PARAFORMALDEHYDE FIXATIVE

1. Weigh out 4g paraformaldehyde powder (BDH: Poole,Dorset,UK) and add to 50ml of distilled water at 60 C.
2. Stir thoroughly.
3. Add 2-6 drops of 0.1N sodium hydroxide until solution clears. Cool.
4. Add 50ml of 0.2M phosphate buffer (see below).

---

## 0.2M PHOSPHATE BUFFER

Solution A:

| | | |
|---|---|---|
| 1. | Monopotassium phosphate (KH2PO4; M.Wt = 136.09) | 13.61g |
| 2. | Dissolve in distilled water | 1 litre |

Solution B:

| | | |
|---|---|---|
| 1. | Disodium phosphate (Na2HPO4.12H2O; M.Wt = 358.15) | 35.82g |
| 2. | Dissolve in distilled water | 1 litre |

Take 19.6ml of solution A and 80.4ml of solution B and mix. Check pH=7.4. Adjust if necessary.

# APPENDIX 2

## HEIDENHAIN'S SUSA FIXATIVE

| | |
|---|---|
| Mercuric chloride (B.D.H., Poole, Dorset) | 4.5g |
| Sodium chloride | 0.5g |
| Trichloracetic acid | 2.0g |
| Acetic acid | 4.0ml |
| Formalin (40% paraformaldehyde) | 20.0ml |
| Distilled water | to 100ml |

Dissolve the constituents in the order listed and filter before use.

## APPENDIX 3

## PROCEDURE FOR PARAFFIN WAX PROCESSING OF SUSA-FIXED MATERIAL

Remove the head and cut off the lower jaw with the aid of a sharp knife. Place the remaining head in fresh fixative to decalcify for a period of not less than 1 week. When decalcification is complete make coronal slices through the head at 1 centimetre intervals. Treat each block as follows:

1. Place in 90% alcohol for 2 hours.
2. Place in absolute alcohol for 6 hours (3 changes of 2 hours duration).
3. Leave in chloroform for 2 days. Ensure that the tissue is completely submerged by placing discs of filter paper on the surface.
4. Impregnate with paraffin wax (Raymond Lamb, London) for 6h (3 changes of 2 hours duration) at 60 C.
5. Embed in fresh molten wax.

# APPENDIX 4

## HAEMATOXYLIN AND EOSIN STAINING PROCEDURE

| | | | |
|---|---|---|---|
| 1. | Remove paraffin wax by placing sections in xylene 2 changes of 5 mins duration. | | |
| 2. | Absolute ethanol | | 5 mins |
| 3. | 70% ethanol | | 5 mins |
| 4. | 0.5% iodine in 80% ethanol | | 4 mins |
| 5. | Rinse in distilled water | | |
| 6. | 3% sodium thiosulphate | | 3 mins |
| 7. | Wash in running tap water | | 5 mins |
| 8. | Place in Ehrlich's haematoxylin * | | 12 mins |
| 9. | Wash in running tap water until sections appear blue | | |
| 10. | Differentiate in 1% acid alcohol | | 5 secs |
| 11. | Wash thoroughly in running tap water | | |
| 12. | Counterstain in 1% aqueous eosin | | 2 mins |
| 13. | Wash in running tap water | | |
| 14. | Dehydrate in 70% ethanol | | 2 mins |
| 15. | Absolute ethanol | | 2 mins |
| 16. | Clear in xylene | 2 x | 5 mins |
| 17. | Mount sections in D.P.X. | | |

## * EHRLICH'S HAEMATOXYLIN

| | |
|---|---|
| Haematoxylin | 6g |
| Absolute alcohol | 300ml |
| Distilled water | 300ml |
| Glycerol | 300ml |
| Glacial acetic acid | 30ml |
| Potassium alum (potassium aluminium sulphate) | in excess |

Dissolve the haematoxylin in the absolute alcohol and then add the other constituents in the order listed above. Allow to ripen at room temperature and exposed to sunlight for 2 months. Staining time should be approximately 12 minutes.

# APPENDIX 5

## KARNOVSKY'S FIXATIVE (MODIFIED)

(Reference: Karnovsky, M.J.(1965) J.Cell Biol., 27, 137.)

Heat 25ml distilled water to 60-70 C , add 2g of paraformaldehyde powder. Stir thoroughly. Add 1-3 drops of 1N sodium hydroxide until the solution clears. Cool. Add 4mls of 25% glutaraldehyde. Make up volume to 50 mls with 0,22M phosphate buffer pH 7.4-7.6.

# APPENDIX 6

## ARALDITE EMBEDDING PROTOCOL

1. After fixation place small pieces of tissue in 0.1M phosphate buffer pH 7.4. Two changes then leave overnight.
2. Postfix in 1% osmium tetroxide for 1 hour.
3. Wash well in distilled water.
4. Dehydrate through graded alcohols over a 2 hour period.
5. Place in 2 changes of propylene oxide - 30 mins each.
6. Place in propylene oxide/Araldite mixture* 2:1 for 1 hour.
7. Place in propylene oxide /Araldite mixture 1:2 for 4 hours.
8. Place in freshly prepared Araldite mixture for 12 hours.
9. Embedd in polythene capsules or silicon moulds with identifying label and polymerize at 60° C for 18-24 hours.

## * ARALDITE RESIN MIXTURE

Araldite CY212 13.75g; DDSA 12.25g; DMP-30 0.9g. Obtained from Taab Laboratories Equipment Ltd. Reading, Berks. Mix thoroughly in a disposable beaker usin a glass rod. Allow to stand before use to allow air bubbles to disappear.

# APPENDIX 7

## KARNOVSKY AND ROOTS METHOD FOR ACETYLCHOLINESTERASE

(Karnovsky, M.J. & Roots, L., 1986)

1. Fix tissue in 10% buffered formalin. Minimum 24 hrs.
2. Place in 0.88M sucrose overnight.
3. Quench tissue in isopentane cooled with liquid nitrogen.
4. Cut sections at 10um thickness on a cryostat and mount onto gelatinised slides.
5. Incubate sections in the following incubation medium for 30 - 60 minutes at room temperature.

| | | |
|---|---|---|
| Incubation medium: | acetylthiocholine iodide (Sigma) | 5mg |
| | 0.1M sodium hydrogen maleate buffer pH 6.0 | 6.5ml |
| | 0.1M sodium citrate | 0.5ml |
| | 30mM anhydrous copper sulphate | 1.0ml |
| | Distilled water | 1.0ml |
| | 5mM potassium ferricyanide | 1.0ml |

For control sections use butyryl thiocholine iodide (Sigma)as a substitute for acetylthiocholine iodide.

Sites of enzyme activity are coloured deep brown.

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