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Neural Mechanisms of Salivary Gland Secretion

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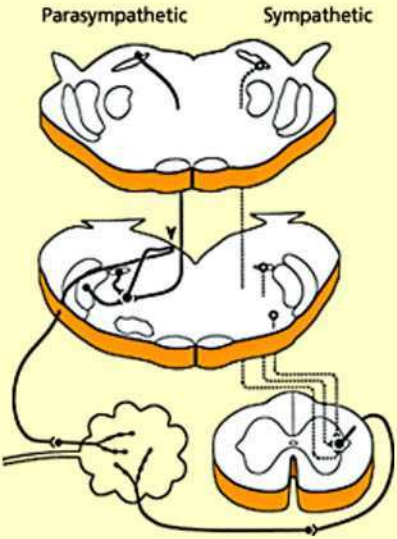

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Frontiers of Oral Biology

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Dedicated to four great pioneers in the subject of salivary glandular innervation

Carl Ludwig (1816–1895)

Claude Bernard (1813–1878)

John Newport Langley (1852–1925)

Nils Emmelin (1914–1997)

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Contents

Preface

> 'There will come a time when things that are now obscure will be brought to light by the dawn of a new day, and as a result of diligent research over a longer period of time.'
>
> Seneca
>
> (Frontispiece – *Adenographia* by Thomas Wharton – 1656)

Wharton described the duct that now bears his name and showed that it delivered saliva from the submandibular gland to the mouth. He considered that the saliva arose directly from the nerves that went to the gland. It took nearly 200 more years before Carl Ludwig discovered in 1850 that electrical stimulation of the autonomic nerves to salivary glands causes the glands to secrete saliva. Since then it has been established that normal salivary secretion is dependent on centrally generated reflex nerve impulses. Extensive studies have continued to explore the different activities of the nerves in the glands and the mechanisms by which they occur. In all this long history, no book has previously been devoted to the roles of nerves in the secretion of saliva. So, it is timely to have a book about neural mechanisms in salivary glands, to reflect progress over the 150 years since Ludwig's great discovery, and to indicate where knowledge stands at this the turn of another century.

This volume is a companion book to volume 10 'Glandular Mechanisms of Salivary Secretion' which included chapters on the neural control of blood vessels and myoepithelial cells. This information will not be repeated in the present volume but is given brief mention in the summary chapter 12, and should be considered in the totality of nerve activities in the glands.

Severe limitations on space have imposed great disciplinary demands on the authors, but this has helped to make for succinct presentations and the avoidance of mere catalogues of references. As previously, within these restraints, authors were encouraged to give personal assessments of knowledge as they perceive it on the subject matter of their chapters.

We have leant towards a generalist approach rather than the now more fashionable reductionist one. Where possible, whole gland whole animal work has been emphasized, particularly as it continues to demonstrate the importance of species differences but, unfortunately, it is an approach that is becoming progressively more difficult to undertake for economic and emotional reasons. This type of investigation also helps create a more balanced outlook than can possibly accrue from studies on single cells from a single species using single agonists and single antagonists, no matter how important such findings may be. Nevertheless, at the end of the day, both approaches should be complementary.

Regrettably, current literature continues to show that a number of misconceptions persist about salivary phenomena. This often relates to extrapolations from one species to others. It also arises from the common, oversimplistic, consideration of only single alternative basic mechanisms, e.g. fluid formation is the sole province of parasympathetic nerves; exocytosis is controlled exclusively by sympathetic nerves, and each is the consequence of a single transmitter. It is hoped that this book will help to redress such erroneous opinions, increase awareness of the collaborative effects of the different nerves and their transmitters, broaden horizons and continue to provide useful information for a long time to come. It is also hoped that the book will assist and perhaps even help to create new research, for there is still a long way to go.

'There are more things in heaven and earth, Horatio, than are dreamt of in your philosophy.'

Hamlet

J.R. Garrett
J. Ekström
L.C. Anderson

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Chapter 1

Nerves in the Main Salivary Glands

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Historical Introduction

The gross anatomy of the nerves to submandibular glands was known to Carl Ludwig in 1850 [1] when he made the momentous discoveries that electrical stimulation of the chorda-lingual nerve caused copious flows of submandibular saliva in dogs and the secretory pressure thereby induced could exceed the blood pressure. He also mentioned that there was a sympathetic supply to the gland, travelling with its artery, but it is not known whether he stimulated this nerve. Claude Bernard [2] followed up Ludwig's discovery by studying reflex salivary secretion and the effects of sectioning the nerves on this secretion. He also assessed the glandular effects of stimulating the cut nerves electrically. Bernard produced a diagram of the gross innervation of dog submandibular glands largely as it is perceived today [2, and see 3]. Later, he showed that each type of nerve has distinctly different effects on glandular blood vessels [4]. Slowly, the gross innervation for the other main salivary glands was worked out and it was recognised that each gland receives a parasympathetic and sympathetic input from essentially separate routes [5].

Knowledge about the microscopic innervation within the glands evolved much more slowly, because the methods were inadequate during the first 100 years after Ludwig's discovery, consisting of silver staining or methylene blue techniques. Each set of artefacts, so produced, differed. Attempts to reconcile the findings of one worker with those of others were doomed because of the impenetrable jungle of ideas being created. As recently as 1953, on the basis of silver staining, it was proposed that only vascular sympathetic nerves exist in the glands [6] and any sympathetic effects on parenchymal cells were thought to be indirect from vascular nerves!

Then in the 1950s came the modern neurohistological revolution from both histochemical and electron-microscopic studies. In 1956, it was found that acetylcholinesterase staining provided a good survey method for demonstrating nerves in rat salivary glands [7], and they appeared more prominent in submandibular than sublingual glands. Subsequent denervation studies showed that the cholinesterase positive nerves were predominantly, if not exclusively, parasympathetic [8]. In 1959, Scott and Pease [9] published an extensive electron-microscopical study of rat salivary glands, and showed that neuroeffector relationships differed between the glands. In the 1960s, catecholamine fluorescence of adrenergic nerves was developed and used to advantage on rat salivary glands by Norberg and Olson [10], who found that the distribution patterns of adrenergic (sympathetic) nerves differed between the 3 main types of salivary gland. In the 1970s immunohistochemical methods were introduced for showing neuropeptides and it was found [11] that they could co-exist with conventional transmitters. This work continues and vast differences are now evident in the neuropeptides detectable in salivary nerves in different glands and different species. Most recently, attention has been attracted to the immunohistochemical detection of nitric oxide synthase which is found in some but not all salivary nerves [12].

Innervation Patterns within the Salivary Glands

Assessments of nerve distributions in the glands requires a twofold approach, starting with light microscopy using highly reproducible histochemical methods to provide surveys of terminal nerves in the glands. However, the details of the neuroeffector arrangements with salivary cells can only be achieved by careful electron-microscopic assessment, using both conventional and special cytochemical methods. For example, Tranzer and Thoenen [13] introduced the use of 5-hydroxydopamine to enhance the ultrastructural visualization of the dense cores in adrenergic vesicles, which enables sympathetic adrenergic axons to be identified more readily.

Light-Microscopic Observations

(i) Acetylcholinesterase (AChE) enzyme histochemistry was used to great avail for many years to provide light microscopical surveys of predominantly parasympathetic nerves in the glands (fig. 1A). This has shown remarkable differences in the extent of the nerves present in different glands. In cat submandibular glands there are extensive networks of AChE-positive nerves around the acini but in their parotid glands these nerves are much less frequent and distributed mainly in the interstitial spaces [14]. In rabbits there is a dense

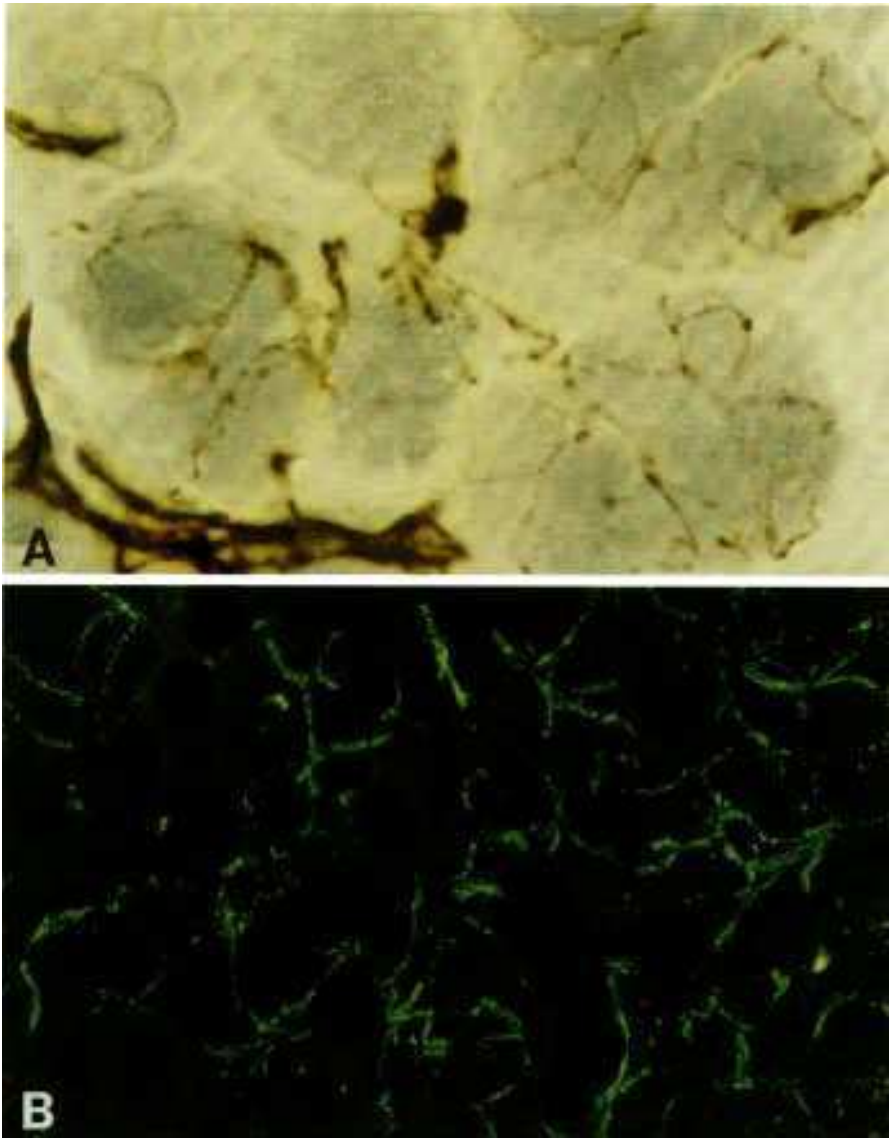

Fig. 1. Sections of human glands. *A* Submandibular gland stained for acetylcholinesterase activity, showing fine networks of nerves around acini. ×659. *B* Parotid gland treated for catecholamine fluorescence, showing abundant adrenergic nerves associated with acini. ×280.

uniform arrangement of AChE-positive nerves around parotid acini but in submandibular glands they are much more irregularly distributed, being particularly dense around intralobular ductal structures and more sparse around acini [15]. In human glands the innervation by AChE-positive nerves was found to be similar in parotid and submandibular glands but surprisingly few nerves were seen around the collecting ducts [16]. In all studies numerous AChE-positive nerves were detected around bloood vessels in the glands. This technique has always had its critics because it is not necessarily specific for cholinergic parasympathetic nerves, and has now largely fallen into disuse because of the greater enthusiasm for immunocytochemical staining on paraffin sections. However, an advantage of using post-fixed cryostat sections of unfixed material for AChE-staining is that adjacent sections can be used for catecholamine fluorescence.

(ii) Catecholamine fluorescence must be one of the most perfect histochemical methods, because it depends on direct visualization of the actual transmitter – noradrenaline. It was used by Norberg and Olson [10] for studying adrenergic sympathetic nerves in rat salivary glands. They found that blood vessels in each gland have dense adrenergic innervations but main ducts were devoid of adrenergic nerves. Acini were shown to receive the densest innervation in submandibular glands by thicker, more eye-catching, nerves than in the parotid glands where the nerves were finer, and in the sublingual gland the parenchyma had an extremely sparse innervation. Striated ducts in all of the glands were wrongfully considered to be devoid of adrenergic nerves. Closer study has since shown that the few adrenergic nerves in the parenchyma of rat sublingual glands are particularly directed to the striated ducts [17]. Furthermore, it was shown that the granular tubules in rat submandibular glands also receive an adrenergic innervation [17]. Catecholamine fluorescence has been used to great effect on many other species including man [16, 18]. In human salivary glands the parenchymal innervation was found to be plentiful (fig. 1B) and considered to be similar in both parotid and submandibular glands in the former paper [16], but the latter paper [18] found it less rich in parotid glands. Despite the immense value of this method it has also fallen from use, largely because unfixed cryostat sections are required, and it has been replaced by more fashionable immunohistochemical methods for enzymes on the metabolic pathway for the formation of noradrenaline, tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH). However, results with either of these methods are more equivocal than those that depend on the actual transmitter itself.

(iii) Immunohistochemistry: (a) Neuropeptides: In recent years immunohistochemical methods for neuropeptides have increased understanding about the presence of other transmitters that can co-exist in salivary nerves, together with acetylcholine in terminal parasympathetic nerves or noradrenaline in

terminal sympathetic nerves. One of the first to be studied was VIP (vasoactive intestinal polypeptide), which has a potential for inducing vasodilatation. VIP-containing nerves were studied in salivary glands from rat, cat and man [19, 20], and a liberal distribution of such nerves was found in the glands around blood vessels, acini and larger ducts. The latter paper [20] found the presence of VIP-containing nerves was greatest in cat glands and less in those from rat and man, as was backed up by radioimmunoassay of tissues. The authors also showed that parasympathetic stimulation of cat submandibular glands caused a big increase of VIP in the effluent blood, especially at higher frequencies such as 20 Hz. A similar finding was made by Bloom and Edwards [21] and the same group subsequently demonstrated that the output of VIP into the effluent blood was optimized by stimulating at even higher frequencies in bursts of 1 s every 10 s [22].

Refined studies by Lundberg and co-workers [11, 23, 24] have shown that the neuropeptides in efferent glandular nerves co-exist with the classical transmitters. Differences were found between glands and species in the amounts and types of neuropeptides in the salivary nerves. They commented in summary [24] that parasympathetic cholinergic nerves are present around acini, ducts and blood vessels and these nerves also contain VIP/PHI (peptide with N-terminal histidine and C-terminal isoleucine) and/or in some instances NPY (neuropeptide Y) and SP (substance P). Sympathetic noradrenergic nerves surround blood vessels and acini (in most glands) and the subpopulation of sympathetic nerves containing NPY were found predominantly close to blood vessels. Subsequently, it was demonstrated in rat salivary glands that perivascular NPY-containing nerves are sympathetic and periacinar NPY-containing nerves are parasympathetic and also contain VIP [25, 26].

Neuropeptides have been found in sensory (afferent) nerves in the glands. Retrograde studies have shown [27–29] that the afferent nerves travel mainly through the trigeminal ganglion, passing from submandibular glands via the chorda-lingual nerve and from parotid glands mainly via the auriculo-temporal nerve. Ekström et al. [30, 31], using nerve sectioning or capsaicin treatment (to destroy sensory nerves), found that the sensory nerves in rat salivary glands contain both substance P and CGRP (calcitonin gene-related peptide) and the nerves containing only substance P or only CGRP were efferent autonomic nerves.

Other neuropeptides have been explored in salivary glandular nerves. Galanin (GAL)-containing nerves were associated with ducts and acini in rat submandibular and sublingual glands [32]. They were considered to be parasympathetic in origin and a subset of submandibular ganglion cells was found to express galanin immunoreactivity. Bombesin was not found in the nerves present in sheep salivary glands [33]. Some neurokinin-A-containing

nerves were found around acini, blood vessels and ducts in rat parotid glands, but they were less abundant in rat submandibular glands [34]. Enkephalin (ENK)-like immunoreactivity was found in some nerves around acini in young rat submandibular glands and in some submandibular ganglion cells, but they were not seen in the sublingual glands [35]. As the ENK-immunoreactive periacinar nerves were less after excision of the superior cervical ganglion it was inferred that enkephalin-containing nerves in the gland can originate from both sympathetic or parasympathetic ganglia. Met-enkephalin-Arg-Gly-Leu [MEAGL] immunoreactivity has been found in rat salivary glandular nerves around acini and ducts [36]. These nerves were most abundant in submandibular glands, a little less in sublingual glands and relatively sparse in parotid glands. Excision of the superior cervical ganglion led to some reduction of these nerves in submandibular glands but not in the other glands. PACAP (pituitary adenylate cyclase activating peptide)-containing nerves have been identified in ferret submandibular glands, particularly around blood vessels and most were distinct from those containing VIP, but VIP and PACAP have similar amino acid compositions and both can cause vasodilatation [37].

Most of the studies so far have been concerned with rat glands but possible strain differences have not been considered. Many other papers exist on the rat and other species; too many to list in an article of this size. Not all of the findings are in accord with each other, and great differences exist between glands, species and probably strains and substrains. The last word has not yet been said on this fascinating subject and other neuropeptides possibly await discovery. To go into further detail at this stage is likely to cause even greater confusion, similar to that which occurred with the earliest nerve staining methods (see 'Historical Introduction').

Attempting a summary comment at this stage, it may be said that neuropeptides can be found in sensory and autonomic secretomotor nerves in salivary glands. The presence of different neuropeptides varies considerably between species and glands. The functions of some of the neuropeptides are considered in chapter 6, but the specific functions of many of the neuropeptides that may possibly be released from salivary nerves are not so far known. It seems likely that the neuropeptide presence in the nerves is adaptable to the functional requirements of the species, the gland and the cell type that is being innervated. Their variability, plus insufficient data, make it impossible to lay down any general patterns about their presence.

It is not unreasonable to end this section with special comment about neuropeptide-like activity in human glands. As mentioned above, VIP-containing nerves have been described in human glands [20]. Nerves containing CGRP immunoreactivity were sparse in human submandibular glands [38]. In parotid glands [39], although differences were observed between regions in the same

gland, NPY-containing nerves were most abundant around acini and greater than TH-containing nerves (inferred as adrenergic) which in turn were more abundant than VIP-containing nerves. Sometimes, but not always, NPY and TH co-localized in these sites. Very few SP- or CGRP-containing nerves were detected. Striated ducts received TH- and NPY-containing nerves with partial co-localization. Few nerves were found by collecting ducts and then only those containing NPY. Numerous NPY-immunoreactive nerves were seen around blood vessels but somewhat fewer TH-containing nerves were present; co-localization with TH occurred to some extent around smaller vessels but not around larger vessels. Surprisingly, VIP immunoreactivity was not found by these authors around arterioles. Substance P and CGRP-containing nerves were identified moderately frequently around blood vessels and smaller ducts and often co-localized, so were then considered as possible afferent fibres. ENK-immunoreactive nerves were scarce. More recent studies [40] on human submandibular glands found that there was a dense distribution of NPY-, VIP- and GAL-reactive nerve fibres around acini and ducts, with such fibres being significantly greater around 'mucous' acini than 'serous' ones. SP- and CGRP-reactive nerves were sparse and no nerves were found to contain somatostatin, Leu- or Met-enkaphalin. Around blood vessels NPY-containing nerves were most abundant, but VIP containing axons were fairly frequent. In the human parotid glands [41] the neuropeptide results were compared with those using 'anti-protein gene product (PGP) 9.5', considered by the authors as the best marker for total nerve density. As in submandibular glands many NPY-containing and VIP-containing nerves were found associated with parotid acini but GAL-containing nerves were less frequent. Percentages compared with PGP-9.5-containing nerves were approximately 75% for NPY, 70% for VIP and 42% for GAL. The densities around intercalated ducts were somewhat lower and even less in association with striated and other ducts. SP- and CGRP-containing nerves were sparse throughout and no activity for somatostatin, Leu- or Met-enkephalin was detected in the glandular nerves. Around blood vessels NPY-containing nerves represented approximately 94% of those detected with the PGP-9.5 antibody. Moderate numbers of VIP-containing nerves were also seen around blood vessels.

(b) Nitric oxide synthase (NOS): This new field of enquiry is attracting a great deal of attention but the full significance of any nitric oxide that is capable of being generated is not yet understood. In rat submandibular glands Ceccatelli et al. [12] found some of the nerves around blood vessels and parenchyma contained NOS immunoreactivity. Similar distributions have been found in pig [42] and cat [43] submandibular glands. Recent work on the major glands from rat and ferret [44] shows that NOS-immunoreactive nerves encircle acini and arteries of various sizes, but there were marked differences

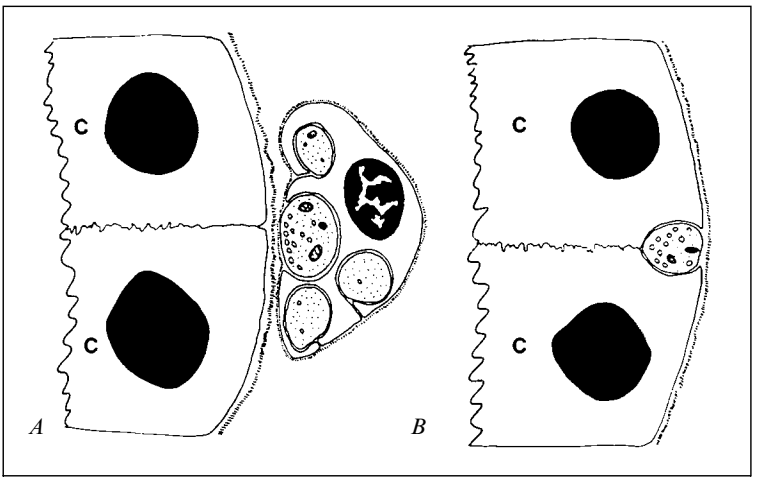

Fig. 2. Diagram of neuro-effector relationships in salivary glands. *A* Epilemmal, *B* Hypolemmal. For details see text. C = Parenchymal cells.

between the two species. In rats the innervation of acinar cells was more abundant, but in the ferret the vascular innervation by NOS-containing nerves was greater. Furthermore, in the ferret the collecting ducts were supplied by NOS-containing nerves but not in the rat. Denervation studies on the parotid glands of both species and the use of capsaicin (a sensory neurotoxin) showed that the NOS-containing nerves were predominantly, if not exclusively, parasympathetic efferent nerves.

Electron-Microscopic Observations

The ultrastructural features of neuro-effector relationships in salivary glands have been reviewed a number of times over the years [45–47]. Although the efferent autonomic nerves pass separately to the glands from their respective ganglia, travelling in Schwann axon bundles present in the post-ganglionic parasympathetic and sympathetic nerve trunks, once in the glands the non-myelinated axons from each type of ganglia intermingle and travel together in association with intraglandular Schwann cells. The ramifications of the numerous Schwann cells create a kind of scaffolding for the transit of axons in the interstices of the glands, along which they reach their final destinations. From the pioneering studies of Scott and Pease [9] in 1959 two types of potential neuro-effector sites have been known to exist in the glands and, using a classification devised by Arnstein [48] in 1895, they were subsequently designated [49]: (1) Epilemmal (fig. 2A), where a non-myelinated axon, still associated with its Schwann cell and contained within its basement membrane,

has a free surface closely adjacent to a glandular cell and contains vesicles, but remains outside the parenchymal basement membrane, with a gap between the axon and the glandular cell of about 100 nm. (2) Hypolemmal (fig. 2B), where a bare axon has penetrated the parenchymal basement membrane, contains vesicles and is in intimate association with adjacent glandular cells, separated by a gap of 20 nm or less.

No morphological evidence has been observed for post-synaptic specialization on the effector cells in salivary glands at potential neuro-effector sites. However, it would be of great interest to learn if there is a special localization of neuro-receptors on the effector cells at potential innervation sites, or whether they are randomly distributed around baso-lateral parts of the cells. It should be appreciated that epilemmal axons are likely to form a number of en passant neuro-effector sites along their length. This may also occur with hypolemmal axons after penetrating the parenchymal basement membrane, if they course alongside more than one cell before terminating and possess vesicle-releasing sites near each cell. It is also possible that some hypolemmal axons may have had epilemmal associations with effector cells at more central parts along their course. These things cannot be told from static thin sections and would require very detailed serial sectioning. Convergence of different axons on the same effector cell is not an uncommon finding (fig. 3).

From a simplistic point of view it seems likely that the intimate hypolemmal relationship will create a more efficient neuro-effector responsiveness than an epilemmal one, where the transmitters have to pass through 2 layers of basement membrane (that of the attendant Schwann cell and that of the parenchyma) before reaching the appropriate cellular receptors. But this has not been tested. Electrophysiological changes in individual salivary cells with different types of innervation, in response to single nerve impulses, may be helpful for evaluating this. Conversely, the responsiveness of different cells may not be the same for similar quanta of locally released transmitters. For instance, it has been found experimentally that myoepithelial cells are more responsive to low frequency stimulation than parenchymal cells [51]. So the possibility exists that some cells may require a hypolemmal relationship in order to achieve what other cells can do with an epilemmal association, and it would be most interesting to learn about the factors during development that cause some nerves to penetrate parenchymal basement membrane whereas others do not.

The misused term varicose (meaning beaded) was already being used to describe the pattern of silver staining by Arnstein [48] in 1895. It has continued in use ever since and now is given religious devotion as representing the sites from which neurotransmitters are actually released. However, electron microscopy shows that not all the widest parts of axons necessarily contain

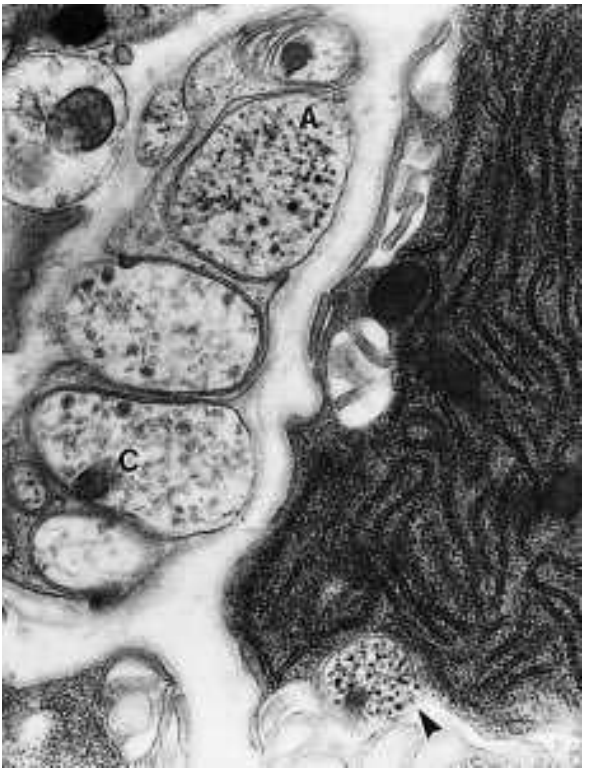

Fig. 3. Normal rat parotid gland after 5-hydroxydopamine treatment. Electron micrograph showing adrenergic (A) and cholinergic (C) axons in epilemmal association with an acinar cell and another adrenergic axon (▲) in hypolemmal association with the same cell. × 23,000. Reproduced from Garrett [50].

the greatest number of synaptic vesicles and, as seen in figure 3, hypolemmal axons may be much narrower than some epilemmal ones. Furthermore, using light microscopy, where the greatest reverence to varicosities is given, one seldom sees single axons in epilemmal situations, but aggregates of axons associated with the same Schwann cell. Static morphology also tends to create immutable concepts, so the possibility is not considered that both beaded sites and collections of synaptic vesicles may be moving objects and not necessarily at the same pace. If such mobility does actually occur, it would suggest that the sites of neurotransmitter release may not be static; in which case, neuro-effector efficiency would be facilitated if the neuro-receptors are widely dispersed on the salivary cells rather than just being in specific positions. The possibility that positional changes in the nerve-cell relationships can occur

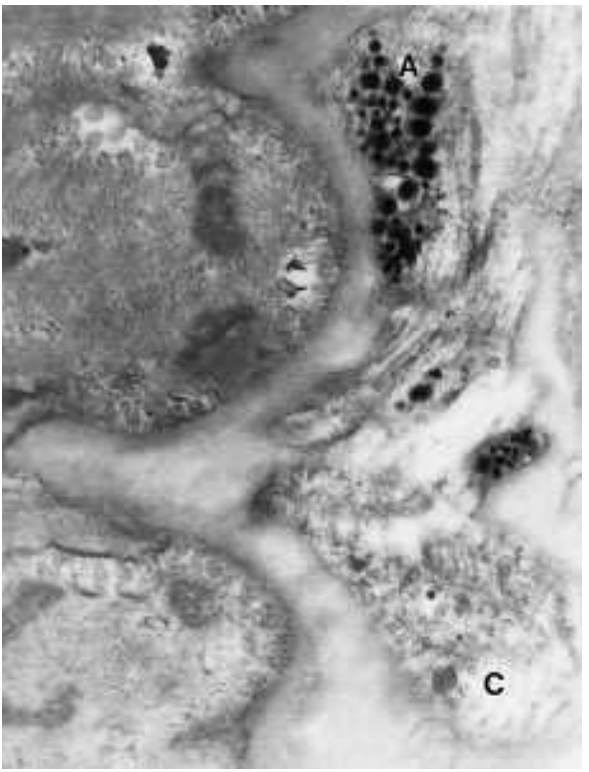

Fig. 4. Normal cat submandibular gland after 5-hydroxydopamine treatment. Electron micrograph showing an adrenergic (A) and a cholinergic (C) axon in epilemmal association with outer smooth muscle cells of an arteriole. ×35,000. Reproduced from Garrett [54].

has been observed after prolonged nerve stimulation [52, fig. 6] and after duct ligation [53].

The axons at potential neuro-effector sites contain a mixture of synaptic vesicles and occasional mitochondria. The majority of vesicles are small, with a diameter of 40–60 nm. After special staining, these vesicles remain clear and agranular in cholinergic parasympathetic efferent nerves but in adrenergic sympathetic nerves the vesicles show a dark granular content (fig. 3, 4). It is generally accepted that these small vesicles contain the conventional transmitters, acetylcholine in cholinergic efferent parasympathetic nerves and noradrenaline in adrenergic efferent sympathetic nerves. In addition to the small vesicles, variable numbers of large dense-cored granular vesicles, often with a clear outer halo and diameters of 80–120 nm, are usually also seen in axons

at potential neuro-effector sites. After 5-hydroxydopamine these larger vesicles become much darker in adrenergic axons but remain of intermediate electron density in cholinergic axons. The large vesicles are usually present at a greater distance from the axonal plasmalemma than many of the smaller vesicles. Since Johansson and Lundberg [55] showed that VIP is localized in the large dense-cored vesicles of cholinergic axons in salivary glands, but not in the small vesicles, it has been generally accepted that the large vesicles contain the various neuropeptide, non-adrenergic non-cholinergic, transmitters that can also occur in axons. Subsequently, Al-Hadithi et al. [56] demonstrated that substance-P and VIP immunoreactivities can co-exist in the same large dense-cored granular vesicles in cholinergic-type axons at potential neuro-effector sites in rat parotid glands. So the possibility that various non-conventional transmitters may co-exist to variable extents in the same large dense-cored vesicles should always be kept in mind.

The distribution of either type of neuro-effector relationship (epilemmal and/or hypolemmal) varies between glands and species [45–47]. Hypolemmal axons are frequent in rat parotid and cat submandibular glands but, so far, only epilemmal relationships have been found in rat submandibular and cat parotid glands. One has to be cautious, however, about saying that hypolemmal axons do not exist in a gland. Two early papers [16, 18] on human submandibular and parotid glands found no hypolemmal axons in the glands, despite Tandler [57] previously finding them occasionally present. Subsequent, more assiduous studies have shown that some hypolemmal axons can be found between acinar, myoepithelial, intercalary and striated ductal cells in human submandibular glands [47]. So the last word about the existence or absence of hypolemmal axons in a salivary gland may not necessarily have been said. In mouse parotid glands hypolemmal axons were found to be abundant [58], but they were not observed within mice submandibular acini and were only occasionally found within intercalated, granular and striated submandibular ducts.

Vascular innervation sites in salivary glands have only been found in an epilemmal relationship and only adjacent to the outer muscle cells. The gap between an axon and the smooth muscle cell tends to be greater than with parenchymal cells and is usually of the order of 200 nm or greater (fig. 4). Both adrenergic and cholinergic axons are found in this situation, and they are often adjacent in association with the same Schwann cell (fig. 4). Axons are also seen in close proximity to capillaries, to plasma cells and mast cells in salivary glands, but any roles that such nerves may have at these sites are not yet known.

Afferent nerves have been detected adjacent to larger blood vessels and ducts using neuropeptide immunohistochemical studies (see previously), but they show no distinguishing features with conventional electron microscopy. Afferent

sensory nerves have been identified immunocytochemically between cells of the main ducts in rat salivary glands, particularly near their oral ends [59].

Ganglionic Connections with Efferent Salivary Nerves

It has been known for a very long time that parasympathetic ganglia tend to be more peripherally placed than sympathetic ganglia. Parasympathetic ganglion cells for the submandibular and sublingual glands occur in association with the chorda as it runs along the glandular ducts and also within the interstices of the submandibular gland itself. For the parotid gland the neurones are present in the otic ganglion but not within the gland. The sympathetic ganglion cells for salivary glands occur in the superior cervical ganglion. Ingenious experiments by Langley [5, 60], using nerve stimulations at different sites and nicotine to block ganglia, showed that the numbers and distributions of ganglia along the chorda nerve, between its emergence from the lingual nerve to its entry into the submandibular gland, vary amongst species [5, 60]. There were more extraglandular ganglia in the rabbit chorda than in the cat and more in the latter than in dogs. These observations were backed up by histological assessment. In a similar way, Langley also showed that all of the sympathetic ganglion cells for the submandibular gland reside in the ipsilateral superior cervical ganglion, and this is supported by post-ganglionic denervation studies and the assessment of adrenergic nerves in submandibular glands. However, in parotid glands of dogs [61] and rats [62], some adrenergic nerves persist after excision of the superior cervical ganglion on that side. In rats some of these parotid sympathetic fibres were found to arise from the contralateral superior cervical ganglion [63], so presumably they reached there by travelling along intracranial vessels. Even so bilateral cervical ganglionectomy does not remove all adrenergic nerves from rat parotid glands [64], so the possibility exists that some sympathetic ganglion cells for this gland reside outside the superior cervical ganglia. Retrograde tracer studies from submandibular glands showed that in young mice some of the sympathetic innervation to the gland originated from neurones in the contralateral superior cervical ganglion, but their numbers decreased with age [65].

Neurohistochemical studies, including the retrograde tracing of nerve markers from glandular nerves to their ganglion cells and the assessment of potential transmitters in these cells, have been invaluable for studying the designation of ganglion cells. As with the peripheral nerves, the neuropeptide profiles differ between species and collectively the information is confusing. Also it has to be appreciated that a chemical found in a cell body may not necessarily be expressed in its axons at effector sites. Furthermore, fetal gan-

glion cells often show a greater pluripotentiality of genetic expression than in the adult and it is thought that the end organ plays some reciprocal part in refining the final genetic expression in the ganglion cells that are supplying it. In rats, submandibular parasympathetic ganglion cells have been shown to express DBH-immunoreactivity [66], as was also found in otic ganglion cells [67], but they do not form noradrenaline. Furthermore, some TH-immunoreactive cells were found within ciliary ganglion cells but not in the otic ganglion [67] and these cells also do not form noradrenaline. It was concluded that the presence of DBH and TH in parasympathetic neurons may be remnants of a noradrenaline expression during ontogenesis that is later suppressed.

Retrograde nerve marker studies by Gonatas et al. [68] indicated that 46–54% of neurones in the ipsilateral superior cervical ganglion were labelled after submandibular gland injection in rats and the marked cells were widely distributed throughout the ganglion. In contrast, Luebke and Wright [69], in similar more recent studies, found the submandibular projecting neurones were widely dispersed but calculated that they constituted only 7% of all neurones present. They also observed that these neurones were larger (ca. 34 μm diameter) than other neurones and considered that the target organ may be influencing their size. However, in mice Lahtiverta et al. [65] also found that about 45% of the neurones in the superior cervical ganglion innervate the submandibular gland on the same side and they formed a sub-population of large neurones (ca. 25 μm diameter). Previously they made the interesting observation that removal of the submandibular gland in mice led to a decrease in the density and size of sympathetic noradrenergic neurones assessed by catecholamine fluorescence [70]. This provides a strong indication of the reciprocal influence that an end organ can have on the neurons innervating it, presumably by means of humoral transfer of trophic factor(s).

Submandibular ganglion cells have been found to contain strong uniform AChE staining in rat [8 (fig. 2) and 35], cat [14, 71] and man [16]. The neurones in rat submandibular ganglia showed immunoreactivity for VIP [19] and MEAGL [36]; sub-populations demonstrated immunoreactivity for NPY [72] and ENK [35], but none showed staining for CGRP [73]. SP immunoreactivity was found in fetal rat submandibular ganglion cells, but after gestation its detectability decreased and few cells expressed such activity in the adult [74]. In cat submandibular ganglia immunoreactivity has been detected for VIP [19, 20, 23] and for PHI in the ganglion cells, for GAL and TH in some cells, but none showed reactivity for CGRP or SP [71]. Immunoreactivity for PACAP has been found in ferret submandibular ganglion cells [37]. In guinea pigs most submandibular ganglion cells showed immunoreactivity to VIP, NPY, ENK and Substance-P [75]. Submandibular ganglion cells in pigs were immunoreactive to VIP, PHI and NPY [42].

Otic ganglia have been less often studied but showed immunoreactivity to substance P in a proportion of neurones retrogradely labelled from the parotid in the rat [29]. Immunoreactivities for VIP and NPY were found in neurones in rat otic ganglia and are thought to co-exist [25] and for VIP, NYP, ENK and substance P in most neurones in the guinea pig otic ganglion [75].

The superior cervical ganglion contains a heterogeneous population of cells going to different target tissues, so it is important to identify neuronal reactivities in those cells identified retrogradely to subserve the glands. In this way the neuropeptide content of neurones projecting to the submandibular gland in rats were found to be heterogeneous with respect to 3 neuropeptides [76], with 57% of these cells claimed to be immunoreactive to VIP, 54% to somatostatin and 24% to NPY. A fascinating recent study by Grkovic and Anderson [77] showed that a percentage of preganglionic nerves to the superior cervical ganglion in rats contained calretinin-immunoreactivity (function unknown), and retrograde marker studies from various target organs showed that these nerves formed arborizations only around the perikarya of submandibular directed neurones. Furthermore these contacts occurred only with those ganglion cells that lacked NPY immunoreactivity and not with submandibular directed neurones that contained it. Of the neurones retrogradely labelled from the submandibular gland, 91% were surrounded by baskets of calretinin-immunoreactive nerve terminals. Only 5% of the retrogradely labelled neurones showed NPY-immunoreactivity, they were consistently smaller than the other submandibular directed neurones and were not surrounded by calretinin immunoreactive fibres. This indicates that there are at least 2 distinct and separate sympathetic nerve populations to the submandibular gland, as will be discussed later.

Numerous studies have now investigated the immunoreactivity of the various ganglia for NOS and showed that most parasympathetic neurones for salivary glands were stained in rats [12, 78, 79a], and all were stained in cats [43] and pigs [42]. In superior cervical ganglia of rats many pre-ganglionic nerves showed NOS-immunoreactivity, but the sympathetic ganglion cells were all negative. Furthermore, the large neurones projecting to the submandibular gland were found to the surrounded by NOS-immunoreactive preganglionic sympathetic nerves whereas the small NPY-containing neurones projecting to the blood vessels were not [79b].

Special Consideration of Vascular Nerves in Salivary Glands

A number of years ago Emmelin and Engström [80] provided physiological evidence that sympathetic vasoconstrictor activity in cat submandibular glands is dissociated from the secretomotor activity. Their findings suggested that

sympathetic vasoconstriction of glandular blood vessels is under central vasomotor control, whereas the secretomotor activity is under salivary centre control, so two separate sets of sympathetic efferent nerves must exist for these purposes. Further physiological support comes from recent electrophysiological studies on sympathetic neurones projecting to submandibular glands in rats [81]. Only 5–10% of these neurones were found to be spontaneously active under anaesthesia and were excited reflexly by nociceptive stimuli, inhibited by baroceptor stimuli but none of them responded to gustatory stimuli. Thus, they behaved like vasoconstrictor neurones and appeared continuously to exercise a tonic effect. The remaining 95% of the submandibular directed neurones, presumably secretomotor, were silent during anaesthesia.

Morphologically, Lundberg et al. [82] observed that in submandibular glands of cats the sympathetic nerves to the blood vessels contained noradrenaline and APP (avian pancreatic polypeptide), whereas those associated with the parenchyma did not contain APP. Similarly they found that the noradrenergic sympathetic nerves innervating cat submandibular blood vessels also contained NPY, but NPY was not found in sympathetic nerves associated with the parenchyma [83], so the concept of separate populations of sympathetic efferent nerves to glandular blood vessels from the parenchymal nerves was supported. Separate organizational control for these two populations of sympathetic nerves is indicated by the recent finding of Grkovic and Anderson [77] that 95% of retrogradely labelled neurones serving submandibular glands were large, did not contain NPY and were associated with pre-ganglionic nerves containing calretinin immunoreactivity; but the remaining 5% of submandibular directed neurones were smaller, contained NPY and were not associated with calretinin immunoreactive preganglionic nerves.

Electrophysiological studies on salivary centre neurones and pre-ganglionic parasympathetic nerves in rats suggested that the firing patterns underlying secretomotor nerves are also different from those relating to the blood vessels [84]. Speculatively, therefore, the parasympathetic vascular nerves may be in a separate population from the parenchymal nerves, nevertheless they all came under salivary centre control.

Surgical nerve section indicated that the domain of the sympathetic vascular nerves in the adventitia around the main artery to the submandibular gland in cats extends for only a relatively short distance (1–2 mm at most) [85]. Neuropeptide profiles of vascular nerves in guinea pig salivary glands showed that the neuropeptide content of the nerves supplying the larger more proximal glandular vessels differed from those in more distal arterioles [86]. This was found with both sympathetic and parasympathetic nerves. It was considered that the post-ganglionic neurones, having similar different profiles, represented different populations for larger than smaller glandular vessels.

This work therefore supports the idea that the domain of vascular nerves is relatively short within the glands as well as outside them, and suggests the different types of vessel may have different neuropeptide requirements.

Development of Salivary Gland Innervation

This important subject has not yet received as much attention as it should have, but interesting pockets of information exist. Coughlin [87, 88], using in vivo and in vitro studies on mice, found that the growth of AChE-positive nerves from parasympathetic ganglia to submandibular glands occurred with the onset of development of the salivary epithelium and depended upon stimulation from the developing target tissue. On the other hand, adrenergic nerves in submandibular glands of rats were not found to express catecholamine until about 5 days post-partum [89], and this coincided with the first indication of a functional sympathetic response, but a parasympathetic response had been found at birth. Reciprocal influences of nerves on the proper development and maintenance of normal salivary parenchyma are accepted for parasympathetic nerves (see chapter 7) but are less evident for sympathetic nerves in the adult. However, neonatal sympathectomy was found to impair the development of parotid acinar cells in rats [90].

There are conflicting reports about neuropeptide-containing nerves during development of salivary glands in rats. Ekström et al. [91], using both immuno -chemistry and -histochemistry, found the total amounts of VIP, SP and CGRP increased in surges in the glands during the first 8 weeks of life. Nerve fibres containing the peptides were present at birth, showing adult-like patterns, and increased in number during the first 4 weeks. However, Virta et al. [92] found nerve fibres immunoreactive for SP and NKA first appeared on the 19th day i.u., the glands were richly innervated up to 16th postnatal day, but the numbers decreased thereafter to adult levels and similar findings were made for CGRP [93].

Hypolemmal axons were found between epithelial cells in developing mouse submandibular acini, but such axons were lost from mature glands [94]. Similarly, hypolemmal axons in cat submandibular acini were less in adults [95], indicating a tendency for the more intimate type of innervation to decrease with age.

Fascinating studies by Landis and co-workers [96] showed that sympathetic nerves to sweat glands in rat footpads normally change their genetic expression during maturation and develop a cholinergic phenotype. Transplantation experiments demonstrated that if these nerves were re-directed to parotid tissues during development this change did not occur and the nerves continued to show strong catecholamine fluorescence. The authors concluded that ‘cellular interactions

between neurons and their targets play an important role in the differentiation of mature neurotransmitter and neuropeptide phenotypes in vivo'.

An interesting developmental distinction occurs between the sympathetic adrenergic innervation of rat submandibular and sublingual glands. The nerves travel together in the same bundles along the main artery and must have migrated together during development. Nevertheless, in the adult there is abundant adrenergic innervation in the submandibular gland but it is sparse in the sublingual gland [10]. Does this mean that initially their innervations were similar but development of sublingual tissue caused a cut back? Or are there other explanations? Furthermore, those sympathetic adrenergic nerves that do exist in association with the sublingual parenchyma appear to be specially directed to the striated ducts [17] and they maintain a functional role in the secretion of secretory proteins from these ducts. Thus, sublingual striated ducts appear to exert an attracting infuence on sympathetic adrenergic nerves encouraging them either to migrate there, or to stay there if the other parenchymal cells are inducing a cut back.

Another interesting developmental query arises about what cellular factors encourage some axons to penetrate the parenchymal basal lamina and take up a hypomemmal position, whereas in other situations such movement is resisted?

Concerning Neurotransmitters and Their Release

It is easy to consider that all axons of one type (sympathetic or parasympathetic) in a gland will contain similar amounts of transmitters, but is this so? Adrenergic trunks normally show less catecholamine fluorescence than the terminal axons and those around glandular blood vessels are often more conspicuous than those associated with the parenchyma and tend to show more dense-cored vesicles by electron microscopy (fig. 4), which suggests that they may contain proportionately more of the transmitters. Immunohistochemical studies indicate that not all nerves of each type in a gland contain the same neuropeptides in the same proportions. Gross chemical assessments of conventional transmitters in salivary glands from mice and rats [97] found that the concentration of noradrenaline was greatest in submandibular glands, less in the parotid and least in sublingual glands. This fits in with the visual impressions from catecholamine fluorescence. The initial difference may relate to the fact that many of the axons in the parotid are in separate intimate hypolemmal association with acinar cells, whereas in submandibular glands they run together and the transmitters have to pass through two layers of basal lamina to activate the cells. Acetylcholine concentrations [97] were high in submandibular and sublingual glands but surprisingly much lower in parotid

glands, despite the fact that parasympathetic nerves are plentiful there and can induce a copious atropine sensitive flow of saliva in rats. It is, therefore, possible that individual axons in the parotid glands may contain less acetylcholine, but its release on impulse formation is highly effective because of the close hypolemmal arrangement with acinar cells.

It has to be appreciated that neurotransmitter release does not occur from all potential neuroeffector sites along an axon with each impulse. Refined studies by Stjärne and Stjärne [98] have revealed that, in selected adrenergic nerves, release of transmitter on each impulse occurs from very few potential releasing sites on each axon. With trains of impulses the releases occur at different sites with each impulse, in a non-uniform manner. Actions of transmitters and the like on inhibitory and facilitatory prejunctional receptors along axons are considered to influence these events [99]. Cholinergic axons are likely to behave in a similar manner. Whereas released acetylcholine is removed by cholinesterase activity, there is uptake of noradrenaline back into adrenergic axons and support for this has been found in rat salivary glands [100, 101]. The latter also showed that on prolonged preganglionic sympathetic stimulation there was an overall reduction of noradrenaline in submandibular/sublingual glands. It was concluded that the vesicles in the terminals can release much of their noradrenaline and during rest the retrieved vesicles can restore noradrenaline by resynthesis, in addition to any reuptake. Similar resynthesis is generally considered to occur in retrieved cholinergic synaptic vesicles. Any migration of small vesicles down the axons is thought to play only a small part, if any, to the presence of small vesicles at the neuro-effector sites. However, migration of the large vesicles from the Golgi apparatus of neurones is essential for delivery of neuropeptides and their other components to the terminals and, once released from the axons, they cannot be resynthesised locally but have to be replenished by axonal flow. Using adrenergic nerves containing numerous large dense-cored vesicles, it was confirmed morphologically that they can undergo exocytosis under suitable conditions of stimulation [102]. Similarly, parasympathetic nerves in rat parotid glands showed a significant depletion of large dense-cored vesicles from hypolemmal neuro-effector sites after prolonged post-ganglionic stimulation at 40 Hz [103] without any obvious change to the small agranular vesicles. The reduction corresponded, in time and magnitude, to the depletion of SP and VIP from the glands under identical conditions [104]. Remaining large dense-cored granules were considered [103] to represent a mixture of those not yet exocytosed and those that had arrived by axonal transport.

Impulse rates affect the amounts of transmitters released. Whereas some release of the conventional transmitters acetylcholine and noradrenaline is likely to occur with every propagated nerve impulse, higher frequencies are

associated with detectable release of neuropeptides [20, 21] and this has been found to be optimized when the impulses are applied in bursts [22]. A greater release of noradrenaline also occurred from adrenergic vascular nerves when the same number of impulses were administered in bursts at higher frequencies [105].

Over recent years a considerable amount of information has accrued about the complex molecular processes involved in priming and docking of axonal vesicles and the exocytosis of their transmitters [99, 106]. The mechanisms differ to some extent for small and large vesicles, that contain the fast and slow transmitters respectively and correspondingly are released rapidly or more slowly [106]. The processes for both are triggered by the presence of ionized calcium but the requirement for Ca^{2+} is greater for small vesicles than for large vesicles. This appears to be influenced by the closer proximity of the small vesicles to the calcium channel but the large vesicles depend on its diffusion. Furthermore release of transmitters from large dense-core vesicles is influenced by a greater need to overcome the actin restraining network [106, 107], starting as they do at a greater distance from the axonal membrane. Thus, many factors influence the differential release of neuropeptides and conventional transmitters.

Concluding Remarks

Study of nerves within salivary glands shows they have diverse characteristics that differ between glands and species, not only in their arrangements but also in the ranges of potential transmitters present and in the manner of their release. It is likely, therefore, that nerve-mediated glandular responses in vivo are considerably more complex than is usually contemplated from studies with single agonists or antagonists and this will be considered further in ensuing chapters.

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Chapter 2

Central Connections for Salivary Innervations and Efferent Impulse Formation

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Introduction

Salivary glands are unique exocrine glands of the digestive tract, whose secretory activity is solely and entirely controlled by the sympathetic and parasympathetic autonomic nervous systems. There is no hormone which normally initiates salivary secretion. Ever since Bernard [1] observed salivary secretion from the dog submandibular and parotid glands when he punctured the floor of the fourth ventricle in 1856, the location of the primary centre and its central connections for the major salivary glands (especially the parotid and submandibular glands) have been explored by histological and physiological methods. Little is known about the connections for the minor salivary glands.

The Primary Centre of Salivary Secretion

Regarding the primary parasympathetic centre of salivary secretion control, Grützner [2] was the first in 1873 to apply electrical stimulation to the dog medulla and produce a copious flow of saliva. The salivation secreted from the submandibular gland was diminished by a transection of the chorda tympani nerve and abolished by an additional transection of the cervical sympathetic trunk. This finding suggested that the chorda tympani contains the efferent fibres from the parasympathetic salivary centre to the submandibular gland, although the electrical stimulation conducted through a pair of needles may have been strong enough to activate the fibres of passage and/or taste relay neurones (the solitary nucleus) which innervate the preganglionic sympathetic

neurones (the intermediolateral nucleus) in the upper thoracic spinal cord (fig. 1). In 1898, Beck [3] utilized a method of serial transverse sections of the brainstem for determining the location of the parasympathetic salivary centre, and found that reflex secretion from the submandibular gland of curarized dogs could be obtained as long as the brainstem rostral to the facial nucleus remained (fig. 1). In the 1900s, chromatolytic changes in the brainstem were explored after sectioning the parasympathetic preganglionic fibres innervating the salivary glands of dogs [4–8]. This histological examination revealed the common finding that the rostral part of the parasympathetic centre (the superior salivatory nucleus) connects with the submandibular and sublingual glands, and the caudal part (the inferior salivatory nucleus) connects with the parotid gland. In 1913, Miller [9] stimulated the dorsal surface of the cat medulla (the floor of the fourth ventricle) with a unipolar electrode, and located two points with a minimal electrical intensity; the electrical stimulation of one caused parotid secretion, and that of the other (the more rostral point) caused only submandibular secretion. Interestingly, the latter point is in accord with the dorsomedial part of the medulla (immediately below the surface) where the bundles of the descending fibres from the superior salivatory nucleus run, making a loop (or a genu; indicated by an arrowhead in fig. 1) [10]. A genu is not identified for the inferior salivatory nucleus. In the 1940s, the brainstems of cats [11, 12] and monkeys [13] were stimulated electrically with a small bipolar electrode which was introduced into the brainstem with the aid of a stereotaxic apparatus. The salivary points for submandibular or parotid secretion, however, were not localized only within the salivatory nuclei; many of them were also distributed along the course of efferent fibres from the salivatory nuclei, or in the solitary nucleus and spinal trigeminal nucleus, which are the relays of taste afferents and of somatosensory afferents from the face and mouth, respectively (fig. 1). Since the end of the 1970s, the method of tracing the retrograde axonal transport of horseradish peroxidase (HRP) has been used extensively in localizing the salivatory nuclei of various animals including rats [14–17], rabbits [18, 19], hamsters [20], cats [10, 21, 22], dogs [23] and monkeys [24, 25]. In these experiments, the tracer HRP was injected into the peripheral course of the parasympathetic preganglionic fibres or into their terminals, i.e. the submandibular ganglion and otic ganglion for the submandibular and sublingual glands and parotid gland, respectively. The HRP studies confirmed the results of the early histological studies utilizing the neuronal degeneration method. Generally, the superior and inferior salivatory nuclei consist of parvocellular cells sparsely scattered in the lateral reticular formation of the medulla at the level of the facial nucleus. The superior subdivision is situated rostral to the inferior subdivision, but there is no anatomical boundary. In the human brainstem, the inferior salivatory nucleus

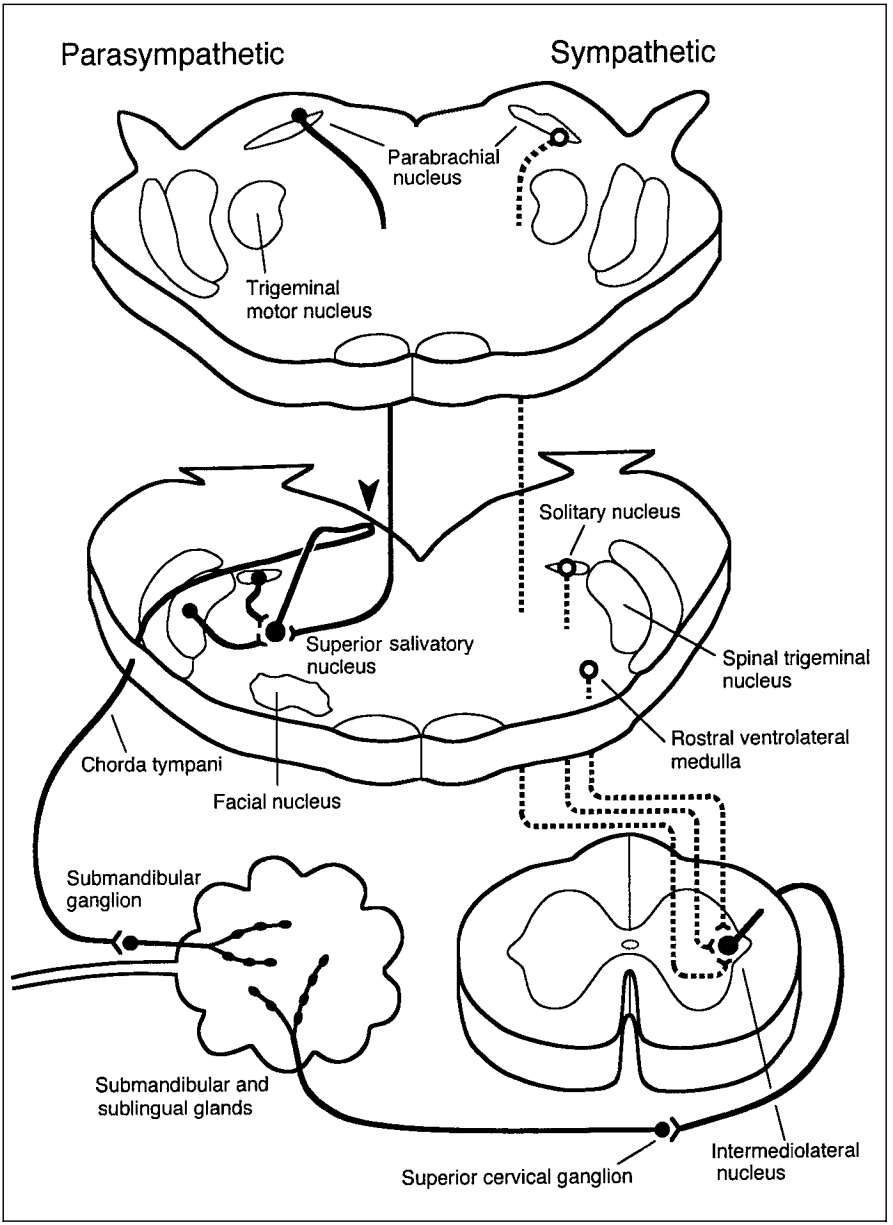

Fig. 1. Schematic diagram of the nervous system control of submandibular salivary secretion, in the brainstem and spinal cord. Parasympathetic and sympathetic connections are shown in the left and right side of the brain, respectively. The upper, middle, and lower right panels illustrate brains near the level of the parabrachial nucleus, superior salivatory nucleus, and upper thoracic spinal cord (sympathetic salivary center), respectively. The solid

is thought to be located medial to the rostral portion of the solitary nucleus; the location of the superior salivatory nucleus has not been identified. This result is based on original evidence from retrograde neuronal degeneration in a patient who died with injuries to the cranial nerves of the glossopharyngeal, vagus, accessory, and hypoglossal nerves [26]; the glossopharyngeal nerve contains efferent fibres from the inferior salivatory nucleus. Recent immunohistochemical studies in humans demonstrated nitric oxide synthase [27] but not acetylcholinesterase [28] in neurones medial to the rostral solitary nucleus.

As for the sympathetic salivary centre, Langley [29] found in 1892 that a secretory response of the cat and dog submandibular glands could be elicited by electrical stimulation of the upper thoracic nerves (from the first to fifth nerve), exclusively by stimulation of the second thoracic nerve. On the basis of this finding, it is known that the sympathetic preganglionic neurones in the upper thoracic segments of the spinal cord connect with the salivary glands (fig. 1). However, the precise location of the sympathetic salivary centre has not yet been identified histologically, partly because its peripheral ganglion (the superior cervical ganglion) consists of the postganglionic neurones innervating various peripheral targets such as the lacrimal gland, skin of the head, pupils, and salivary glands. Nevertheless, recent immunohistochemical studies suggest that the pre- and postganglionic neurones may be coded with particular neuropeptides depending on their target organs or functions. The rat postganglionic neurones projecting to the submandibular gland lack immunoreactivity to neuropeptide Y, and are exclusively surrounded by calretinin-immunoreactive terminals [30]. This histochemical characteristic was not seen in the neurones innervating the lacrimal gland, thyroid gland, eye, and skin of the head [30]. Thus, this finding suggests that a group of calretinin-immunoreactive preganglionic neurones may be the sympathetic salivary centre.

Central Connections for Salivary Innervations

A method using the transneuronal transport of a virus has recently been utilized for determining the central neurones projecting to the superior salivatory nucleus. Jansen et al. [31] injected pseudorabies virus into the submandibular gland of sympathetically ganglionectomized rats, and labeled the central neurones which would be the second, third or higher order of the neurones. The subsequent labeling of the higher centres of the parasympathetic

lines connecting the panels represent axons documented; dashed lines, possible axons without histological confirmation. An arrowhead indicates a small genu of descending fibres from the superior salivatory nucleus. Produced from data from rats [15, 31, 41, 43] and cats [10].

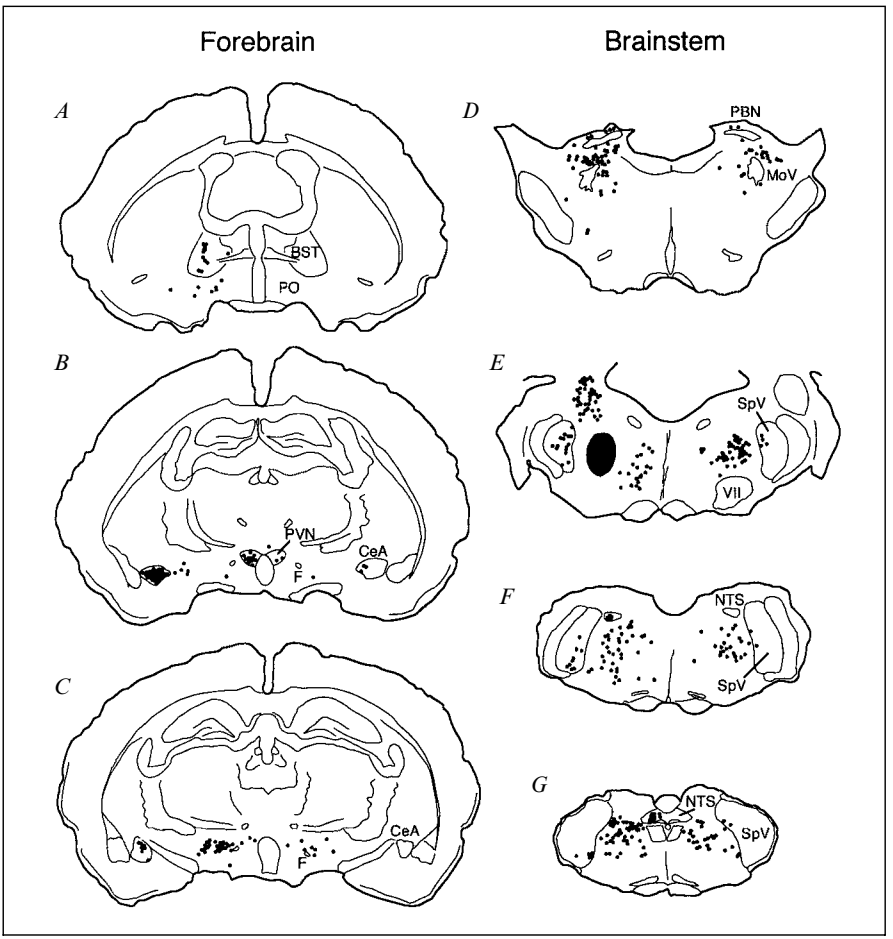

Fig. 2. Distribution of retrogradely labeled neurones (solid dots) after a horseradish peroxidase injection into the left superior salivatory nucleus in rats (dark area in *E*). The forebrain (left panels) and brainstem (right panels) sections are arranged in a rostral (*A*) to caudal (*G*) sequence. BST = Bed nucleus of the stria terminalis; CeA = central nucleus of the amygdala; F = fornix; MoV = motor trigeminal nucleus; NTS = solitary nucleus; PBN = parabrachial nucleus; PO = preoptic area; PVN = hypothalamic paraventricular nucleus; SpV = spinal trigeminal nucleus; VII = facial nucleus. Unpubl. observ.

nervous system is essentially similar to that obtained by the injection of HRP into the superior salivatory nucleus; the HRP method labels only the central neurones directly innervating this nucleus. Figure 2 shows the distribution of the neurones retrogradely labeled as a result of injecting HRP into the nucleus of rats (fig. 2E, dark area). At the forebrain level, the labeled cell groups

were seen mainly in the bed nucleus of the stria terminalis, hypothalamic paraventricular nucleus, central nucleus of the amygdala, and lateral hypothalamic area (especially lateral to the fornix) (fig. 2A–C). In the midbrain, a small number of labeled cells were found in the central grey matter and deep mesencephalic nucleus (not shown in the figure). In the lower brainstem (pons and medulla), the labeled cells were observed in the parabrachial nucleus, pontine and medullary reticular formation, spinal trigeminal nucleus, and solitary nucleus (fig. 2D–G). These labeled cells were distributed mainly on the ipsilateral side of the HRP injection site.

As shown in figure 2E, there were many labeled cells in the contralateral side in the same position of the injection site, i.e. the superior salivatory nucleus. This suggests that interneurones connecting both sides exist within the nucleus. If this is the case, it is not clear whether the HRP-labeled cell groups provide direct synaptic inputs so the superior salivatory neurones or indirect inputs via the interneurones. As for only the neurones in the rat lateral hypothalamic area [32] and the cat central nucleus of amygdala [33], it appears that their efferent fibres (which are anterogradely labeled with HRP or radioactive amino acids) form synaptic contacts with the superior salivatory neurones identified with an HRP injection into the chorda-lingual nerve (containing the efferent fibres from the superior salivatory nucleus).

Moreover, the HRP-labeled cell groups in figure 2 are responsible for not only salivary secretion but also for other autonomic functions. For example, anatomical studies in rats employing the transneural transport of viruses have found that the same cell groups provide inputs to the pterygopalatine parasympathetic preganglionic neurones [34], vagal preganglionic neurones [35], and pancreatic parasympathetic preganglionic neurones [36]. In view of the functional role of saliva, the HRP-labeled cell groups are implicated in regulating feeding and drinking behaviour. The lateral hypothalamic area is well recognized as the feeding centre. The amygdala relates to memory and taste preference (see chapter 10). The paraventricular nucleus participates in the regulation of body water balance and drinking behaviour. The solitary and parabrachial nuclei are the first and second order of relay neurones, respectively, of visceral and taste afferents (see chapter 10). The HRP-labeled cells in the pontine and medullary reticular formation may contain motor- and/or taste-related neurones such as the premotor neurones for the trigeminal and hypoglossal motor nuclei [37–39].

Central connections for the sympathetic nerves of salivary glands have not yet been explored. However, viral injections into the adrenal medulla [40] or into various sympathetic ganglia including the superior cervical ganglion [41] of rats result in the labeling of neurones in the rostral ventrolateral and ventromedial medulla, in addition to the paraventricular nucleus, lateral hypo-

thalamic area, and central grey matter. The rostral ventrolateral medulla is well recognized as playing an important role in the integration of cardiovascular and respiratory reflexes [42]. In addition to these cell groups infected by viruses, HRP studies in rats have shown that the solitary nucleus (mainly the caudal portion where visceral afferents input) and parabrachial nucleus (mainly the ventrolateral portion, electrical stimulation of which evokes blood pressure changes) each send a descending projection to the sympathetic preganglionic neurones in the spinal cord (fig. 1) [43].

The above-mentioned higher centres may send both excitatory and inhibitory projections to the primary salivary centres. Recent electron-microscopic immunohistochemical studies in rats [44, 45] have specified various kinds of neurotransmitters in the synaptic terminals contacting with the superior salivatory neurones; the salivatory neurones examined in these studies were retrogradely labeled from the pterygopalatine ganglion, which innervates the nasal and palatal mucosa, lacrimal glands, and cerebral blood vessels, but not the salivary glands. A majority of the synaptic terminals were immunoreactive to glutamate, γ-aminobutyric acid (GABA), and glycine; about 45, 21 and 20% of the total synaptic terminals, respectively [45]. A smaller number of terminals contain substance P, enkephalin, neuropeptide Y, somatostatin, vasoactive intestinal polypeptides, tyrosine hydroxylase, thyrotropin-releasing hormone, and serotonin [44]. This implies that a major part of synaptic inputs to the superior salivatory nucleus is comprised of a glutamatergic excitatory input and GABAergic and glycinergic inhibitory inputs. Similar excitatory and inhibitory synaptic inputs were also found in the rat sympathetic preganglionic neurones, including those that project to the superior cervical ganglion [46–48]. Among the higher centres for salivary secretion, the glutamate-immunoreactive neurones were densely distributed in the hypothalamic paraventricular nucleus, lateral hypothalamic area, spinal trigeminal nucleus, and medullary reticular formation, although they were detected in all of the higher centres for salivary secretion in rats [49–51]. GABA-immunoreactive neurones were demonstrated in the bed nucleus of the stria terminalis, central nucleus of amygdala, central grey matter, spinal trigeminal nucleus, solitary nucleus, and medullary reticular formation in rats [49, 52, 53]. Glycine is thought to coexist with GABA in neurones of the central nervous system [46, 48]. These histological findings suggest that salivary secretion is controlled by both excitatory and inhibitory neural mechanisms. However, the inhibitory effect of the higher centres on salivary secretion remains largely unexplored in animal experiments. To evaluate this effect, we should pay attention to the effect of anaesthesia; recent studies show that GABA receptors are the dominant target for various anaesthetic agents [54–56].

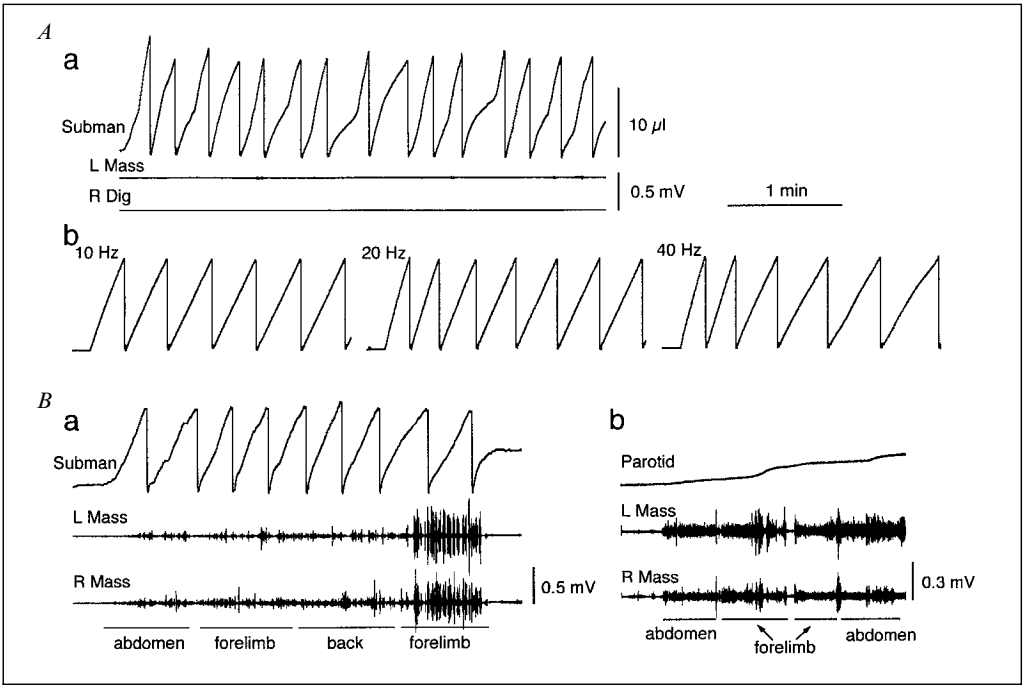

Fig. 3. Salivation during heat exposure and grooming in rats. *Aa* Vigorous submandibular salivary secretion (Subman) at an ambient temperature of 42 °C. The jaw muscle activities of the left masseter (L Mass) and right digastric muscles (R Dig) were very small. *Ab* The flow rate of saliva induced by heat exposure was similar to, and sometimes greater than, that produced by electrical stimulation of the chorda-lingual nerve, i.e. the preganglionic parasympathetic fibres, at frequencies of 10, 20 and 40 Hz. *Ba* submandibular salivary secretion during grooming various parts of the body. *Bb* A far smaller amount of saliva was secreted from the parotid gland. *A* Unpubl. observ. *B* Data from Matsuo et al. [61].

Control of Various Types of Salivation

It is well known that vigorous salivation occurs when an animal chews food, drinks taste solutions, grooms, or is exposed to heat. Analyses of these various types of salivation in behaving animals provide information regarding their neural mechanisms. The simplest salivation in terms of neural mechanisms may be seen during heat exposure. Many mammals that do not sweat or pant increase salivary secretion as the ambient temperature increases, and they sometimes groom and spread saliva on their fur for evaporative cooling [57]. The recording shown in figure 3Aa is the flow rate of submandibular saliva and jaw muscle activity obtained from a rat after 40 min of exposure

at about 42 ºC; the rat extended its body on the floor after grooming. Since no jaw muscle activity was recorded, sensory inputs from the oral region may be negligible. Thus, the salivation observed depended exclusively on the activity of the hypothalamic heat-loss centre, i.e. the preoptic and anterior hypothalamic area [58], without participation of the salivary reflexes evoked by oral sensory inputs. Concerning the neuroanatomy, only a few HRP-labeled cells were found in the preoptic and anterior hypothalamic area (fig. 2A). According to a behavioural experiment in rats [59], destruction of the lateral hypothalamic area impaired the heat-induced saliva. This suggests that the heat-loss centre may activate the salivatory nucleus via the lateral hypothalamic area.

Grooming behaviour in rats can be elicited at room temperature by local stimulation of the hypothalamus including the paraventricular nucleus and dorsal hypothalamic area [60]. During grooming, rats lick various parts of their body surface. Depending on the licking site, different magnitudes of taste afferent responses were recorded from the chorda tympani nerve, while essentially the same flow rate of submandibular saliva was observed regardless of the site of grooming [61] (fig. 3Ba). This suggests that activity of the hypothalamic grooming centre affects the grooming-induced salivation more so than do oral sensory inputs. Interestingly, the grooming-induced saliva was secreted mainly from the submandibular gland, but not from the parotid gland (fig. 3Bb). The submandibular saliva is also thought to be more important for thermoregulation than is the parotid saliva [62, 63]. It is likely that the hypothalamic heat-loss and grooming centres connect exclusively with the superior salivatory nucleus; however, the neuroanatomical pathway from these hypothalamic centres to the salivatory nucleus has not yet been fully elucidated.

Chewing and drinking are integrated behaviours consisting of jaw and tongue movements with associated autonomic responses, which are controlled by neural commands processed in both the lower brainstem and higher brain structures. As for salivation, in the lower brainstem, oral sensory inputs such as taste, thermal, and mechanical stimulations activate the sympathetic and parasympathetic salivary centres, via the nucleus of the solitary tract, parabrachial nucleus and spinal trigeminal nucleus (fig. 1). This salivary reflex is simultaneously facilitated by an efferent command from the higher brain, mainly the feeding centre (lateral hypothalamus). This efferent command may be formed or modulated by oral sensory inputs. Accordingly, unlike the relatively constant flow rate of saliva seen during grooming and heat exposure, the quantity and quality of this saliva depend on changes in oral sensory inputs associated with the chewing side, consistency of food, and quality of taste (see chapter 10).

Anatomical studies in rats [43] indicate that the lateral hypothalamus projects to the nucleus of the solitary tract, the parabrachial nucleus, and the

pontine and medullary reticular formation including the salivatory nuclei and premotor neurones of the jaw and tongue motoneurones. Electrical stimulation of the rat lateral hypothalamic area changes the rhythmical sequence of masticatory movements, by modulating the excitation level of trigeminal motoneurones [64]. This stimulation also enhances the taste-elicited responses of the neurones in the nucleus of the solitary tract [65], and of the efferent fibres of the superior salivatory nucleus [66]. These findings suggest that the hypothalamus modulates the activities of motor, sensory, and autonomic components in the lower brainstem.

Impulse Formation of the Salivary Autonomic Nerves

Activities of the sympathetic and parasympathetic nerves supplying the salivary glands have been recorded from anaesthetized animals, and the pattern and frequency of impulse discharges were analysed. These analyses have provided information regarding impulse formation in the lower brainstem. In a few studies, impulse discharges in response to electrical stimulation of the higher brain including the cerebral cortex and hypothalamus were examined with reference to the effects of taste and chewing on salivary secretion. Therefore, impulse formation in the higher brain is covered in chapter 10; we will concentrate here on that in the lower brainstem.

Parasympathetic Nerves

Due to technical difficulties in the approach to and identification of parasympathetic neurones, most researchers have recorded neural activity chiefly from the superior salivatory neurones, their axons (the parasympathetic preganglionic fibres), or the submandibular postganglionic neurones in rodents and cats. Carr [67, 68] recorded neural activity from the parasympathetic postganglionic fibres innervating the sheep parotid gland.

Impulse discharges in the superior salivatory nucleus reach the submandibular and sublingual salivary glands via the submandibular ganglion cells. The preganglionic neurones, i.e. the superior salivatory neurones, can produce impulses at maximal frequencies of up to 30–70 Hz in rats [69]. The ganglion cells of rats have an ability to transmit the preganglionic impulses at frequencies of up to 20–60 Hz [70]. These cells generally lack dendrites, and most of them are innervated by a single preganglionic axon in mice, hamsters, and rats [71, 72]; there are no interneurones or other sources of intrinsic synaptic input to these cells in rats [73]. Moreover, the submandibular ganglion cells of hamsters [74] and rats [72] showed the fast excitatory postsynaptic potential (EPSP) with a high safety factor for evoking spikes, in that a single fast EPSP

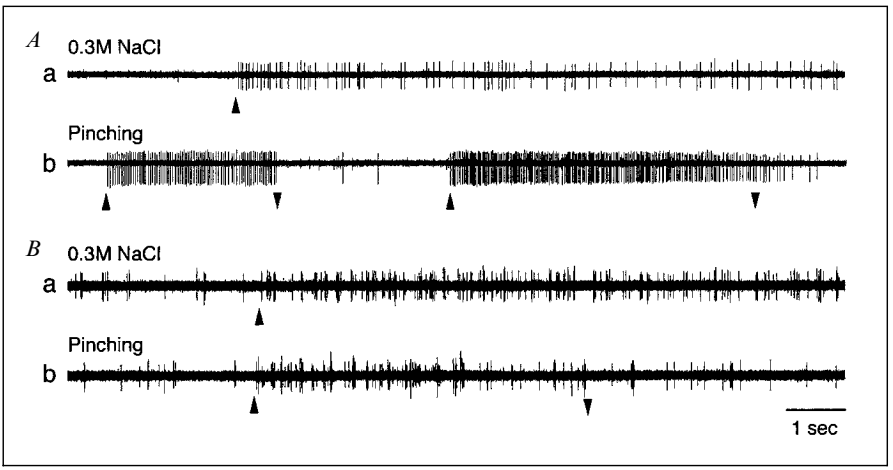

Fig. 4. Activities of the parasympathetic preganglionic (*A*) and sympathetic postganglionic (*B*) fibres innervating the submandibular and sublingual salivary glands in hamsters. *Aa* This fibre responded to taste stimuli (e.g. 0.3 *M* NaCl), but not to the pinching of the tip of the tongue with a pair of forceps. *Ab* This fibre responded mainly to the pinching of the tongue. *B* Multi-unit activity of the sympathetic fibres showed spontaneous discharges in burst, and increases of bursts during taste stimulation (*a* 0.3 *M* NaCl) and pinching of the tongue (*b*). Data from Matsuo and Yamamoto [78].

is able to evoke an action potential. These findings thus indicate that a single preganglionic action potential is immediately transmitted to the postganglionic neurone with a one-to-one relationship. However, there is a species difference in this transmission. For example, rabbit ganglionic cells are multiply innervated, receiving synapses from 3–8 separate preganglionic fibres [71]. It is conceivable that such a multiple innervation is an underlying mechanism for yielding impulse discharges in doublets or triplets, as observed in the postganglionic fibres innervating sheep parotid gland [67].

When the preganglionic neurones were reflexly activated by electrical stimulation applied to various parts of the oral region or the branches of the trigeminal sensory nerve, more than half of them responded to inputs from a confined area of the oral region in rabbits [75] and cats [76]. In addition to this regional dependency, the parasympathetic reflex activity also depends on the sensory modality in rats [66, 77], hamsters [78], and rabbits [79]. As shown in figure 4A, the hamster preganglionic fibres did not respond to light mechanical stimulation (e.g. tactile), but did respond mainly to either taste or noxious mechanical (e.g. heavy pressure or pinch) stimulation applied to the oral region. Both types of preganglionic fibres showed spontaneous discharges

at a low firing rate (about 0.2 Hz) and reflex responses in a tonic or phasic-tonic discharge pattern at relatively low frequencies (5–18 Hz, average value over 10–15 s) in rats [66, 77] and hamsters [78].

The above-mentioned reflex discharges are formed in the lower brainstem involving the sensory nuclei which relay the potent sialogogue inputs, i.e. taste and strong mechanical stimulation. These sensory relays of rats show tonic or phasic-tonic firing patterns at slightly higher impulse frequencies compared to the preganglionic neurones. The taste relay neurones in the solitary and parabrachial nuclei discharge at average frequencies of 5–40 Hz in response to various kinds of taste stimuli at moderate concentrations [80, 81]. Noxious mechanical stimulation produces tonic impulse discharges at frequencies of 5–30 Hz in the nociceptive neurones of the trigeminal spinal nuclei. In contrast, most of the trigeminal spinal neurones responsive to the light mechanical stimulation (the weak sialogogue input) discharge transiently and cease firing within 2 s of the maintained stimulus [82]. Such a rapidly adapting firing pattern may not be suitable for activation of the superior salivatory neurones. However, there is a possibility that some of the rapidly adapting firing patterns may change to a reflexogenic firing pattern when the receptors are repeatedly stimulated during rhythmical chewing movements [83].

The above-mentioned centrally formed firing patterns are transmitted to the various structures of the salivary glands via the ganglion cells. A single postganglionic fibre may contact, en passant, various targets such as acinar cells, myoepithelial cells, and perhaps blood vessels. The nerve terminals contain various kinds of neurotransmitters. The release of the transmitters may depend on, or be potentiated by, the firing pattern of the neurones; for instance, a greater amount of vasoactive intestinal peptide was released from the submandibular gland of atropinized cats, and stronger vasodilation occurred in the gland, when the same total number of electrical stimuli were delivered to the preganglionic fibres in the form of a burst at 20 Hz rather than in a continuous form at 2 Hz [84]. However, as mentioned above, the reflex activity of the preganglionic neurones depends on the sensory modality, taste and mechanical inputs. This suggests the existence of functionally different types of neurones. Thus, one can speculate that, even if a single postganglionic neurone innervates many targets, the salivary gland would be differentially activated during reflex activation depending on the firing pattern and/or type of preganglionic neurones recruited. To test this speculation, it is necessary to analyse the relationship between the impulse discharge pattern of different types of neurones and events in the salivary gland (e.g. the flow rate and composition of saliva, and the blood flow rate). It may also be informative to analyse the pattern of vasodilator impulses in the anterior part of the

tongue, where there are no salivary glands and blood flow is controlled by some of the superior salivatory neurones [69, 85].

Sympathetic Nerves

Activity of the sympathetic nerves has been recorded from the superior cervical ganglion and its postganglionic fibres innervating the submandibular and sublingual salivary glands in anaesthetized rodents. Whole nerve recordings from the hamster postganglionic nerve at the hilus of the submandibular gland have shown characteristics of sympathetic impulse discharges; irregular burst spontaneous discharges and an increase in the number of bursts during taste or heavy mechanical stimulation (fig. 4B). These multi-unit activities may be responsible for various functions including vasoconstriction, the secretion of protein, and the contraction of myoepithelial cells [86–88]. Therefore, for the determination of the patterns of impulse discharges regulating such different functions, the recording of single-unit activities is needed. A single-unit recording technique was recently applied to rat sympathetic nerves, and it was found that 5–10% of the neurones displayed spontaneous discharges at a relatively constant rate at 0.1–0.7 Hz, and the rest were silent [89, 90]. Bartisch et al. [90] also showed that the spontaneously active neurones were inhibited by baroreceptor stimulation and exhibited respiratory modulation; those authors suggested that these are vasoconstrictor neurones. However, Bartisch et al. [90] failed to detect reflex activity induced by taste stimulation, due partly to the suppressive effect of anaesthesia on the reflex pathway. These single-unit analyses suggest that at least the vasoconstrictor neurones have very regular spontaneous discharges. The following questions remain: Are the vasoconstrictor neurones reflexly activated by gustatory inputs? Do the silent or spontaneously inactive neurones relate to functions other than vasoconstriction such as the secretion of protein or the contraction of myoepithelial cells? If so, do these neurones display burst discharges during reflex salivation, as observed in the study of multi-unit recording? Concerning the last question, Garrett et al. [91] electrically stimulated sympathetic nerves of rats, and found that the acinar cells of the submandibular gland require low-frequency stimulation (say 2 Hz) to induce a maximal output of protein, whereas the granular tubules require short sharp-burst stimulation (50 Hz in burst of 1 s every 10 s) to produce an explosive but exhaustible secretion of their prepackaged proteins. Garrett et al. [91] suggested that the two main types of secretory cells, acini and granular tubules, may be innervated by separate populations of sympathetic nerves that fire at different rates. To answer the above-mentioned questions, single-unit activity evoked by oral sensory inputs should be further analysed with the monitoring of blood pressure and respiratory movements.

Concluding Remarks

Sympathetic and parasympathetic efferent impulses are responsible for the activation of salivary glands. These impulses are outflows from the preganglionic neurones in the medulla (the parasympathetic primary centre) and those in the upper thoracic spinal cord (the sympathetic primary centre). Recent histological studies have shown that these primary centres receive excitatory and inhibitory synaptic inputs from neural structures in the lower brainstem and forebrain. The brainstem structures involve relay neurones of oral sensory inputs, whereas the forebrain structures related to the regulation of feeding, drinking, and body temperature. Functionally, the oral sensory inputs and descending signals normally converge on the primary centers simultaneously, in an excitatory or inhibitory fashion. Considering such multiple convergences of synaptic inputs from many brain loci, we can speculate that the primary salivary centres may be able to produce various patterns of impulse discharges. However, in electrophysiological experiments using anaesthetized animals, relatively simple firing patterns have been recorded from the sympathetic and parasympathetic neurones innervating salivary glands. Most of these experiments were designed to focus on only a certain reflex pathway that was activated by oral sensory inputs, and an inhibition of the primary centres has not yet been detected.

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Chapter 3

Receptors in Salivary Glands

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Introduction

It has been recognized for many years that adrenergic and cholinergic neurotransmitters, following binding to their cognate receptors, have a primary role in physiologically mediating saliva secretion from mammalian salivary glands [1, 2]. Many laboratories, including our own, have spent considerable effort to characterize these receptors in membranes from salivary glands from a variety of species, including man [e.g. 3; see table 1, ref. 4]. Both acinar and ductal cells possess autonomic neurotransmitter receptors [5]. However, receptor studies typically focus on those present in acinar cells as acinar cells are the primary cell type involved in fluid and protein secretion. Unless otherwise stated, the descriptions provided in this chapter will refer to receptors associated with acinar cells.

Classically, three autonomic neurotransmitter receptors (α-adrenergic, α-AR, β-adrenergic, β-AR, muscarinic-cholinergic, mAChR) have been examined by means of biochemical, pharmacological and physiological approaches. This chapter will not provide an exhaustive review of the data supporting the importance of these three receptor types to salivation. Rather, the reader is referred to one of a number of earlier review papers which provide such descriptions [e.g. 1, 3, 5–7]. Of these three receptor types, the βARs and mAChRs, and their associated coupling events, are particularly important. Most exocrine protein secretion occurs subsequent to βAR activation, while most fluid secretion follows mAChR activation. Much less, however, is known mechanistically about the former process [8].

In considering what to cover in this chapter, the authors have concluded that the past ~5 years (i.e. approximately the time since the last substantive reviews were written on this subject) have seen three areas of significant prog-

Table 1. Abundance of adrenergic and muscarinic-cholinergic neurotransmitter receptors in rat parotid membranes (modified from Baum et al. [4])

	Ligand	K_d n*M*	B_{max} fmol/mg protein
α_1-Adrenergic	[^{3}H]-prazosin	0.8	13
β-Adrenergic	[^{3}H]-dihydroalprenolol	9.0	207
Muscarinic	[^{3}H]-qunuclidinyl benzilate	0.3	364

ress. The first involves the classical neurotransmitter receptors, the α-ARs, β-ARs, and mAChRs. Part of this progress has involved elucidating finer levels of receptor characterization, and part has involved the recognition of new postreceptor amplification and cellular activation steps that positively regulate secretory responses. The second emphasized area addresses progress in neurotransmitter receptors long considered of lesser importance to physiologic secretion, those controlling the so-called nonadrenergic, noncholinergic secretory responses. Finally, the last area covered will briefly mention recent studies from diverse disciplines that have demonstrated the presence in salivary glands of receptors for a wide variety of other 'factors' (e.g. growth factors, cytokines, steroid hormones). These other receptors do not mediate salivary secretion, but rather can be viewed as modulating the metabolic state of the epithelial cells, and in this regard influencing secretions.

Classical Neurotransmitter Receptors

Muscarinic-Cholinergic Receptors

These receptors are quite abundant in salivary cells (table 1) and, as noted above, appear to mediate the primary fluid secretory response (parasympathetic) from salivary glands in mammals. Studies with the rat parotid gland have shown that ~40% of the mAChRs present are 'spare', unnecessary for a maximal functional response [9]. This means that activation of a submaximal fraction of existing receptors in the acinar cells will result in a maximal physiological response, in this case the formation of the second messenger, inositol trisphosphate (IP_3). As such, this contributes to an inherent amplification process which seeks to achieve, at multiple levels, a maximization by the acinar cell of the initial neurotransmitter secretory signal. Detailed examinations of mAChRs in other species for evidence of such extra receptors have not yet been done.

There are 5 known subtypes of mAChR [10], each of which is encoded by a distinct gene (m_1–m_5) and manifests particular pharmacological responses (M_1–M_5). Like other neurotransmitter receptors, mAChRs belong to a large family of plasma membrane proteins that transmit their signals via a guanine nucleotide-binding regulatory protein (G-protein). All of these receptors have 7 transmembrane α-helical domains, an extracellular N-terminus, and an intracellular C-terminus. The 5 mAChR subtypes generally couple differentially to distinct signaling systems (G-proteins; effector enzymes or channels). Previously, we reported that the vast majority of mAChRs in rat parotid gland were of the M_3 subtype, ~93% by immunochemical criteria [11]. With two major muscarinic signaling systems operative in these glands (intracellular Ca^{2+} mobilization; inhibition of cAMP accumulation), it appeared from this work that individual rat parotid m_3AChRs can couple to both systems.

Recently, similar studies have been performed with two other rodent salivary glands, the rat sublingual and the mouse parotid [12, 13]. Both studies yielded results generally similar to each other but slightly different from those with the rat parotid. Each of these other glands displayed both M_3 and M_1 receptors. However, the majority of these mAChRs were of the M_3 subtype and apparently activation of M_3 receptors alone in these two other glands can yield maximal cellular responsiveness. Thus, a unifying concept emerging from these three independent investigations suggests that the M_3AChRs are the functionally relevant subtype involved in fluid secretion, at least in these rodent glands.

An important point for the reader to recognize is that most studies of neurotransmitter receptor characteristics, whether in salivary or other tissues, have been performed in vitro by studying the binding of relatively specific radiolabeled ligands to membrane or cell preparations. Such experiments, while highly informative, may introduce some variability or artificiality compared to the results obtained in the true, in vivo situation. Because of the clinical importance of mAChRs, particularly in the brain and heart, considerable attention has been devoted to developing methods for evaluating mAChRs in these tissues using pharmacokinetic techniques and radionuclide ligands [14, 15].

Recently, we applied such approaches to the study of mAChRs in rat and human salivary glands [16–18] using two enantiomers of quinuclidinyl iodobenzilate, one of relatively high affinity (so called RR−) and the other of relatively low affinity, (termed SS−). The latter provides an index of nonspecific binding while the former gives an indication of total ligand binding (specific and nonspecific). In rats the binding potential (kinetically equivalent to B_{max}/K_d) for receptor specific sites was roughly similar for all major salivary glands: sublingual (575), submandibular (345) and parotid (380), and inter-

mediate between that seen for the parietal cortex (930, a tissue source rich in mAChRs) and cerebellum (10, a tissue source poor in mAChRs). These results confirm in vitro estimations that suggest rat salivary glands are a fairly abundant source of mAChRs. In man these two ligands also proved useful, but the SS− form did not function ideally as a general probe for nonspecific ligand distribution [18]. While it can certainly be argued that better ligands are needed for such in vivo pharmacokinetic studies, clearly these experiments demonstrated the feasibility of evaluating salivary gland mAChRs in vivo. Correspondingly, the value implied by these studies for pathological diagnosis is considerable.

The fundamental process following neurotransmitter activation of cell surface receptors is one of signal amplification. Both the G-protein activation step and the activation of the subsequent effector protein (e.g. phospholipase C) typically result in an amplified final response, fluid secretion. Ultimately, the neurotransmitter binding of mAChRs on acinar cells leading to secretion of an isotonic primary fluid requires the transcellular movement of anions (fig. 1), Cl^- and to a lesser extent HCO_3^-. Cl^- entry is promoted by the basolateral membrane-localized, loop diuretic-sensitive, $Na^+/K^+/2Cl^-$ cotransporter, and by the parallel operation of Cl^-/HCO_3^- and Na^+/H^+ exchange across this same membrane [19]. Recent studies show that subsequent to neurotransmitter (acetylcholine) binding to the mAChRs, a profound upregulation of the $Na^+/K^+/2Cl^-$ cotransporter and the NHE1 isoform of the Na^+/H^+ exchanger occurs [20, 21]. While not a direct receptor effect, these findings are novel and, as indicated above, such activation processes function in a positive manner to facilitate fluid secretion. The upregulation of the former leads to increased Cl^- accumulation intracellularly, while the latter prevents acidification of the cytoplasm subsequent to HCO_3^- extrusion from the cell. Neither process is mechanistically well understood, although no phosphorylation event is involved [20, 21]. Interestingly, Evans and Turner [20] suggest that the activation of the cotransporter is Ca^{2+}-dependent and involves an as yet unidentified metabolite of arachidonic acid operating via the cytochrome P450 pathway.

Adrenergic Receptors

Norepinephrine released from sympathetic nerves activates α- and β-adrenergic receptors (α-ARs and β-ARs, respectively) on the basolateral surface of salivary acinar cells (fig. 1) [3, 5, 7, 22]. Rat parotid acinar cells have been studied most extensively, and the results of pharmacological competition studies indicate that they possess α_1- [23], α_2- [23], and β_1- [24] subtype receptors. There is evidence that parotid acinar cells might also possess β_2-subtype receptors [25]. Physiological responses to salivary α- and β-AR activation have

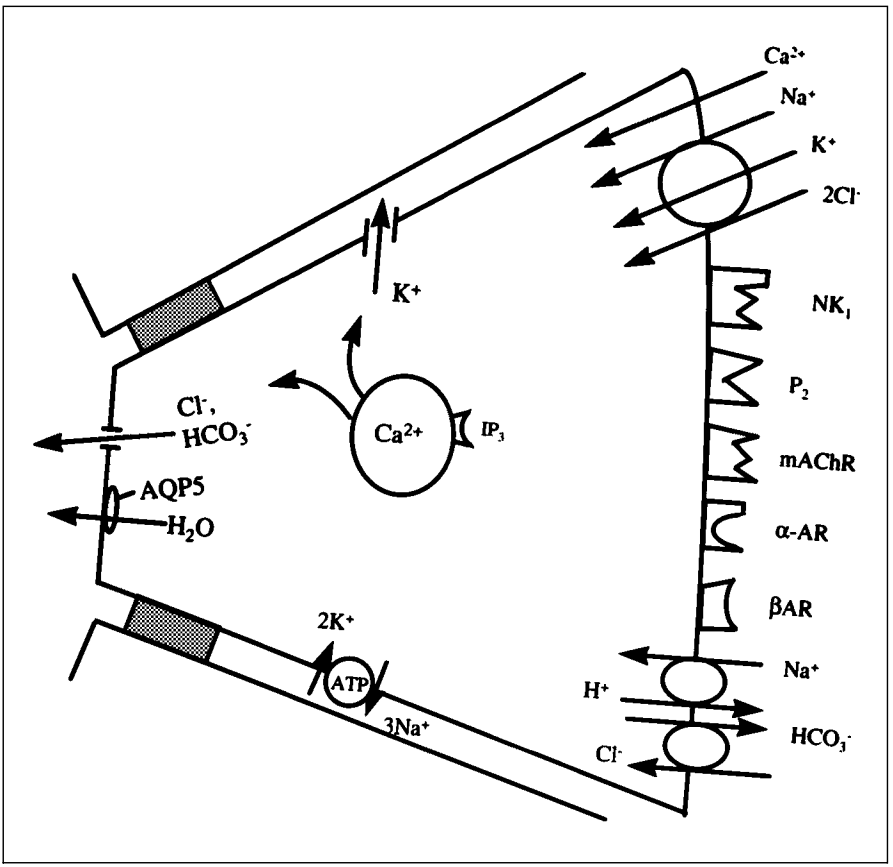

Fig. 1. Schematic depiction of several key transport molecules and neurotransmitter receptors in a rat salivary acinar cell. Modified from [7, 19]. Shown are the following transport pathways: a Ca^{2+}-activated K^+ channel in the basolateral membrane (BLM); a Ca^{2+}-activated anion channel (shown here as permeant to both Cl^- and HCO_3^-, for simplicity) in the apical membrane (ApM); the water channel aquaporin-5, AQP 5, in the ApM; the Na^+/K^+ ATPase in the BLM; the Na^+, K^+, $2Cl^-$ cotransporter in the BLM; paired Na^+/H^+ and Cl^-/HCO_3^- transporters also in the BLM; and an inositol trisphosphate (IP_3) sensitive, Ca^{2+} release pathway in an intracellular membranous store (likely a specialized part of the endoplasmic reticulum). For clarity, Ca^+ release from the endoplasmic reticulum is shown by arrows directly towards the two Ca^{2+}-sensitive ion channels activated by this process. In actuality, the IP_3 receptor also functions as a Ca^{2+} channel. Ca^{2+} enters the cell from the interstitium through an as yet unidentified pathway (shown as an arrow in the BLM). The following receptors are shown in the BLM: NK_1 (tachykinin), P_2 (purinergic), mAChR (muscarinic), α-AR (α-adrenergic), and β-AR (β-adrenergic). Secretion would proceed from right to left (in a serosal to muscosal direction). The shaded regions adjacent to the apical region of the cell represent tight junctions separating the ApM from the BLM.

received extensive review in recent years [3, 5–7, 19, 22, 26]. Below we discuss briefly salivary α- and β-AR activation, paying particular attention to recent developments in β-AR-activated protein secretion.

α-Adrenergic Receptors

In general, activation of α-ARs in various tissues evokes either a Ca^{2+} signal (α_1-ARs) or an inhibition of adenylate cyclase (α_2-ARs). The role of α_2-AR activation in parotid acinar cell physiology is uncertain, but it has been reported that cAMP accumulation is not inhibited in rat submandibular glands [27]. However, the Ca^{2+} signal generated by α_1-AR activation stimulates fluid and electrolyte secretion, modest protein secretion (as compared to that obtained by β-AR activation), and augmentation of β-AR-stimulated protein secretion.

The generation and transduction of an α_1-AR-stimulated Ca^{2+} signal occurs in several steps similar to that by mAChRs (above). Initially, norepinephrine binds to the α_1-AR and elicits a G-protein-(pertussis toxin insensitive)-mediated activation of plasma membrane-bound phospholipase C. Consequently, membrane-bound phosphatidylinositol-4,5-bisphosphate is hydrolyzed to inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 then binds to an intracellular receptor, releasing Ca^{2+} from an internal Ca^{2+} store. This is followed by the release of another internal Ca^{2+} store (Ca^{2+}-induced Ca^{2+}-release), and Ca^{2+} entry from the extracellular fluid. The Ca^{2+} entry pathway appears to be activated by the depletion of the IP_3-sensitive internal Ca^{2+} store. The Ca^{2+} signal can be transduced in several ways, including (i) direct interaction with effector proteins; (ii) with DAG, by direct activation of protein kinase C, and (iii) with calmodulin, either to phosphorylate or directly interact with effector proteins. Ultimately the increased $[Ca^{2+}]_i$ is returned to the resting level, at least in part, by (i) a thapsigargin-sensitive, Ca^{2+}-ATPase-mediated uptake of Ca^{2+} into the IP_3-sensitive store, and (ii) a Ca^{2+}-ATPase extrusion of Ca^{2+} across the plasma membrane.

β-Adrenergic Receptors

In diverse tissues β-AR activation (all receptor subtypes) triggers a G-protein-mediated cAMP signal [28]. There are indications that an elevation in $[cAMP]_i$ might not be required for salivary β-adrenergic-stimulated protein secretion to occur [reviewed in 5]. However, the results of numerous studies have shown that cAMP can act as a second messenger in this response. β-AR activation elevates rat parotid acinar $[cAMP]_i$ [22], and addition of cAMP or cAMP analogues to intact or permeabilized cells evokes protein secretion in a dose-dependent manner [29, 30].

Several lines of evidence suggest that cAMP mediates its effect by activating cAMP-dependent protein kinases (PKAs) which phosphorylate endogene-

ous proteins involved in exocytosis. Thus, in rat parotid acini (i) there is a close dose-dependent correlation between the extent of protein secretion and protein phosphorylation by cAMP analogues in permeabilized cells [30]; (ii) protein secretion can be evoked by direct introduction of PKA subunits into permeabilized cells [31], and (iii) protein secretion is inhibited by some inhibitors of PKAs [31–34]. Several parotid acinar cell proteins are phosphorylated as a result of β-AR activation [22, 29, 30, 33, 35], including a 26-kD protein which displays a phosphate turnover rate that is compatible with a direct role in regulating β-adrenergic-stimulated exocytosis [22].

In addition to cAMP, PKAs, and one or more unidentified phosphoprotein substrates, other components of the β-AR-activated exocytic machinery have been suggested. These components include tyrosine kinase(s), type 2C phosphatase, vesicle-associated membrane protein 2 (VAMP-2), and Ca^{2+}-independent phospholipase A_2.

A role for tyrosine kinase has been inferred from the results of tyrosine kinase inhibitor studies. However, the results obtained by two separate groups have differed. In studies of rat parotid acinar explants it was found that the inhibitors augmented β-adrenergic-stimulated protein secretion [36]. In contrast, in studies of acutely prepared acini the inhibitors reduced β-adrenergic- or cAMP analog-stimulated protein secretion [37]. Clearly, the role of tyrosine kinase(s) in the regulation of β-adrenergic-stimulated protein secretion remains to be elucidated.

Type 2C phosphatase is found in the cytosol and secretory granule fractions of rat parotid acinar cells, and its activity is increased by a β-adrenergic-stimulated increase in $[cAMP]_i$ [38]. Results of inhibitor studies suggest that β-adrenergic-stimulated PKA activates the phosphatase [38]. Interestingly, this phosphatase dephosphorylates the 26-kD protein possibly involved in β-AR-stimulated protein secretion [35].

The final steps in salivary β-AR-activated exocytosis involve interactions between secretory granules and plasma membranes. In Ca^{2+}-stimulated exocytotic systems (e.g. neurotransmitter secretion), such interactions are brought about by protein complexes which dock the secretory granules to the plasma membrane [39]. Proteins involved in the formation of such complexes include syntaxin-1, SNAP-25, rab3A, VAMP-2, α-SNAP, and NSF. In rat parotid acinar cells, VAMP-2, but not syntaxin-1 or SNAP-25, appears to be involved in cAMP-stimulated exocytosis [40, 41]. VAMP-2 has been localized to secretory granule membranes of rat parotid acini, and its cleavage by botulinum neurotoxin B inhibited cAMP-stimulated protein secretion in permeabilized cells [40]. In contrast, SNAP-25 and syntaxin were not detected in rat parotid acini [40, 41]. Furthermore, when acinar cells were treated with botulinum neurotoxin serotypes which specifically cleave SNAP-25 or syntaxin, cAMP-

stimulated secretion was not inhibited [40]. The GTPase rab3A [40], α-SNAP [41], and NSF [41] have also been detected in rat parotid acinar cells. Rab3A was detected almost exclusively in the cytosol. α-SNAP and NSF were detected as co-immunoprecipitates of VAMP-2. Based on studies of Ca^{2+}-stimulated exocytosis in other systems, it was suggested that, in rat parotid acinar cells, α-SNAP and NSF bind indirectly to VAMP-2 via other unidentified proteins [41]. Possibly VAMP-2, α-SNAP, NSF, rab3A, and other proteins participate in the formation of an exocytotic complex between secretory granules and the plasma membrane of rat parotid acinar cells.

β-AR-stimulated exocytosis from parotid acini undoubtedly requires the fusion of secretory granule membranes with plasma membranes. Results of cell-free studies have demonstrated that phospholipase A_2 (PLA2) can cause such fusions, which are accompanied by a release of the parotid secretory granule contents [42]. Results of inhibitor studies suggest that, in intact acini, a cytosolic Ca^{2+}-independent PLA2 is required for β-AR-stimulated protein secretion [43]. It has been hypothesized that, in vivo, this PLA2 activity (i) is part of the putative β-adrenergic-, cAMP-responsive exocytotic protein complex cited above, and (ii) catalyzes a fusion of the secretory granule membranes with the plasma membrane [43].

Nonadrenergic, Noncholinergic Transmitter Receptors

Despite the recognition that adrenergic and cholinergic receptors are primary in mediating salivary secretory events, numerous studies, over many years, have clearly shown that other neurotransmitter receptors can regulate reflex secretion [44]. In this regard, three families of neurotransmitters have been significantly investigated; VIP and related peptides, the tachykinins (substance P) and purines.

Vasoactive Intestinal Polypeptide (VIP) Receptors

VIP is a 28 amino acid peptide hormone which is released, along with acetylcholine, from parasympathetic neurons. VIP binding to receptors on the basolateral surface of acinar cells leads to adenylate cyclase activation and increased cAMP levels. Rat parotid [45] and submandibular [46] gland receptors have been demonstrated using pharmacological approaches, and VIP causes amylase and mucin release from rat parotid [47] and submandibular [48] glands, respectively. This effect is less than that caused by β-AR activation, however. VIP receptors have been cloned from several sources, including rat lung and anterior pituitary, human intestine and placenta, and HT-29 and NG-108 cells [49]. As with other G-protein-linked receptors, the VIP receptors

contain 7 membrane-spanning domains [49]. PACAP-38 and PACAP-27 are peptides structurally related to VIP. Recently, they have been shown to stimulate secretory responses from all three major salivary glands of the rat [50].

Tachykinin Receptors

The tachykinins are a family of peptides sharing a common carboxyl sequence [51]. Currently, 3 homologous tachykinin receptors are known: NK_1, NK_2, NK_3. The former preferentially binds substance P while the latter two receptors preferentially bind neurokinins A and B, respectively. Substance P receptors have been characterized in rat salivary cells [52]. Substance P also has been used as an agonist in many studies of signal transduction in rat salivary glands [53, 54], and is now well accepted as a secretion-inducing neurotransmitter capable of activating the generation of IP_3 and subsequent intracellular Ca^{2+} mobilization. Recent studies using ^{125}I-labeled substance P (for autoradiographic localization and kinetic experiments), or an ^{111}indium-labeled substance P analog (for in vivo visualization of receptors via gamma camera scintigraphy), have further documented the significant presence (and by implication function) of substance P tachykinin receptors in rat salivary glands [55].

Purine Receptors

In 1982, Gallacher [56] first demonstrated that extracellular ATP increased the permeability of the mouse parotid plasma membrane and stimulated a Ca^{2+}-dependent secretion of amylase. Since that time, many studies have confirmed the important role of purinergic receptors in nonadrenergic, noncholinergic salivary secretion [57, 58]. Purinergic receptors are roughly divided into two categories, P_1 receptors which preferentially bind adenosine and P_2 receptors which can bind other nucleotides [59]. Within the P_2 grouping are two distinct subclasses with unique molecular characteristics. P_{2y}, P_{2t} and $P_{2\mu}$ receptors are G-protein-coupled and have 7 transmembrane domains as for mAChRs (above). P_{2x} and P_{2z} receptors, on the other hand, have two transmembrane domains and function as cation channels [60]. Studies with salivary epithelial cells provide considerable evidence for multiple purinergic receptor types in both acinar and ductal cells of these glands [58, 61, 62], mediating a diverse array of responses (metabotropic and ionotropic). For example, in rat submandibular duct cells there exists a P_{2y} receptor which can increase IP_3 formation and a P_{2x} receptor which is coupled to kallikrein secretion [58]. Based on the progress made recently with purinergic and tachykinin receptors, there is good reason to expect a much clearer description to emerge in the near future of the mechanistic role of these receptor types in nonadrenergic, noncholinergic salivary secretion.

Other Potential Neurotransmitter Receptors

In addition to VIP, tachykinins and purines, relatively recent studies have pointed to the possibility that other neurotransmitters and their cognate receptors may potentially function in part in controlling or modulating the observed nonadrenergic, noncholinergic salivary gland secretion. Generally, studies of these other neurotransmitters have been less extensive, so that an appreciation of their physiological relevance is not yet clear. Mention will be made here of a few such recent studies as an indication of possible directions work in this area may take.

Studies by Kawaguchi and colleagues [63–65] have demonstrated the presence of benzodiazepine (BDZ) receptors in rat salivary gland membranes. Specific binding sites for ligands of both peripheral-type and central-type BDZ receptors were found, and diazepam was able to reduce salivary secretion induced by pilocarpine in vivo, as well as reduce $^{36}Cl^-$ fluxes in parotid cell aggregates in vitro. A specific role for these receptors in gland physiology and their salivary cell type localization, have not yet been established. Kawaguchi et al. [63], and Shida et al. [66], also demonstrated the presence of $GABA_A$ receptors in rat salivary glands and showed effects of GABAergic agents on secretion.

Turner et al. [67] have recently added to a small body of literature suggesting that serotonin (5-hydroxytryptamine; 5-HT) receptors may also have a place in modulating mammalian salivary gland secretion. Their studies have provided evidence for the presence of mRNA for these receptors in a human salivary cell line, and for effects of 5-HT on cyclic AMP accumulation and salivary flow in isolated or perfused cells, respectively, from rat submandibular glands in vitro. Additional, detailed studies are needed before the true place of BDZ, $GABA_A$ and 5-HT receptor-mediated steps are understood in the physiology of salivary secretion.

Receptors for Other Factors in Salivary Glands

It is widely recognized that salivary glands are highly reactive tissues to diverse pharmacological and endocrine stimuli. Indeed, clinically about 400 drugs are suggested to cause dry mouth, and numerous systemic diseases have been shown to have secondary effects on salivary glands [68, 69]. The last, brief section of this chapter will mention some relatively recent studies that have demonstrated the presence of receptors for other factors (including growth factors, cytokines and steroids) in mammalian salivary gland cells. The precise role of these different receptors in normal salivary cellular physiology is not

Table 2. Other receptors found in salivary gland cells

Receptor	Reference
Fibroblast growth factor	Myoken et al. [70], 1996
Platelet-derived growth factor	Palman et al. [71], 1992
Prolactin	Garcia-Caballero et al. [72], 1996
Folate	Antony [73], 1996
Interferon-γ	Wu et al. [74], 1996
Interleukin-2	Coll et al. [75], 1995
Androgen	Laine et al. [76], 1993
Progesterone	Ozono et al. [77], 1992
Mineralocorticoid	Sasano et al. [78], 1992

known and the mention made herein is primarily to alert the reader that such potential control mechanisms should be considered.

Table 2 provides a brief list of some such receptors demonstrated to be present in certain salivary cells. The function of these receptors can only be the subject of conjecture. However, it is particularly worth noting that relatively little is understood about the regulation of cell growth and differentiation in salivary epithelial cells. Conceivably, the occurrence of so many growth factor/cytokine receptors in salivary cells may provide an indication of such mechanisms. Clearly, the demonstration of a variety of steroid hormone receptors in salivary cells points to a likely important regulatory role for this class of hormones in salivary gland biology. It is also reasonable to expect that during the next 5–10 years some significant clarification of these roles will be achieved.

Concluding Remarks

This chapter has reviewed recent areas of progress made in receptor control of the secretory functions in salivary glands. In addition to the well-known classical autonomic neurotransmitter receptors (α-adrenergic, β-adrenergic, muscarinic-cholinergic) it clearly has been recognized that nonadrenergic, noncholinergic neurotransmitters and their cognate receptors play an important role in salivary physiology. These include VIP and related peptides, the tachykinins, and purines. Further, many other signaling molecules, i.e. other neurotransmitters and hormones, are suggested to have modulatory roles in salivary cells. For most of this century, salivary glands have been studied in part because of their considerable responsiveness to various neural and pharmacological stimuli. As the new century begins, it is fitting to see such continued and vigorous investigative activity in the area of salivary gland receptors.

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Chapter 4

Effects of Autonomic Nerve Stimulations on Salivary Parenchyma and Protein Secretion

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Historical Introduction

Heidenhain [1, 2] pioneered attempts to wed the histological changes in salivary glands with the secretory events caused by electrical stimulation of their nerves. This approach was encouraged by contemporary improvements in histological procedures, both with regard to fixation and staining, and he produced beautiful illustrations of carmine-stained sections. He showed that when prolonged stimulation was associated with a large output of organic matter, it was accomplished by a decrease in gland weight and a reduction in secretory cell size. He established that increasing the rate of secretion initially caused increasing amounts of organic matter to enter the saliva, but in time this became exhausted and the percentages decreased, though the increased secretion of salts continued. His interpretations of the mechanisms for secretion of organic matter from the cells and the separate roles of sympathetic and parasympathetic nerves [3] seem quaint by present-day standards. However, he emphasized that, within secretory cells, the cyclical changes of synthesis, storage and secretion occur.

In 1877 Nussbaum [4] showed that fixation of rabbit submandibular glands with osmium tetroxide demonstrated 'transition' cells between intercalated ducts and acini (now called neck cells or granular tubules), containing dark-stained granules in histological sections, and he found that these granules were lost from the cells on cranial nerve stimulation.

Langley [5, 6] continued with this type of approach but often used unfixed, fresh preparations and thereby could identify granules in parotid

acinar cells. He used hand-cut thin sections and commented that the microscopic appearances were best when mounted in saliva. Studying rabbit parotid glands in this way, he observed in 1879 [5] 'If the gland be thrown for some time into a state of activity either by stimulating the sympathetic in the neck, or by feeding, the alveoli alter their appearance, and instead of being granular throughout become clear at their outer portion near the basement membrane, and thus show an inner granular and an outer clear, non-granular zone.' He indicated that with prolonged stimulation the cells became smaller, the granules were heavily depleted and arranged around more conspicuous lumina. This was beautifully illustrated and gave a clear indication of the progressive effects of exocytosis, though this word was not used at that time. Langley [6] later made the interesting observation that loss of secretory granules causes a gland to become less white and less opaque to the eye.

Babkin [7] was also keen on attempting to correlate microscopic structure with physiological function and, in the 1930's, he gave his PhD student Rawlinson the task of assessing the microscopical changes in cat submandibular glands associated with secretion, induced by electrical stimulation of its sympathetic or parasympathetic nerve supplies. Using conventional histological staining, Rawlinson [8] observed that the copious secretion on parasympathetic stimulation was accompanied by a marked irregularity in size and shape of the alveolar (central-acinar) cells, but the demilunes showed no definite change. In contrast sympathetic stimulation caused a smaller output of saliva, and the alveolar cells showed 'practically no change' but the demilunes developed cytoplasmic vacuolation. He concluded that the 'parasympathetic and sympathetic nerve supplies to the gland each primarily effect different gland elements'. This idea was enthusiastically embraced by Babkin [7] thereby creating an extreme view, which became widespread and lasted for a long time. Further support for such dichotomy came from primative biochemical testing of parasympathetic and sympathetic saliva from cat submandibular glands by Komarov and Stravraky [9]. Each type of saliva formed different kinds of coagula with an acetone/acetic acid solution, and they concluded that this was due to the mucous cells secreting a specific type of glycoprotein under chorda stimulation, differing from that secreted by the demilunes under sympathetic control. A pioneer electrophoretic study by Kahn et al. [10] in 1969 showed corresponding differences between parasympathetic and sympathetic saliva from cat submandibular glands. This approach laid the foundations for subsequent more-refined electrophoretic studies for identifying the secretory components that can be mobilized from glandular cells by either type of nerve, so helping to improve our understanding of their roles in the secretion of salivary proteins.

Rawlinson [11] had a less clear understanding of changes in the striated ducts; largely because of the inadequacies of the methods used and a consequent lack of appreciation that these cells contain secretory granules on their luminal sides. He described changes after parasympathetic stimulation that we would now regard as ballooning disruption from secretion against peripheral resistance [12]. With sympathetic stimulation he observed that the luminal zone becomes smaller [11] and concluded that the striated duct cells of cat submandibular glands showed signs of secretory activity under both sympathetic and parasympathetic stimulation.

As with modern improvements in our understanding of innervation patterns in salivary glands (see chapter 1), histochemical, histological and electron-microscopic developments from 1960 onwards have been integral in the development of understanding about the roles of nerves in the secretion of proteinaceous components from parenchymal cells in salivary glands. This has been coupled with modern biochemical developments for identifying such components in saliva and has often included electrophoretic procedures.

Methods for Correlating Nerve-Induced Structural Changes with Secretory Events

Nerve Stimulations: Their Limitations and Advantages

As with all experimental methods, their limitations need to be appreciated. Electrical stimulation of nerves for the purposes of this chapter aims to be of sufficient voltage to stimulate all of the nerves in the bundle within the electrode synchronously – an event that is never likely to occur in life. One is also aiming to stimulate the nerves with sufficient frequency for enough time to cause detectable secretory changes in the gland and provide sufficient saliva for analysis. Again, events that are seldom likely to occur in life. Sympathetic stimulation also unavoidably causes indiscriminate activation of vasomotor as well as secretomotor fibres, so causing a vasocontriction which may prejudice the cellular responses. Nevertheless, electrical stimulation of the nerves induces salivary parenchymal responses to the actual neurotransmitters that may be released in life. The frequency of stimulation can also be altered to cause differential release of certain transmitters (see chapter 1). So, the ranges of activities witnessed reflect those that can occur in life, and certainly more so than the administration of single agonists and/or antagonists whether given in vivo or in vitro. Furthermore, with nerve stimulations the responses are limited to the gland being studied. The main salivary glands have the advantage of accessible nerves that can be sectioned for stimulation of their peripheral ends and ducts that can be cannulated for the collection of the resulting saliva. Another advantage exists in glands which clearly show their lobular nature (as in cat and rat submandibular glands) of being able to separate the lobes, so that one can be excised prior to stimulation and others can be taken at different phases during stimulation. Thus sequences of events can be monitored in the same gland. The glands being paired, one can be used for test purposes and the other as the unstimulated control, or for other nerve stimulations. With care, the effects of stimulating either nerve separately or

together can be studied on the same gland. Throughout these procedures the glands receive normal blood constituents as in life, but this can never be imitated in vascular perfusion studies that do not use blood. The ultimate aim of this type of work is to obtain sufficient understanding of nerve-mediated events so that experimental reflex studies (if practical) can be interpreted satisfactorily.

Microscopic and Biochemical Methods

Light microscopy tends to give good survey indications of morphological changes. Conventional staining is satisfactoy for demonstrating changes in cell sizes and sometimes for studying granules, especially in relatively thin section of plastic embedded tissues. This can be correlated biochemically with a known constituent from the granules, e.g. amylase in rat parotid acini, by studying amounts entering the saliva and amounts remaining in the glands. Electron microscopy is essential for studying details of the changes occurring in the cells but, as the material examined is so selective, should be done in conjunction with light microscopy to create a balanced view. Ultrastructural assessment is necessary for monitoring the fine details of exocytosis, preferably using perfusion fixation.

Simple mucosubstance histochemistry, e.g. staining by Alcian blue (for acidic mucins) plus periodic acid-Schiff (PAS for neutral mucins) of appropriate tissue sections can often give clear indications of any depletion of secretory material from the cells and this can be crudely aligned to similar straining of electrophoretic preparations.

Enzyme histochemistry may be useful for monitoring changes in secretory constituents microscopically and especially if correlated with biochemical measurement of the same enzymes in the saliva and the glands. This type of procedure could be exploited in electrophoretic preparations but, so far, this has not been much used for salivary work. Lectin histochemistry helps to identify the glycosylation patterns of secretory materials in microscopial preparations and this may give clear indications of differences between cell types in a gland and of their secretory changes. Electrophoretically separated protein bands in the saliva can then be assessed for binding affinities with the same lectins in blot preparations. The DMAB (*p*-dimethlaminobenzaldehyde) method for the detection of tryptophan [13] in light-microscopic sections is very useful for detecting stored tissue kallikreins in submandibular glands of a number of species [14], as was confirmd elecrophoretically, and is a simple method for studying their depletion from cells in microscopical sections.

Immunohistochemistry of specific secretory constituents has a great potential use for correlating the microscopical changes in the cells with the secretion of the same constituents into saliva, using electrophetic separations and blotting. However, this type of procedure has received little attention so far in nerve stimulation studies. Comparisons between enzyme-histochemical/-biochemical type assessments with immuno-histochemical/-biochemical results for the same enzymes may be useful for indicating whether the enzymes present are in an active or an inactive form.

Glandular and Secretory Changes on Nerve Stimulations

For convenience, events will be considered under selected individual glands, in a semi-historical order, using major salivary glands from different species to exemplify concepts.

Parotid Glands

These glands have the advantage of containing essentially monomorphic secretory cells, so differences between stimulating either type of nerve are considered to reflect different responses from the same cells.

Rat Parotid

Changes in rat parotid glands in response to pharmacological agents received early ultrastructural attention when it was found that the B-adrenoceptor stimulating agent isoprenaline would cause depletion of secretory granules from the acinar cells [15, 16]. In fact, the former paper became a prototype for exocytotic secretion [15]. It was also shown that α-adrenoceptor stimulation caused K^+ and water transport but little amylase secretion [17]. Schneyer [18] subsequently found that parsympathetic nerve stimulation of rat parotid glands caused a copious secretion low in amylase, whereas sympathetic stimulation caused a small secretion with a high amylase content.

Working with Anders Thulin [19], who developed a technique in rats for stimulating the auriculotemporal nerve on the medical side of the mandible near the base of the skull, we confirmed Schneyer's [18] findings about flow rates and amylase concentrations in the saliva. We also showed morpholigically that sympathetic stimulation tended to cause extensive depletion of parotid acinar granules (see fig. 1). In light-microscopic preparations these effects looked very similar to those discovered by Langely [6] in 1879 in rabbit parotid glands. Conversely, with low-frequency parasympathetic stimulation there appeared to have been little or no degranulation, but there was a hint of its occurrence at 10 Hz. Another finding emerged with parasympathetic stimulation in that it tended to be associated with vacuolation in some acinar cells. Thulin [20] went on to reveal that after muscarinic blockade, an atropine-resistant flow of saliva would surprisingly occur from rat parotid glands. Subsequent awareness of neuropeptide transmitters afforded the opportunity for Ekström [21] to provide an explanation for this interesting phenomenon. The subject continues to be explored and new information about widespread participation of neuropeptides, in addition to conventional transmitters, in nerve-mediated secretory events is given elsewhere [see chapters 1 and 6].

Ekström et al. [22] used high frequency stimulation of the postganglionic, parasympathetic auriculotemporal nerve (at the base of the skull) at 40 Hz for up to 80 min in the presence of adrenoceptor blockade and in the absence or in the presence of atropine. They found that there was a progressive depletion of VIP (vasoactive intestinal polypeptide)and substance-P from the gland and only 25% remained after 60 min. A similar protocol was subsequently used for studying the parenchymal changes [23]. In the absence of atropine there was a large flow of saliva that decreased a little in the second 10-min period

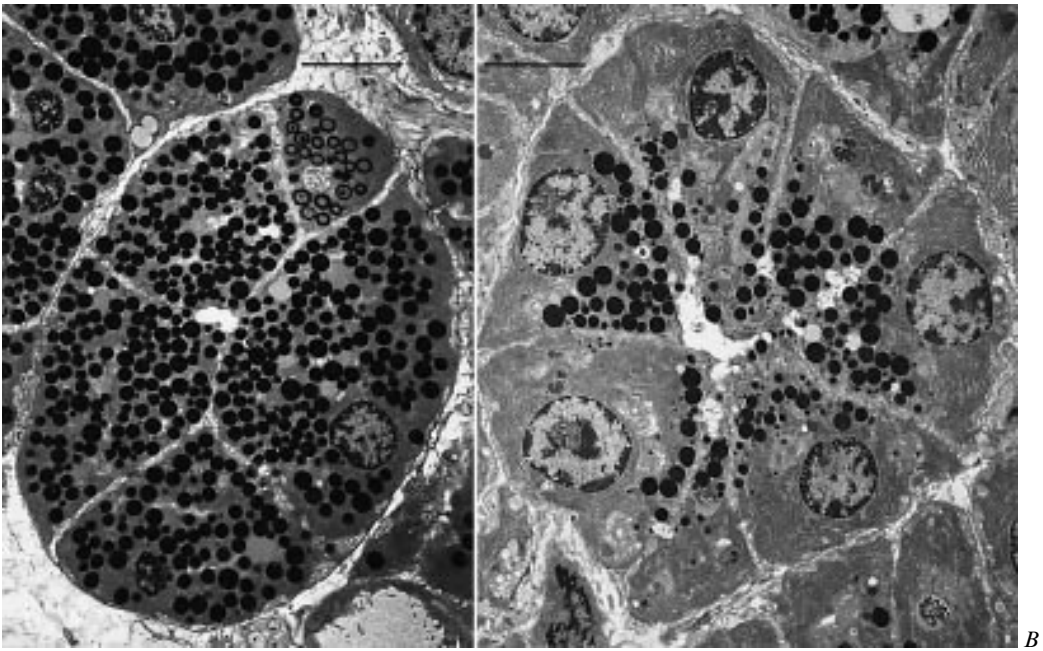

Fig. 1. Electron micrographs of parotid glands from the same rat. *A* Control unstimulated left gland showing acini packed with secretory granules. *B* Right gland, sympathetically stimulated at 10 Hz for 60 min, showing a big depletion of acinar granules (contrast with fig. 2B). Bar = 5 μm. Reproduced from Garrett and Thulin [19].

and then carried on fairly steadily, remaining at about 60% of the initial rate in the eighth 10-min period. Amylase output was relatively high in the initial 10-min period and thereafter decreased more rapidly than the flow. At the end of stimulation a moderate degree of acinar degranulation was evident in the stimulated gland. After atropine the flow of saliva was initially 40% of that from non-atropinized glands. This was followed by a rapid progressive decline in flow so that after 80 min the total amount of saliva was only 15% of that from normal glands, however, the amounts of amylase secreted were similar to those in the absence of atropine, and a similar loss of acinar granules had also occurred. From these studies it was concluded that some of the fluid and most of the amylase secreted during parasympathetic nerve stimulation at 40 Hz were attributable to the release of non-adrenergic, non-colinergic transmitters, and likely to involve both VIP and Substance-P. Morphometric support for the degranulation of rat arotid acinar cells on parasympathetic stimulation at 40 Hz came from a light-microscopic study [24] that showed the granule content was reduced by 30% after 40 min and 39% after 80 min

in the absence of atropine. After atropine the depletions were 30% after 40 min and 27% after 80 min of stimulation. These differences suggest that no further secretion of granules occurred in the second 40 min when atropine was present, but a further small secretion of granules occurred in its absence, so some interaction of the peptide transmitters with acetylcholine may have taken place.

Large dense-cored vesicles present in hypolemmal parasympathetic axons in the glands also showed a depletion after 80 min stimulation at 40 Hz [25]. Bilateral post-ganglionic sympathectomy had been undertaken 4–6 weeks previously to eliminate sympathetic nerves. The overall reduction of axonal large vesicles was about 80% but the depletion was greater in the presence of atropine than in its absence, suggesting that presynaptic muscarinic receptors may have some inhibitory effect on exocytosis of these large vesicles during impulse formation. Since the time scale for the depletion of VIP and Substance-P ran a similar course [22] to the reduction of large dense-cored granular vesicles, this supports the concept that these neuropeptides, stored in the large axonal vesicles, are released from them during high frequency stimulation and are then involved in the parenchymal responses. Concerning the small amounts of amylase secreted during low-frequency parasympatheic stimulation with an apparent lack of degranulation, it is likely that some of this secretion had occurred from a non-granule pool by a constitutive-vesicular type of mechanism [26, 27] and reflected concurrent synthesis. More attention to this type of protein secretion, which probably occurs universally, will be given in the section dealing with rat submandibular glands.

Emmelin [28] showed that, in general, synergistic effects occur between the transmitters released from each type of autonomic nerve on the secretory responses from salivary cells, particularly when stimulation of both nerves is close to threshold levels and not maximal. This has been tested in rat parotid glands by Asking and co-workers [29–30] and it was found that with a low background of parasympathetic activity even subthreshold levels of sympathetic stimulation would cause some augmentation of the flow of saliva and a large augmentation of the secretion of amylase. This is likely to occur in nature if, as is believed [28], sympathetic activation takes place on cells already receiving some parasympathetic drive.

As mentioned above, there was an erratic tendency during parasympathetic stimulation for scattered rat parotid acinar cells to form intracellular vacuoles, which was not seen with sympathetic stimulation [19]. Vacuolation was also seen occasionally in unstimulated glands and was moderately increased by reflex stimulation from chewing [31]. So it seems to be a natural phenomenon that can occur in life. Exploring factors influencing vacuole formation during parasympathetic stimulation, it was dramatically increased if there had been preceding

sympathetic degranulation of the parotid acinar cells [32]. Thus, this type of vacuolation appears to be a pathophysiological phenomenon, mainly reflecting parasympathetic drive, that can be affected by the metabolic state of the cells. In vitro studies suggest that the vacuolation is likely to be due to elevations of intracellular Ca^{2+} [33]. Ultrastructural features indicated that continuities between vacuoles and lumina can develop, so it is possible that a limited movement of proteins to saliva may arise from this unconventional source, as well as from both classical exocytosis of secretory granules and a constitutive secretion of vesicles from rat parotid acini.

Conclusion. The concept of an absolute dichotomy between sympathetic and parasympathetic responses, simplistically and often tenaciously believed for rat parotid glands, is no longer tenable. Normal secretory events from the same cells depend on complex interactions between the two types of autonomic nerves present, involving their complex arrays of neurotransmitters and the influences of different impulse rates on their release.

Cat Parotid Glands

Although the cat parotid gland does not secrete amylase or contain any known secretory enzymes that can be used reliably to monitor secretoy events, study of the nerve-induced changes in acinar granules plus electrophoretic and ion exchange chromatographic analysis of the saliva have been very informative [34, 35]. In contrast to the rat parotid gland parasympathetic stimulation at 10 Hz caused an extensive degranulation of cat parotid acini (see fig. 2) and the saliva had a high protein content. Perfusion fixation during the early stages of stimulation occasionally captured granule exocytosis, as it was occurring, and confirmed that it followed a classical pattern. Sympathetic stimulation per se induced only a very small flow of saliva and no obvious degranulation of the acini was detected in the sections examined. Graded sympathetic stimulation was undertaken on a background of parasympathetic stimulation (10 Hz) and even 0.1 Hz sympathetic stimulation caused a reduction in flow, but there was a progressive augmentation of protein concentration on increasing the stimulation rate. No consistent electrophoretic differences were detected in saliva from parasympathetic stimulation with or without sympathetic stimulation. This suggests that most of the protein present had come from the exocytosis of acinar secretory granules. There seemed to have been a greater degree of exocytosis as a result of dual-sympathetic, parasympathetic stimulation, but the protocols did not permit exact comparisons.

The parasympathetically induced secretion of protein has been analysed further by Ekström et al. [36] and found to involve non-adrenergic, non-cholinergic mechanisms. In the presence of atropine, despite no overt movement of fluid, parasympathetic stimulation at 10 Hz for 90 min induced a degranula-

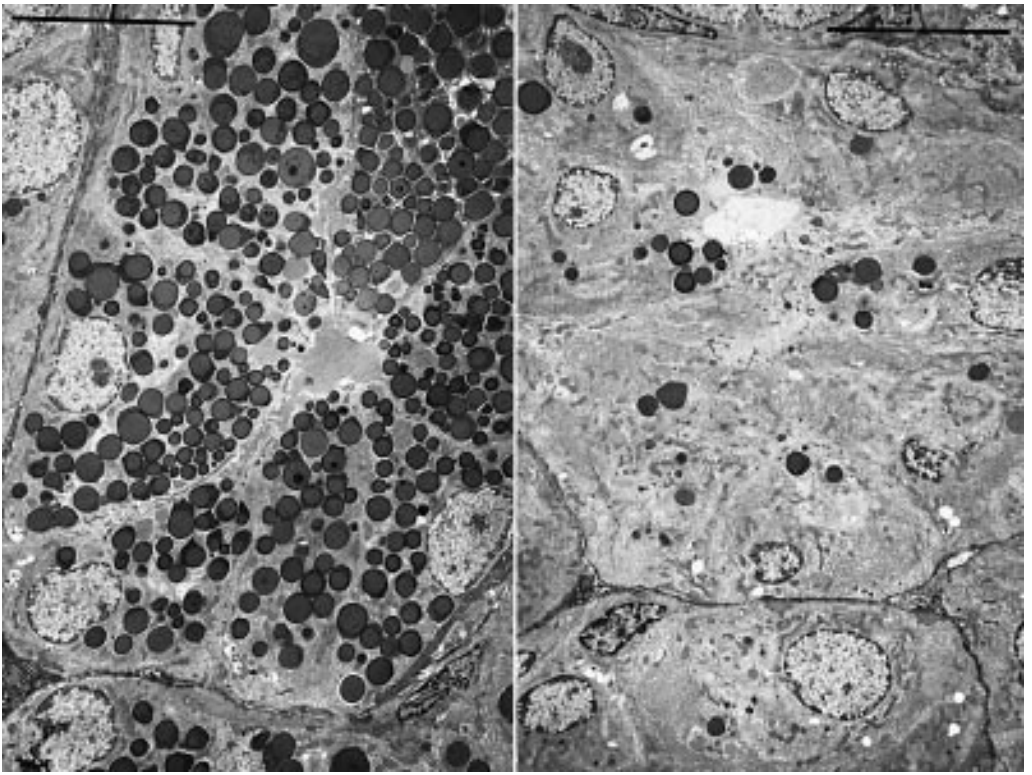

Fig. 2. Electron micrographs of parotid glands from the same cat. *A* Control unstimulated left gland showing acini packed with secretory granules. *B* Right gland, parasympathetically stimulated at 10 Hz for 90 min, showing extensive depletion of acinar granules (contrast with fig. 1B). Bar = 10 μm. Reproduced from Emmelin and Garrett [34].

tion, but it was not as extensive as in the absence of atropine. Correspondingly, it was found that intra-arterial VIP would induce a degree of degranulation as would also an acetycholine analogue.

Conclusion. From these studies it can be concluded that in cat parotid glands secretion of protein by exocytosis of prepackaged secretory material is predominantly a parasympathetic function, involving both acetylcholine and neuropeptide release, which appear to have potentiating effects. Sympathetic transmitters also appear to have some influence, so long as there is a background parasympathetic activity.

Submandibular Glands

These glands have the advantage of containing different secretory cell types, so differential effects of either type of nerve on each type of cell can be assessed.

Cat Submandibular Glands

We extended the classical experiments of Rawlinson [8, 11] see 'Historical Introduction' by studying specific cellular enzymes, histochemically and biochemically. Exploratory enzyme histochemistry had revealed that a peroxidase was confined to the demilunar cells, a diffuse acid-phosphatase was confined to the central acinar cells [37], and kallikrein occurred in the secretory granules of striated ducts [38, 39]. This facilitated a correlative functional assessment of nerve-induced changes in the 3 different secretory cell types histochemically in the glands and biochemically in the saliva [40, 41]. For these experiments, different stimulation frequencies with either nerve were tested, separately or together. In addition, a ploy suggested by Langley [6] of using interrupted periods for sympathetic stimulation per se, to obtain greater volumes of saliva, was also employed. In general terms parasympathetic stimulation caused a large depletion of secretory material from central acinar cells but little change in demilune cells or striated ducts. Correspondingly, there was a high output of acid phosphatase in parasympathetic saliva with low outputs of peroxidase and kallikrein. On the other hand, sympathetic stimulation caused morphological changes in demilune cells and depletion of kallikrein-staining from striated ducts, but no obvious change in central acinar cells. These structural changes correlated with the high outputs of kallikrein and peroxidase in sympathetic saliva and a low output of acid phosphatase. With dual stimulation there was enhancement of peroxidase and kallikrein secretion but not of acid phosphatase compared to parasympathetic stimulation. Increasing the rates of stimulation tended to increase the enzyme outputs. However, kallikrein was found to be secreted dramatically during sympathetic stimulation, rapidly reaching a very high peak then declining steeply but always remaining above outputs with parasympathetic stimulation. This work shows that there are wide differences between the rates of protein secretion from each cell type in response to either type of nerve stimulation, nevertheless each nerve always caused some secretion of protein from each cell type. Thus, transmitters released from either sympathetic or parasympathetic nerves stimulate each type of secretory cell but, with respect to the secretion of prepackaged proteins, sympathetic impulses have greater effects on demilunes and striated ducts, whereas parasympathetic impulses mainly affect central acini.

The secretion of kallikrein was also analysed during low-frequency dual nerve stimulation experiments before and after β- and/or α-adrenoceptor

blockade [41]. This showed that, in the presence of parasympathetic neurotransmitter release, sympathetic impulses caused a largely α-adrenoceptor secretion of kallikrein but β-adrenoceptor responses were also making a contribution. This is in conflict with purely parmacological assessments in which β-adrenoceptor stimulation per se causes no secretion of kallikrein. However, a β-adrenoceptor contribution is probably evoked from sympathetic transmitter release in life since it is likely to affect cells already receiving a parasympathetic stimulation [28].

Subsequent use of lectin histochemistry on glandular sections and of lectin binding on blots from electrophoretic separations of the proteins in sympathetic and parasympathetic saliva have helped to expand our understanding of nerve-induced cellular secretions of proteins into saliva by cat submandibular glands [42]. Without going into detail about the types of glycosylations or the different lectins used, it was found, as previously, that secretory material from central acinar cells was secreted on parasympathetic stimulation and that from striated ducts on sympathetic stimulation. However, the demilune cells now showed secretory changes with either parasympathetic or sympathetic stimulation. The electrophoretic patterns of saliva with lectin staining were more complex than might simplistically be anticipated from the histochemical features but were supportive of the above beliefs and showed that some proteins were common to each type of saliva and some were distinct. The cellular origin for some of the bands still remains obscure but this could probably be unravelled by correlative immunohistochemcal, immuno-biochemical investigations.

Special studies have indicated that VIP probably makes a contribution to the protein release on parasympathetic stimulation [43]. Later experiments showed that parasympathetic stimulation in high frequency bursts enchanced the outputs of protein into the saliva as well as the overflow of VIP into the effluent blood. The increased protein output was blocked by inhibiting nitric oxide formation [44], and further experiments support the idea that the potential for VIP to increase protein output is dependent on the presence of nitric oxide [45].

Conclusion. Both parasympathetic and sympathetic impulses have effects on each of the 3 types of secretory cell in cat submandibular glands. However, with respect to prepackaged protein secretion, that from central acinar cells is predominantly evoked by parasympathetic stimulation and, at higher frequencies, this is likely to involve the neuropeptide transmitter VIP. Protein secretion from striated ducts is largely a sympathetic phenomenon that is probably enhanced under natural conditions by an associated release of parasympathetic transmitter(s) and then involves β- as well as α-adrenoceptor responses. Demilunes on the other hand can secrete protein in response to either parasympathetic or sympathetic impulses.

Rabbit Submandibular Glands

As mentioned in the 'Historical Introduction', Nussbaum [4] studied the effects of intermittent lingual nerve stimulation on rabbit submandibular glands in 1877. He found that prolonged stimulation caused all the cells to become smaller and the 'interstitial' (granular tubule) cells to lose their granules. More recently, the effects of stimulating the chorda lingual nerve on the submandibular duct, at frequencies of 1–10 Hz for 30–60 min, have been studied morphologically by means of simple mucosubstance histochemistry and electron microscopy [46]. Saliva flow and cellular changes increased with the frequency and duration of stimulation. Acinar cells and granular tubular cells showed progressive degranulation and the effects were usually more dramatic in the tubules.

Sympathetic stimulation of rabbit submandibular glands caused only a small initial flow, which then gradually ceased. The former may relate to myoepithelial cell contraction. Yet, the glandular parenchyma receives a widespread and complex adrenergic innervation [47], and when the saliva at the end of a cannula in the duct was examined after stimulation it was found to be very thick. So, interrupted sympathetic stimulation was tested on rabbit submandibular glands at 8–10 Hz [48] without cannulating the duct, to avoid any obstruction. Microscopic assessments showed that prolonged sympathetic stimulation caused a substantial depletion of secretory material from acinar cells which, in the absence of water secretion, accumulated in lumina and distended them. No changes were seen in the granular tubule cells. Use of adrenoceptor blocking drugs indicated that the acinar protein secretion was mainly a β-adrenoceptor response.

Recently, lectin histochemistry on sections of rabbit submandibular glands has been used to study cellular changes after nerve stimulations [49]. Results support the previous findings. Parasympathetic stimulation caused secretion of secretory glycoproteins from both granular tubules and acini. After 1 h of parasympathetic stimulation a different pattern of lectin-binding was seen in the Golgi apparatus of acini indicating that resynthesis and glycosylation of protein had begun. Sympathetic stimulation caused secretion of secretory glycoproteins from acinar cells but no evidence of resynthesis was witnessed. Secretory material was also revealed in intercalated duct cells but, unlike in acinar and granular cells, it showed no evidence of being secreted either by parasympathetic or sympathetic stimulation.

Conclusion. Secretion of prepackaged secretory proteins from rabbit granular tubules appears to be predominantly a parasympathetic function despite an intimate association with adrenergic nerves. Secretion of protein from acinar cells can be mediated by both parasympathetic and sympathetic impulses and this is likely to involve collaborative effects in life. Secretory

proteins in intercalated ducts appear not to be mobilised by either type of nerve so it is wondered whether some are sporadically released during the slow 'spontaneous secretion' from these particular glands, that occurs independently of nerve impulses, and this is balanced by resynthesis.

Rat Submandibular Glands

Pharmacological studies on rat submandibular glands in vivo, including electrophoretic analysis of the saliva, led Abe and Dawes [50] to comment in 1978 that 'the acinar cells secrete protein in response to cholinergic, α- and β-adrenergic stimulation, with β-adrenergic stimuli being most effective, whereas granular tubules secrete proteins – only in response to α-adrenergic stimulation'.

When we started to investigate the effects of nerve stimulations on the protein secretion from the cells in rat submandibular glands an unsuspected problem emerged in that continuous sympathetic stimulation at 5 or 10 Hz invariably caused a pathological oedema of the gland. This often started soon after stimulation had begun and was accompanied by a decrease in the flow of saliva thereafter. Manipulating the protocol, so that stimulation was delivered in bursts of 50Hz 1 s every 10 s, overcame this problem and led to a greater secretion of saliva [51]. Our prediction that this type of stimulation may change the sustained vasoconstriction, that results from continuous stimulation (and with which the damage appears to be associated), into an overall vasodilation was confirmed recently [52]. Burst type of sympathetic stimulation (50 Hz 1 s every 10 s) for 1 h caused a reasonable flow of saliva with a high protein content [53]. Morphometrically, there was a 47% reduction in acinar secretory granules after 1 h and 52% depletion of granules from granular tubules. Parasympathetic stimulation at 10 Hz for 1 h, on the other hand, produced more than a 10-fold greater flow of saliva but the protein output was only 8% of that from sympathetic stimulation and morphometrically there was no evidence of any depletion of granules from acini or granular tubules.

Enzyme markers secreted into rat submandibular saliva have been used as indicators of secretion from the different secretory cell types; kallikreins from the granular tubules and peroxidase from the acini [54, 55]. These studies show that there is a small output of both enzymes into parasympathetic saliva despite the absence of morphometric change. So it appears that parasympathetic impulses have an influence on protein secretion from both types of cell without causing a detectable exocytosis of prepackaged granules. Graded parasympathetic stimulation [56] caused a moderate increase of peroxidase (acinar) secretion with increasing stimulation frequency. However, the concentration of kallikrein (granular ductal) showed little change. Graded sympathetic stimulation applied on a background of parasympathetic stimulation at 4 Hz caused

a greatly increased secretion of peroxidase even at the lowest sympathetic stimulation of 0.1 Hz and there was a progressive increase up to 2 Hz. No further increase occurred when the sympathetic stimulation was added in bursts of 10 or 20 Hz, 1 s every 10 s, but the outputs were significantly greater than with burst sympathetic stimulation by itself. In contrast, addition of continuous sympathetic stimulation up to 2 Hz caused no increase in kallikrein output. However, when the additional sympathetic stimulation was applied as 20 Hz in bursts of 1 s every 10 s there was a dramatic increase in kallikrein output, which was considerably greater than when the same sympathetic stimulation was applied by itself. These findings confirm the importance of sympathetic impulses for causing protein secretion into saliva from both types of submandibular cells. They also indicate that the sympathetic effects are greater when occurring in conjunction with the release of parasympathetic transmitters, as seems the likely situation in life. Nevertheless, the patterns of response by the two cell types were very different. Whereas additional low frequency sympathetic stimulation increased acinar peroxidase outputs that soon peaked, the secretion of kallikreins from granules in granular tubules required high-frequency stimulation. These differences indicate that a complex central integration is required to induce granule secretion reflexly from the granular tubules.

In order to trace sequential events in the secretion of peroxidase from acini and kallikrein from granular tubules, a new sympathetic stimulation protocol was devised. This consisted of stimulating at 50 Hz 1 s every 10 s using an initial stimulation period of 1 min followed by 2-min periods, with rest pauses for 2 min in between each to enable the collecting tubes, etc. to be changed with ease [57]. Surprisingly, this interrupted protocol produced a dramatic increase in flow rates giving a mean that was more than twice that when there had been no pauses. Furthermore, the secretion of granules from the granular tubules was even more dramatic and the depletion of kallkrein from the glands was 85% after a mere 9 min of actual stimulation time, compared to 52% granule depletion after 1 h of similar stimulation without interruption [53]. Secretion of kallikrein was most dramatic in the initial two periods of stimulation [57] and reached very high levels, it then fell steeply but the amounts of secreted always remained much higher than in parasympathetic saliva. This sequence of a rapid initial large mobilization of kallikrein then a decline is similar to that seen in cat submandibular glands when continuous sympathetic stimulation was used [41]. In the rat a small increase in kallikrein outputs occurred after 1 h rest pauses, but then it fell again. A very different pattern of secretion occurred for peroxidase from acini which remained within narrow limits throughout, and the acinar degranulation was far less extensive. There were no peaks at the beginning and no changes after 1-hour pauses.

These findings indicate that peroxidase secretion from acinar cells will continue steadily and undiminished for long periods of time, whereas kallikrein secretion from granular tubules occurs most efficiently with short sharp bursts of high-frequency sympathetic stimulation, but diminishes. This suggests that, for whatever purposes it is required, the glands may be able to mobilize large outputs of kallikrein as circumstances demand.

Thulin [58] showed that some parasympathetic secretion of submandibular saliva would persist after large doses of atropine. This was studied further by Ekström et al. [59] who showed that this non-adrenergic, non-cholinergic flow involved the release of neuropeptides. The amounts of saliva were less than with parotid glands and declined markedly. Although the protein concentrations were high, the outputs were less than in the absence of atropine. Analysis of acinar peroxidase and granular tubular kallikrein in parasympathetic saliva [60] in the presence or absence of atropine indicates that the non-adrenergic, non-cholinergic transmitters released from parasympathetic nerves have little influence on protein secretions from the submandibular cells when acting in isolation. This contrasts with the findings in the parotid glands (see previously). Addition of VIP or substance P intravenously during low frequency parasympathetic stimulation [61] showed that VIP had no overall influence on flow rate but increased the output of acinar peroxidase. Substance-P, on the other hand, increased the flow rate but only caused a small increase in peroxidase output. Neither neuropeptide had any influence on the secretion of kallikrein from the granular tubules.

Exocytosis from granular tubules has been studied sequentially in biopsied lobes from rat submandibular glands during interrupted periods of sympathetic stimualtion at 50 Hz, 1 s every 10 s [62, 63]. This showed some unusual features. In the very early stages there was an alignment of granules with the luminal plasma membrane, and a limited amount of classical exocytosis was detected. Soon, however, microvesicles appeared in granule membranes near lumina and they were associated with gross fusions between granules, forming large irregular intracellular aggregates which often opened into lumina, or were even protruded from the cells. The bulk of the secretion of granule protein appeared to derive from aggregates rather than from classical exocytosis. Cytoplasmic blebbing from the luminal surface of granular cells also led to a merocrine secretion of such cytoplasm and it often contained glycogen. Despite the fact that this sympathetic stimulation procedure caused extensive degranulation, scattered cells always persisted as if they had lost no granules, suggesting that a refractoriness may occur at times.

Throughout our studies on rat submandibular glands we have been aware that the relative proportions of the different types of kallikrein secreted into the saliva differ in parasympathetic saliva from those in sympathetic saliva,

which contains the same proportions as in the granules [54]. This suggests that the kallikreins in parasympathetic saliva must come from a non-granule pool and so are likely to be transported via the constitutive vesicular route. Strong support for this idea came from the finding of a progressive accumulation of the kallikreins in glandular lumina with time, in the absence of stimulation [64]. The constitutive secretion of kallikreins was increased by parasympathetic stimulation, and this may have involved an increased synthesis. Constitutively secreted true tissue kallikrein also has a different molecular form from that in the secretory granules [64]. For further information about the fascinating subject of constitutive secretion, which is likely to occur with other glandular secretory proteins to greater or lesser degrees from all salivary cells, the reader is referred elsewhere [27, 66].

Another fascinating feature emerged from the work on rat submandibular glands. Classically, water secretion is considered to occur from the acinar end pieces. If this is the case, results from interrupted high-frequency sympathetic stimulation in bursts [57] suggest that it has a greater water secretory (hydrokinetic) effect on acinar cells than on their protein secretory (proteokinetic) ability, as judged by peroxidase outputs. This indicates that wider divergences can occur between water secretion and protein mobilization from secretory cells, under similar conditions of stimulation, than is generally appreciated.

Conclusion. There can be little doubt that in rats sympathetic transmitter release is the main contributory stimulus for secretion of proteins from both submandibular acini and granular tubules. Concomitant parasympathetic stimulation enhances this capacity. The mode of sympathetic stimulation required by the two cell types differs dramatically. Acinar cells require only low-impulse frequency to reach maximal protein secretion that can continue at a steady pace indefinitely. The granular tubules, on the other hand, require high-frequency sympathetic stimulation that is most effective when applied intermittently and this causes an explosive but exhaustible secretion of their prepackaged proteins. Such cellular differences indicate that there must be complex central neuronal integration to implement their different requirements, providing low-frequency impulses for the acini and high-frequency impulses for the granular tubules. One realistic possibility is that these different functions are subserved by separate populations of sympathetic efferent axons.

Lack of space precludes attention to other glands and it should be mentioned that the minor glands warrant greater investigation by the types of study used in this chapter. However, one other important aspect must be mentioned briefly.

Influence of Nerves on Salivary Secretion of Immunoglobulin A

This has only recently received any attention [67] despite the generally considered importance of IgA for mucosal health. IgA is formed by plasma cells in the interstices of the glands, and its secretion into saliva is compex [68]. There is specific uptake by receptors on basal surfaces of certain secretory cells followed by transcytosis across the cell in vesicles and final release into lumina. Studies on rat submandibular glands [67] have now shown that there is a continuous movement of IgA into lumina in the absence of stimulation, but the rate of secretion of IgA into saliva can be increased by nerve impulses, with high frequency sympathetic stimulation inducing greater increases than parasympathetic impulses. The mechanism(s) by which this occurs await further investigation.

General Summary

Comparative experiments using nerve stimulations show that there are no universal rules for the roles of sympathetic or parasympathetic nerves in salivary protein secretion. The respective influences differ between cell types and gland types, between species and even within the same species. Effects of the nerves on water mobilization and protein secretion do not necessarily run in tandem. Interactions between the various transmitters that can be released from the two types of secretomotor nerves have been demonstrated experimentally and are likely to occur under natural conditions. Mobilization of secretory granules that contain kallikrein and related substances seems to depend on sympathetic impulses, but this activity is assisted by concomitant parasympathetic drive. Neurotransmitter outputs and their effects are variably influenced by impulse rates. In extreme circumstances, such as rat submandibular glands, different frequency-dependent outputs of transmitter from the sympathetic nerve supply are required to induce protein secretion from the two main secretory cell types. This suggests that there may be an anatomical and functional separtation of axons from the same source to different parenchymal cells in the same gland. Thus, there must be complex central integration of efferent outputs from the neurones in the salivary centres to meet the differing requirements for protein secretion from different cell types under natural reflex conditions. In addition, there is an ongoing vesicular (constitutive) secretion of many salivary proteins in low concentrations into saliva, that may be increased to some extent by nerve impulses, and this may reflect an increased synthesis at such times. Transcytosis of secretory IgA into saliva is also an ongoing process in

rat submandibular glands and can be increased by parasympathetic and sympathetic impulses.

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Chapter 5

Autonomic Transmitters and Ca^{2+}-Activated Cellular Responses in Salivary Glands in vitro

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Introduction

The onset and rate of salivary secretion has to be acutely regulated to accommodate the rapidly changing conditions inside the oral cavity. This minute to minute regulation is achieved by the autonomic secretomotor innervation to these glands. Both sympathetic and parasympathetic nerves exert acute regulation of salivary acinar cell activity. In an earlier chapter in this series [1], we described the ion channels, secondary active and active transports which give rise to the unidirectional transport of fluid and electrolytes across the acinar epithelium. We explained that the acute activation was achieved by the binding of neurotransmitters to surface membrane receptors. The effect of this was to promote release of Ca^{2+} from intracellular stores and ultimately to allow Ca^{2+} influx from the extracellular to intracellular, cytosolic, compartment. Within a few hundred milliseconds of neurotransmitter release there is a pronounced elevation in intracellular Ca^{2+} which in turn activates those ion channels which initiate and support sustained fluid secretion. The scheme may seem straightforward but it is becoming increasingly apparent that the intracellular signalling mechanisms in exocrine acinar cells, including salivary acinar cells, are highly complex and involve several interacting and integrated systems. The result is that Ca^{2+} signalling patterns are specialised, with distinct spatial and temporal signatures. These complex signals almost certainly play a significant role in generating the unidirectional fluxes in these polarised cells. This review will concentrate particularly on the transduction mechanisms and intracellular second messengers regulated by the muscarinic cholinergic and

alpha-adrenergic receptors. These receptors are classical autonomic receptors, comprised of seven transmembrane domains coupled via heterotrimeric G-proteins to the enzyme phospholipase C (PLC) [2]. PLC, upon activation hydrolyses the membrane phospholipid, phosphatidylinisitol-4,5-bisphosphate (PIP_2) to produce inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol. It is now understood that IP_3 acts at specific IP_3 receptors (IP_3Rs) on the internal Ca^{2+}stores, the endoplasmic reticulum, and that this receptor is also a Ca^{2+} release channel.

Upon binding of IP_3 to IP_3R the integral Ca^{2+}channel is opened, releasing the Ca^{2+}sequestered in these stores. It is also now known, in salivary glands [3, 4] as in other exocrine acinar cells, that another second messenger, cyclic adenosine 5′-diphosphate ribose (cADP ribose) can act on receptors different from the IP_3R, to release $Ca^{2+}$from internal stores. Cyclic ADP ribose acts on ryanodine receptors (RyRs), formerly associated with Ca^{2+}-induced Ca^{2+} release in contractile tissues such as skeletal and cardiac muscle. The ryanodine receptors are presumably also located on the membranes of the endoplasmic reticulum. Most recently, though there is as yet no evidence in salivary glands, it is being recognised that another intracellular second messenger, nicotinic acid adenine dinucleotide phosphate (NAADP) can, by acting at an NAADP-specific receptor (NAADPR), release $Ca^{2+}$from intracellular stores [5]. The stores accessed by NAADP are apparently distinct from those regulated by IP_3 and cADP ribose. A second messenger role for NAADP was first suggested in marine invertebrate eggs, but very recently it has been shown to be effective in mobilising $Ca^{2+}$from internal stores in mammalian pancreatic acinar cells. We will review the evidence and make suggestions as to how interactions between the different intracellular second messenger systems might operate to produce spatial and temporal specialisation in intracellular Ca^{2+}signalling.

Ca^{2+}Signalling in Salivary Acinar Cells

In a chapter in the previous volume [1], we showed that the Ca^{2+}-activated membrane currents, due to the opening of Ca^{2+}-activated ion channels, could present as a series of short transient spikes (fig. 1). It was explained that current models of secretion require the simultaneous activation of K^+ and Cl^- permeability pathways. At high concentrations of the agonist ACh, while there was a K^+ current sustained throughout stimulation, the Cl^- current was inactivated during the first one or two minutes of stimulation. By contrast, during the current spikes induced at low concentrations of ACh the Cl^- currents were larger than those for K^+ and, importantly, persisted, though repetitively, throughout stimulation. It is likely that these repetitive transients

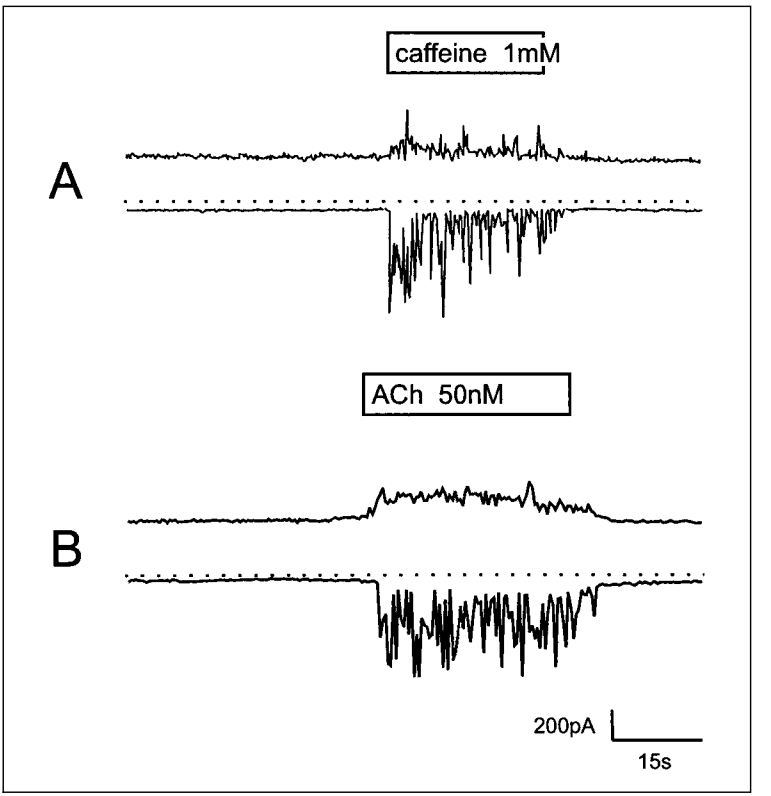

Fig. 1. K^+ (upper trace) and Cl^- (lower trace) currents stimulated by (*A*) 1 m*M* caffeine and (*B*) 50 n*M* ACh measured in acutely isolated mouse submandibular cells. The dotted line indicates zero current.

will better serve secretion than sustained current activations. Indeed, the current transients bear a remarkable resemblance to the electrophysiological responses recorded in vitro in salivary glands following electrical stimulation of the intrinsic nerves to release endogenous neurotransmitters [6]. The spatial and temporal patterns of Ca^{2+} signalling in exocrine acinar cells has been most extensively studied in pancreatic acinar cells where these current spikes have been shown to be due to transient repetitive rises in Ca^{2+} which are localised exclusively to the secretory pole of the acinar cells [7]. Models of secretion in salivary glands require the Ca^{2+}-activated Cl^- channels to be localised to the luminal membrane of the acinar cells and the marked activation of Cl^- currents observed in the submandibular acinar cells during spiking activity is consistent with a release of Ca^{2+} at the secretory pole, i.e. under

the luminal membrane. At higher concentrations of agonists the Ca^{2+} signals in pancreatic and salivary acinar cells spread and cytosolic Ca^{2+} is raised across the cell. Although the rise in Ca^{2+} during sustained stimulation is now global, it is still initiated at the secretory pole from where it spreads as a wave across the cell. Even in such a relatively small cell then, the agonists that promote activation of ion channels to initiate secretion, do so by complex methods which involves spatial and temporal patterning in the signal.

It has not been well investigated in the salivary acinar cells, but in exocrine pancreatic acinar cells, as in hepatocytes, it has been shown that Ca^{2+} signalling can be agonist specific. In the pancreas the different agonists, acetylcholine (ACh) and cholecystokinin (CCK), both of which are considered to be coupled to the generation of IP_3, give rise to distinctive and different Ca^{2+} signalling patterns [8]. The patterns of the Ca^{2+} signals differ both spatially and temporally. ACh, at low concentrations, evokes in the pancreas current spikes similar to those we have described for the salivary acinar cells, mediated by Ca^{2+} transients localised specifically to the secretory pole of the cells. CCK normally evokes much slower, longer-lasting (1–2 min) sinusoidal oscillations in Ca^{2+}, with Ca^{2+}returning to resting levels between each Ca^{2+} rise. The changes in Ca^{2+} in response to CCK are initiated at the secretory pole, but spread globally throughout the cell. It should be remembered that these two agonists are both considered to be coupled to PLC hydrolysis and the generation of IP_3, yet it is clear that they do not activate identical transduction mechanisms. This difference in the response to the two agonists is not a concentration-dependent phenomenon, each has a different Ca^{2+} signature when applied at submaximal (physiological) concentrations. The differences probably reflect the manner in which these agonists are delivered to the pancreatic acinar cells in vivo. ACh is a neurotransmitter released acutely from nerve terminals. If acinar cell metabolism is to reflect activity in the nerves, i.e. the arrival of action potentials, then it must be capable of rapidly developing and adapting responses. CCK by contrast is a circulating hormone, elevated in the bloodstream for tens of minutes after a meal, rapid adaptations in Ca^{2+} signalling are not essential and here the slow, long-duration, sinusoidal oscillations probably represent a mechanism to prevent a sustained elevation in Ca^{2+} over tens of minutes. CCK stimulation is also known to be involved in the long-term regulation of growth and development of the pancreatic acinar cells. In the salivary acinar cells the acute regulation of secretion is nervous and it remains to be established whether there is, or is any need for, any signalling mechanism other than the rapid Ca^{2+} spikes already demonstrated for ACh in the submandibular gland.

IP_3-Mediated Ca^{2+} Signals

Acetylcholine (ACh) and noradrenaline (NA) are the classical autonomic neurotransmitters regulating for fluid and electrolyte transport in salivary acinar cells (fig. 2). Acetylcholine acts at the muscarinic cholinergic receptor. NA acts on both alpha- and beta-adrenergic receptors. Beta-adrenergic receptors regulate adenylate cyclase generating cyclic AMP as an intracellular messenger promoting kinase activity and phosphorylation of proteins. The neuropeptide VIP (vasoactive intestinal polypeptide) is another adenylate cyclase-activating transmitter. The adenylate cyclase transduction pathway is primarily associated with exocytosis of proteins stored in acinar secretory granules. Exocytosis and protein secretion will not be discussed in this article but it will be seen that the cyclic AMP pathway has a regulatory role in Ca^{2+} signalling (fig. 3). The muscarinic and the alpha-adrenergic receptors are both coupled via G-proteins to PLC activity, generating IP_3 and diacylglycerol and both of these autonomic receptors regulate for changes in cytosolic Ca^{2+}. Substance P is another neuropeptide transmitter and its mechanism of action can be considered to be similar, if not identical, to that of ACh, i.e. stimulating PLC to generate IP_3 and mobilise Ca^{2+}. The IP_3 receptor is now well characterised and is known to function as a receptor with an integral Ca^{2+} channel [2]. The IP_3Rs are tetramers and have three characteristic domains, the ligand-binding domain, a regulatory domain and the Ca^{2+} channel containing domain. The sensitivity of the IP_3R is regulated by Ca^{2+}, by phosphorylation sites and by calmodulin. There is then considerable scope for dynamic modulation of the sensitivity of the IP_3R. In addition, there are multiple isoforms of the IP_3 receptor and three of these have been well characterised, IP_3R1, IP_3R2 and IP_3R3. In most cell types, including exocrine acinar cells, more than one isoform has been detected and the IP_3R tetramers are most likely to comprise of subunits of the different isoforms. The different isoforms have different sensitivities to IP_3 and to Ca^{2+}. Ca^{2+} feedback is an important feature in the regulation of Ca^{2+}release mediated by the IP_3R. The type 1 IP_3R shows a bell-shaped [9] dependence on cytosolic Ca^{2+}, initially there is positive feedback such that the rising Ca^{2+} potentiates further release but as the cytosolic Ca^{2+} continues to rise this changes to a negative feedback such that Ca^{2+} release is now inhibited. These positive and negative feedbacks could in themselves provide an explanation for an elementary, transient Ca^{2+} signal. Not all isoforms behave in this manner and the Ca^{2+} dependence of the IP_3R3 has been shown to be sigmoid, i.e. initial positive feedback which plateaus out rather than exerting negative feedback [10]. There is also convincing evidence that the Ca^{2+} concentration within the lumen of the endoplasmic reticulum alters the sensitivity of the IP_3R. A very recent report has described how differential

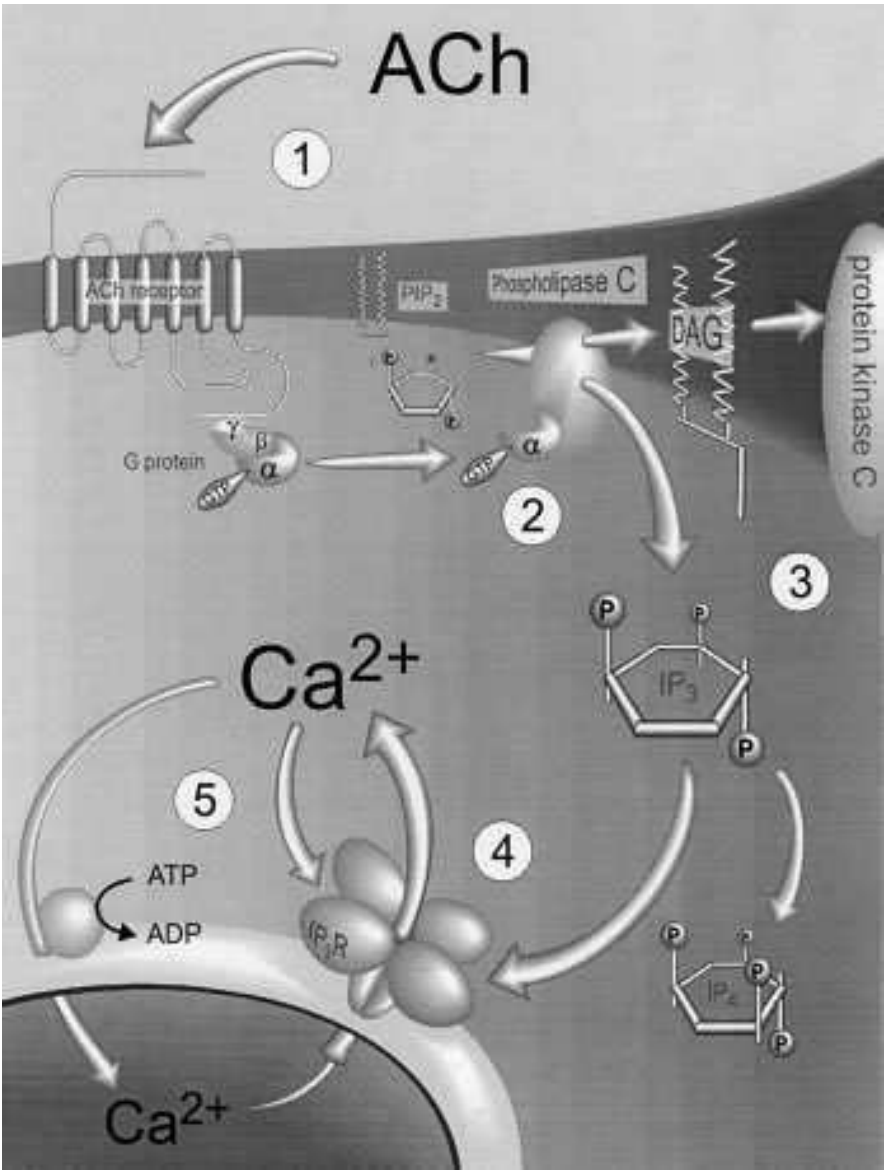

Fig. 2. 1 = Acetylcholine (ACh) binds to its receptor and activates a G-protein which binds GTP. 2 = The α-subunit of the G-protein in turn activates PLC. 3 = PLC splits PIP_2 into DAG which activates protein kinase C and IP_3 which diffuses into the cytoplasm. 4 = IP_3 binds to tetrameric IP_3 receptors on the intracellular Ca^{2+} stores and causes Ca^{2+} release into the cytoplasm. There are at least 3 isoforms of IP_3R (I, II & III), the complete receptor may be a homo- or a heterotetramer. 5 = Ca^{2+} release from the stores feeds back onto the IP_3 receptor to stimulate Ca^{2+}-induced Ca^{2+} release.

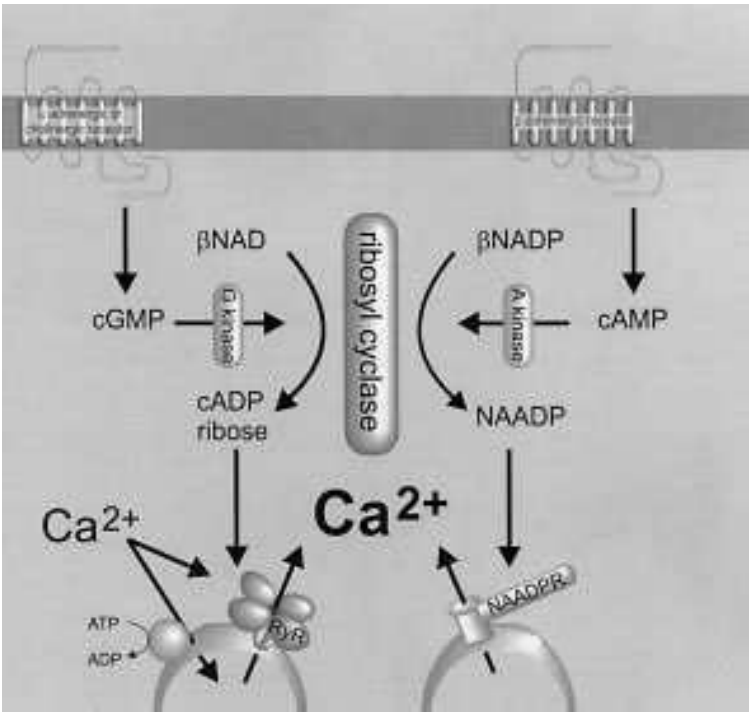

Fig. 3. Ribosyl cyclase generates cADP ribose and NAADP. The scheme shows the possible mechanism(s) by which autonomic receptors could differentially regulate this enzyme's activity.

expression of IP_3R subtypes encode for IP_3-mediated Ca^{2+} signalling in genetically engineered beta cells [11]. One simple explanation for the Ca^{2+} spikes localised to the secretory pole, or the fact that responses are initiated at this site, could be that this region contains the IP_3Rs that are most sensitive to IP_3 and hence are the first to release their Ca^{2+} stores. However, this is probably an oversimplistic interpretation.

The endoplasmic reticulum acts as an internal Ca^{2+} store by virtue of Ca^{2+} pumps which operate to sequester Ca^{2+} into the lumen of this organelle. These are the sarco/endoplasmic Ca^{2+} ATPases (SERCA) pumps. They are distinct from the plasma membrane Ca^{2+} ATPases (PMCA) pumps that extrude Ca^{2+} across the surface membrane. The SERCA pumps can be selectively inhibited by the agent thapsigargin, which results in depletion of the IP_3-sensitive Ca^{2+} stores. There are in fact different isoforms of the SERCA pumps and a recent study in rat submandibular acinar cells has

revealed that there is a polarised expression of the SERCA isoforms [12]. The same group revealed, similarly, that there was a polarised expression of the IP_3R isoforms [3]. The exact mechanism giving rise to the polarisation of Ca^{2+} signalling in salivary acinar cells is then unclear and multiple mechanisms are possible. It could be due to modulation of IP_3R receptor sensitivity, e.g. by cytosolic or stored Ca^{2+}, or by phosphorylation. It could reflect polarisation in the different IP_3R isoforms or their local activity or polarised expression of the SERCA pumps.

Sustained secretion of fluid and electrolytes requires a prolonged elevation in intracellular Ca^{2+}. Ca^{2+} release from internal stores is not sufficient to support sustained secretion and ultimately Ca^{2+} influx from the extracellular to intracellular fluid is required. The mechanism underlying the activation of this Ca^{2+}influx is not well understood and the mechanism for activation of this influx pathway is unknown. It is established that depletion of Ca^{2+} from internal stores is in itself a trigger for Ca^{2+} influx, i.e. calcium-release-activated channels (CRAC). Ca^{2+}-release-activated Ca^{2+} entry was first proposed by Putney [13] who coined the phrase, capacitative Ca^{2+} entry, based on his work on salivary acinar cells. The manner in which the depletion of internal Ca^{2+} stores activates Ca^{2+} entry is still unclear and in fact controversial. It has been suggested that store depletion results in the generation of some diffusible messenger which activates Ca^{2+} channels in the surface membrane. Other suggestions are that store depletion results in a conformational change leading to an interaction between the proteins of the endoplasmic reticulum and surface membrane. IP_4 is another inositol polyphosphate, produced from IP_3 by the action of a specific IP_3-3-kinase. In lacrimal acinar cells it has been shown that a combination of IP_3 and IP_4 are required to activate calcium influx [14]. Specific IP_4-binding proteins (receptors) have been identified and it has been suggested that IP_4 operates to regulate for Ca^{2+} influx. Arguing against this was data from another exocrine acinar cell, the pancreatic acinar cells, where it had been demonstrated that IP_3 alone could entirely mimic the effects of the agonists, ACh , in all respects including Ca^{2+} influx. Not only was there no requirement for IP_4, but IP_4 had no effect in this tissue. However, it has since been demonstrated that if arachidonic acid production is inhibited in pancreatic acinar cells, by applying blockers of phospholipase A_2, then IP_4 itself can mimic ACh and activate Ca^{2+} influx [15]. It has also been shown that the isolated IP_3R is susceptible to blockade by arachidonic acid. The mechanism of action of IP_4 is not established but the evidence in lacrimal and pancreatic acinar cells clearly indicate that it has a significant role to play in Ca^{2+} signalling.

Cyclic ADP Ribose and Ryanodine Receptors

Ryanodine receptors (RyRs) are the principal Ca^{2+}-release channels in excitable tissues responsible for Ca^{2+}-induced Ca^{2+} release, whereas the IP_3Rs have been considered to be responsible for Ca^{2+} release in nonexcitable tissues such as exocrine, salivary, acinar cells. In 1992, Smith and Gallacher [16] reported on the current spikes evoked in submandibular acinar cells by low concentrations of the agonist ACh. In the same study we reported that caffeine, at low concentrations could mimic ACh in generating these current spikes which are now known to be due to localised Ca^{2+} spikes (fig. 1). Caffeine is a classical agonist at the ryanodine receptors. This was the first indication that there might be an involvement of RyRs in Ca^{2+} signalling in salivary acinar cells. Caffeine is not a a physiological agonist but there is a naturally occurring activator (other than Ca^{2+}) of RyRs. The effect of cADP ribose was first demonstrated in invertebrate marine eggs [5] but the Ca^{2+}-mobilising effects of cADP ribose have now been extended to a number of vertebrate tissues, including pancreatic [17] and salivary acinar cells [3, 4]. Cyclic ADP ribose activates RyRs to release Ca^{2+} from intracellular stores. The RyR is similar to the IP_3R in many respects and is both receptor and Ca^{2+} channel. In 1994, cADP ribose was shown to be effective in releasing Ca^{2+} from stores in pancreatic acinar cells [17] and since then it has been demonstrated to mobilise Ca^{2+} from permeabilised rat [3] and canine [4] salivary acinar cells. Ryanodine receptors have now been identified in pancreatic and salivary acinar cells. As with IP_3R there are several isoforms, RyR1, RyR2 and RyR3. Expression of the different isoforms varies in different tissues but in rat submandibular acini it was RyR1 which dominated [3] while in pancreatic acinar cells RyR2 is predominant [18]. The possibility exists then that the RyRs could serve simply to mediate Ca^{2+}-induced Ca^{2+} release, perhaps spreading or amplifying the IP_3R-mediated release of Ca^{2+} that arises at the luminal pole of the cells. Cyclic ADP ribose, however, can be produced within cells by the action of ADP-ribosyl cyclase, converting NAD into cADP ribose. ADP-ribosyl cyclase activity has been demonstrated in canine salivary acinar cells. Cyclic ADP ribose could well be an intracellular second messenger triggering Ca^{2+} release from intracellular stores, and thus the RyRs may not merely function to amplify or spread IP_3-mediated responses by means of Ca^{2+}-induced Ca^{2+} release but operate in direct response to a second messenger, cADP ribose, signal. ADP-ribosyl cyclase activity is stimulated by cyclic GMP-dependent G kinase activity. Calmodulin is a requirement for cADP ribose-induced Ca^{2+} release at the cADPR. There is also a suggested inhibitory role for protein kinase C, this kinase is activated by diacylglycerol which is produced concomitantly with IP_3 by PLC hydrolysis of PIP_2, providing a possible link to estab-

lished receptor-regulated transduction mechanisms. The SERCA pump inhibitor, thapsigargin results in depletion of Ca^{2+} from the cADP ribose stores indicating that the cADPRs regulate for Ca^{2+} release from stores that are the same or very similar to those accessed by IP_3 (fig. 3).

ADP-ribose cyclase activity is found to be present in many cell types. The first known was the ADP-ribosyl cyclase in the invertebrates, the *Aplysia* enzyme which is localised within granules, though there is a soluble cADP ribose-producing enzyme which is stimulated by cyclic GMP but this has not been characterised. In vertebrates there are membrane-bound proteins with NADase activity which show homology with this *Aplysia* enzyme. One of the most extensively investigated is the CD38 protein [5]. CD38 is a type II transmembrane glycoprotein which is an ectoenzyme first detected on human thymocytes but now shown to be expressed in a wide range of vertebrate cells. The protein is bifunctional, its extracellular domain acts both as an ADP-ribosyl cyclase and as an ADP-ribosyl hydrolase, i.e. it can generate and hydrolyse cADP ribose. Any role for this protein in Ca^{2+} signalling would depend on whether the extracellular cADP ribose generated by the ectoenzyme could ever access the internal RyRs. It has been demonstrated, however, that this protein can also function as a transporter for cADP ribose, and in vesicles it could concentrate cADP ribose against a concentration gradient. The intracellular domain of CD38 has a sequence that would suggest it could be a substrate for phosphorylation by cyclic GMP-dependent kinases, allowing for the possibility of intracellular regulation of the ADP-ribose cyclase activity. Other CD38-like proteins, also NADases, have now been found in vertebrates, associated with internal membranes, e.g. on the sarcoplasmic reticulum of cardiac muscle [19]. This ADP-ribosyl cyclase activity was inhibited by protein kinase C. In canine parotid and submandibular gland ADP ribosyl cyclase activity was found in a particulate fraction and the enzymatic activity was potentiated by cyclic AMP-dependent and calmodulin-dependent phosphorylation [4]. These studies suggest a direct link between cADP ribose production and receptor-mediated cell signalling.

NAADP and Ca^{2+} Mobilisation

The ADP-ribosyl cyclase which generates cADP ribose from NAD can utilise NADP (nicotinic acid-adenine dinucleotide phosphate) as an alternative substrate to generate NAADP rather than cADP ribose. As for cADP ribose, it was first demonstrated in invertebrate marine eggs that NAADP had Ca^{2+}-mobilising properties. The effect of NAADP is mediated at a specific receptor distinct from the IP_3R or RyR which IP_3 and Ca^{2+} ADP ribose act upon,

respectively. While the SERCA pump inhibitor thapsigargin depletes the IP_3 and cADP ribose sensitive stores the NAADP-mediated release of Ca^{2+} persists, suggesting that the NAADPRs are located on distinct and different stores. In the invertebrate eggs the NAADP effects are different from those of either IP_3 or cADP ribose in other respects. NAADP is capable even at subthreshold doses of desensitising the NAADPR, blocking NAADP-mediated Ca^{2+} release. NAADP applied at concentrations which mobilise Ca^{2+} give rise to Ca^{2+} release followed by a profound and very long-lasting (at least hours if not longer) inhibition. To our knowledge there has as yet been no investigation of the possible Ca^{2+}-mobilising effects of NAADP in salivary acinar cells. Very recently, however, NAADP has been shown to release intracellular Ca^{2+} in pancreatic acinar cells [20]. Importantly, this report also provided evidence that NAADP could be involved in mediating the response of the pancreatic acinar cell to the low concentrations of the agonist CCK. The evidence was that when desensitisation of the NAADPR was induced, the responses to low concentrations of CCK were inhibited. The inhibition of the CCK responses was not total and could be overcome by increasing the concentration of CCK. So desensitisation of the NAADPR inhibits CCK-induced Ca^{2+} mobilisation but does not block it. The requirement for NAADPRs in CCK Ca^{2+} signalling in the pancreas is not absolute but could well be important at low levels, possibly the physiological levels, of CCK stimulation. In invertebrate eggs, the physiological stimulus mobilising Ca^{2+} is fertilisation by sperm, a one-off event. In the eggs the long-term desensitisation of the NAADPR that is associated with activation is not a problem. Exocrine acinar cells must be capable of rapid and dynamic responses to repeated stimuli from agonists. If NAADP, and the Ca^{2+} store accessed by NAADP, are to be physiologically significant in terms of mediating Ca^{2+} responses to agonists they would have to have developed to overcome the dramatic and long-term desensitisation of the NAADPR that is a predominant characteristic in the marine eggs. There is evidence that the ribosyl cyclases which generate cADP ribose and NAADP are not identical and that they can be differentially regulated [21]. In see urchin eggs the cADP ribose-producing enzyme was soluble and most sensitive to regulation by cyclic GMP. In contrast NAADP synthesis was promoted by a membrane-bound enzyme which was potentiated by cyclic AMP. As mentioned above, the cyclase activity in canine salivary glands was also stimulated by cyclic AMP. This differential regulation of cADP ribose and NAADP production implies that they need not be considered as having to act in synchrony but could each act independently as Ca^{2+}-mobilising second messengers. We consider that the study on pancreatic acinar cells make it most likely that NAADP will be revealed to have Ca^{2+}-mobilising properties in salivary acinar cells (fig. 3).

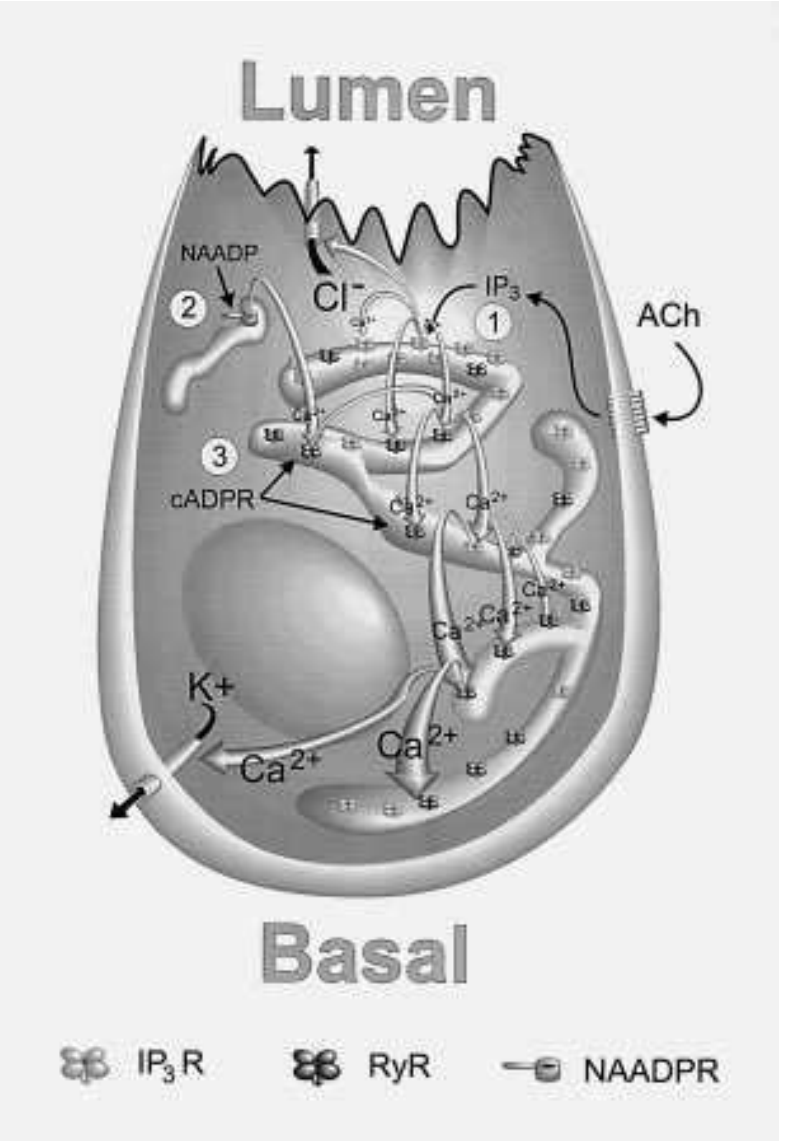

Fig. 4. 1 = Initiation of a calcium cascade at the lumen of the cell following release of calcium from intracellular stores triggered by binding of IP_3 to its receptor. 2 = Initiation of a calcium cascade at the lumen of the cell following release of calcium from intracellular stores triggered by binding of NAADP to its receptor. This calcium store is distinct and separate from the IP_3-dependent store. 3 = Propagation of a calcium cascade across the cell by calcium-induced calcium release mediated by IP_3 receptors and cADPR-dependent ryanodine receptors.

Conclusions

In addition to IP_3-mediated Ca^{2+} release we must now consider roles for cADP ribose and NAADP (fig. 4). The cADP ribose acts at the Ca^{2+}-induced Ca^{2+} release sites regulated by ryanodine receptors. NAADP accesses a different store which is as yet uncharacterised. The ADP-ribosyl cyclase activity is regulated by cyclic GMP, cyclic AMP, calmodulin and protein kinase C, though there may be variation in different tissues. Protein kinase C is activated by the diacylglycerol which is produced concomitantly with IP_3. Cyclic GMP production is reported for both muscarinic cholinergic and alpha-adrenergic receptor stimulation [22, 23]. Cyclic AMP production is regulated by the beta-adrenergic receptor and by VIP receptors. There is clearly a role for autonomic receptor regulation in the production of all three Ca^{2+}-mobilising second messengers. Another promoter of cyclic GMP is nitric oxide (NO). Salivary glands have the nitric oxide synthase (NOS) [24] which generates NO and it could act to promote G kinase activity to stimulate ADP-ribosyl cyclase activity, generating either cADP ribose and or NAADP. The scheme and the number of possible interactions are complex. What remains clear is the special role of the secretory pole of the salivary acinar cells. It is under the luminal membrane of these cells that Ca^{2+} signals arise. This most probably reflects a concentration of the most sensitive IP_3 receptors. The Ca^{2+} signals generated at this region can be contained there as discrete localised spikes or the signals can spread globally. Ca^{2+}-induced Ca^{2+} release via RyRs is almost certainly implicated in this spread or wave of Ca^{2+}. Cyclic ADP ribose can sensitise this system and promote Ca^{2+} release in its own right. The stimulus for cADP production is most probably cyclic GMP-dependent phosphorylation via G kinase. Other kinases are also involved in the regulation of the cyclase. NAADP accesses a unique store. In the pancreas it is suggested that Ca^{2+} release from this store could feed back onto IP_3 Rs and RyRs to sensitise them for further Ca^{2+} release. It is clear that Ca^{2+} signalling in the salivary acinar cells is the result of a complex and dynamic interplay of signalling pathways. The integration of these different mechanisms may well explain the variation in the sensitivities of the cells under certain circumstances and provide the mechanisms for synergism between different neurotransmitters.

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Chapter 6

Role of Nonadrenergic, Noncholinergic Autonomic Transmitters in Salivary Glandular Activities in vivo

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Introduction

During the past 20 years, the view on the autonomic nervous system has been revolutionized. In the late 1970s it became apparent that peptides may serve as autonomic transmitters and in the early 1990s nitric monoxide (NO) was found to be involved in the transmission of autonomic impulses. Largely due to the work by Burnstock and his colleagues in the early 1960s and onwards, the awareness of autonomic responses depending on other transmitter mechanisms than the classical cholinergic and adrenergic ones has been growing [1]. What we now call nonadrenergic, noncholinergic (NANC) phenomena were, in fact, already described before the turn of the past century as various 'atropine-resistant' responses to stimulation of the parasympathetic innervations. These observations include the increase in salivary gland blood flow by Heidenhain [2] in 1872, the contraction of the urinary bladder by Langley and Anderson [3] in 1895, and the relaxation of the stomach by Langley [4] in 1898. The characteristic effect of atropine in abolishing responses to stimulation of the parasympathetic innervation was first described by von Bezold and Bloebaum [5] in 1867, who showed the muscarinic receptor blocker to prevent the cardiac response to electrical stimulation of the vagal nerve. Heidenhain's observation of a parasympathetic nerve-evoked vasodilatatory response in the submandibular gland of the dog resistant to atropine seems to be the very first NANC response on record, and it was confirmed soon after in cat submandibular glands by Langley [6] and Barcroft [7]. In these early experiments on the classical laboratory animals the dog and the cat, the

copious flow of saliva evoked by stimulation of the parasympathetic innervation was completely abolished by atropine, giving rise to the general idea that the effects of parasympathetic secretory nerve impulses are easily abolished by atropine [8]. This opinion was given further support by the common experience of mouth dryness as a side effect in patients treated with atropine or drugs showing atropine-like actions [9].

In the middle of the 1960s peptides belonging to the tachykinin family were found to be powerful secretagogues in some species, despite the presence of traditional autonomic receptor blockers. Physalaemin of nonmammalian origin [10, 11] and substance P of mammalian origin [12, 13] caused, upon injection into the bloodstream, secretion of saliva in dogs and rats but not in cats, rabbits and guinea pigs. The possibility of a NANC regulation of the secretory cells was, however, not considered, and tachykinin-induced salivation was regarded as a pharmacological pecularity. Because the concept of acetylcholine being the sole transmitter of the parasympathetic secretory innervation was deeply rooted, the tackykinins were thought to cause salivation 'by a different mode of action than the chemical transmitters' [14]. In 1976, Thulin [15], working in the laboratory of Emmelin in Lund, studied the parasympathetic atropine-resistant nerve-evoked vascular response of the submandibular gland in the rat, and he noticed that a small flow of saliva occurred from the gland despite the presence of muscarinic receptor blockade. However, this finding, which was in contrast to previous observations on the gland in rats [16], was reported in passing without comment. The present author [17], working in the same laboratory, published in 1974 the observation that while parasympathetic denervation of the rat parotid gland caused a profound fall in gland weight over a period of time, prolonged treatment with an antimuscarinic agent did not. On the contrary, a gain in weight occurred regardless of whether the sympathetic innervation was intact or not. Although this finding hinted at trophic actions of nonconventional transmitters of parasympathetic origin no such connexion was made at that time. Within a few years the picture has changed dramatically.

Today parasympathetic NANC mechanisms have been demonstrated to influence secretory activities in a number of glands and species, and a variety of neuropeptides as well as the NO-synthezising enzyme have been observed histochemically in nerves innervating the acinar and ductule cells. This chapter focuses on these mechanisms and their contribution to the regulation of fluid and protein secretion and their roles in reflex secretion as well as to their regulation of gland size and cell metabolism.

Secretory Nonadrenergic, Noncholinergic Responses Evoked by Electrical Stimulation of the Parasympathetic Innervation

Secretion of Saliva

An overt flow of saliva in response to electrical stimulation of the parasympathetic innervation in the presence of muscarinic receptor blockade as well as of α- and β-adrenoceptor blockade occurs in the parotid, submandibular and sublingual glands of the rat [18–21], also in the parotid and submandibular glands of the ferret [22, 23] and the mink [24], while in the parotid gland of the sheep [25], the continuous flow of parotid saliva is accelerated. The phenomenon was first studied systematically in the rat parotid gland [19]. In the presence of adrenoceptor blockade and before atropine, the gland secreted at 0.2 Hz stimulation of the auriculotemporal nerve and the maximal response occurred at 40 Hz. When the frequency-response sequence was repeated in the presence of atropine, the gland did not respond until 5–10 Hz was reached. The maximal response again occurred at 40 Hz and was 13% of that before atropine. In addition, the latency in onset of secretion after atropine was about ten times that before the administration of the muscarinic receptor blocker; it was 30 s at 10 Hz and 15 s at 40 Hz. In similar types of experiments, where the nerve was stimulated at a range of frequencies before and after atropine, the amylase output was markedly decreased after atropinization. In relative terms the volume of saliva was, however, more affected, which resulted in a 2- to 3-fold increase in the salivary amylase concentration [26]. However, it soon became apparent that by keeping the period of nerve stimulation that preceded the administration of atropine to a minimum, the latency in onset of secretion, in the presence of atropine, decreased, and the amounts of fluid and amylase secreted increased [27–29]. The threshold frequency for eliciting the NANC fluid response was, however, about the same. At 40 Hz the volume of parotid saliva secreted, in the presence of atropine, was initially as much as 40–50% of that in the absence of the blocker (calculated over periods of 2, 5 or 10 min) and the amylase output was either the same or even higher than that in the nonatropinized animals. Prolonged stimulation of the nerve at a high frequency (40 Hz), however, revealed that the secretory NANC phenomenon fatigued markedly and rapidly over time. If the flow rate during the initial 10 min of stimulation was set to 100% (in both atropinized and nonatropinized rats), the gland in the nonatropinized rats secreted at a fairly steady rate after an initial drop to 80% (10–20 min), 70% (30–40 min) and 65% (70–80 min). In contrast, the response in the atropinized rats during the second (10–20 min), fourth (30–40 min) and eighth (70–80 min) 10-min period of stimulation was roughly 40, 15 and 5% of the initial response. A rest for several hours was not enough to allow for recovery of the secretory response. The amylase output

also declined markedly in response to prolonged stimulation. In the atropinized rats the output during the second 10-min period was roughly 30% of the initial output, and 5 and 1% during the fourth and eighth periods, while the corresponding figures in the nonatropinized rats were 60, 30 and 25%.

Some general features of the parasympathetic NANC fluid response emerge from the glands studied in the various species: the relatively high frequency required to elicit salivary flow, the long latency in onset of secretion, the fading response, and the lack of early recovery. A high salivary protein concentration (or amylase concentration) seems, however, not to be a general characteristic as judged from the ferret submandibular gland. Compared to the rat parotid gland, the ferret submandibular gland shows the next-largest NANC fluid response amounting to 30–35% of that in the absence of atropine [23]. By studying the vascular as well as the fluid responses in ferret submandibular glands to stimulation of the chordalingual nerve, the time course of the two was shown to dissociate [30]. In the atropinized ferret, the salivary flow, evoked by continuous stimulation at a frequency of 20 Hz, almost ceased within 20 min whereas the increase in blood flow, being as great as in the nonatropinized animals, was maintained throughout a whole 80-min period of stimulation. This result shows that the blood flow was not a limiting factor for the NANC-induced salivary flow and, further, implies the involvement of different NANC mechanisms in the fluid and vascular responses, one more exhaustible than the other.

'Occult' Release of Protein

Stimulation of the parasympathetic innervation in the presence of atropine and adrenoceptor blockers may cause release of proteins without being accompanied by any overt fluid secretion. In ferret submandibular glands substance P-induced flow of saliva was used to expel proteins released in response to a preceding period of nerve stimulation at low frequencies (0.2–2 Hz) subthreshold for eliciting an overt flow of saliva in atropinized and adrenoceptor-blocked animals [31]. Care was taken to avoid possible interactions, and when the tachykinin was injected 10–15 min after the end of a 5-min period of continuous stimulation of the chordalingual nerve, the protein output, but not the volume of saliva, was increased, by about 40% (0.2 Hz), 60% (0.5 Hz), 90% (1 Hz) and 170% (2 Hz) as compared to the basal response to substance P. A protein release in response to parasympathetic stimulation was also revealed in the submandibular gland of the cat, which in the presence of atropine, is a completely 'silent' gland with respect to the secretion of fluid [32]. In contrast to the ferret, substance P evokes no secretion of saliva in cat submandibular glands; however, by implying an intermittent mode of sympathetic stimulation [33], a highly reproducible flow could be obtained to

expel the released protein. The sympathetically induced wash-out 15 min after a 5-min period of chordalingual stimulation at frequencies of 20 and 50 Hz delivered intermittently for 1 s at 10-second intervals resulted in a 25 and 50% increased protein output, respectively, without any increase in the sympathetic volume response.

Exocytosis of Secretory Granules

A great loss in the number of acinar secretory granules of the cat parotid gland is induced by electrical stimulation of the parasympathetic innervation, but not of the sympathetic innervation [34]. Morphometric assessments showed that it amounted to 60% after a stimulation period of 90 min of the auriculotemporal nerve at a frequency of 10 Hz applied continuously [35]. However, in the presence of atropine and adrenoceptor antagonists, the same experimental protocol, which resulted in no overt fluid response, elicited as much as a 40% loss in number of granules. Similar evidence for a role of the NANC mechanisms in the exocytosis can be found in the parotid glands of some other species, which like the cat parotid gland consist of a single type of acinar cell containing clearly delineated secretory granules. In ferret parotid glands the NANC fluid response to continuous stimulation of the parasympathetic innervation at 40 Hz for 40 min was only 5% of that in the absence of atropine and adrenoceptor antagonists, but the loss in granules amounted to 27% compared to 52% in the absence of atropine [36]. For comparison, the depletion in response to sympathetic nerve stimulation was 10%. In rat parotid glands stimulation of the parasympathetic innervation at 10 Hz or less was found to cause no loss of granules [37, 38]. However, at maximal stimulation (for fluid secretion) of 40 Hz the number of granules was reduced by 30 and 39% after 40 and 80 min of stimulation (in the presence of adrenoceptor blockade) and in atropinized rats the corresponding reductions were 30 and 27% [39]. Thus, these observations from parotid glands of various species show that parasympathetic NANC mechanisms are potentially responsible, at high frequencies, for half or more of the parasympathetic exocytotic responses in the absence of atropine.

Neuropeptides and Their Secretory Actions

Effects of Parasympathetic Denervation on the Neuropeptide Gland Content

In the rat parotid gland almost all the postganglionic cholinergic nerve fibres reach the gland via the route of the auriculotemporal nerve. Following section of this nerve, and allowing time for nerve degeneration, only 1–3% of the activity of the acetylcholine synthesizing enzyme, choline acetyltransferase,

remains in the gland [17, 40]. In the cat, the residual enzyme activity is 10% [41, 42] and in the dog as much as 30% and here, secretory cholinergic nerve fibres travelling along the internal maxillary artery and the facial nerve contribute to the activity of choline acetyltransferase [43]. In the parotid gland of the rat, the content of a number of peptides is also affected by the section of the auriculotemporal nerve. The total amounts of substance P and VIP are reduced to 7–8% and 5% [44, 45], respectively, neuropeptide Y (NPY) to 30% [46], pituitary adenylate cyclase activating peptide (PACAP) to 56% [47] and calcitonin gene-related peptide (CGRP) to 77% [45]. Immunohistochemistry shows that the nerve fibres containing these peptides disappeared around acinar cells (the distribution of PACAP following parasympathetic denervation has not yet been examined). The residual NPY content as well as the NPY-containing nerve fibres innervating the blood vessels are of sympathetic origin as judged by the effect of the removal of the superior cervical ganglion [46, 48]. The persisting CGRP-containing nerve fibres, also contain substance P and innervate blood vessels and ducts, and are considered to be of sensory origin since they disappear in response to treatment with the sensory neurotoxin capsaicin [49]; their pathways to the gland occur via the facial nerve and the dorsal root nerves C3 and C4 and by some unknown routes [45]. The facial nerve also contributes to the persisting substance P content after section of the auriculotemporal nerve. In search of a source for the relatively large remaining content of PACAP, the facial nerve was sectioned, the superior cervical ganglion removed or the animals were treated with capsaicin but these procedures did not affect the content of this peptide. This might indicate the existence of nonneuronal sources for PACAP in the gland [47], and this peptide has been found in endocrine cells of various tissues [50].

In submandibular glands, the relay between many pre- and postganglionic nerve fibres are located within the gland. However, by dissecting the chorda tympani nerve fibres and cutting them deep into gland hilum in rats a partial denervation can be achieved as shown by an 88% fall in the choline acetyltransferase activity [Ekström, unpubl. observation]. In this case, the total amount of substance P was reduced by 92%, whereas the total amount of VIP was only reduced by about 50% [44], a dissociation suggesting that the nerve cell bodies harbouring these two peptides may differ in localization or in their ability to synthesize the peptides after loss of their preganglionic input.

Effects of Prolonged Electrical Stimulation of the Parasympathetic Innervation on the Gland Content and Release of Neuropeptides

The NANC-induced portion of parasympathetic evoked flow of saliva fatigued rapidly. The fading response was not due to decreased responsiveness of the gland, as judged by the secretory response to substance P injected

intravenously after the end of the stimulation period. The volume of saliva secreted to the tachykinin was, in fact, greater. A likely explanation to the fatiguing response was the depletion of the stores of releasable peptides in the nerve terminals [27, 45, 46, 51]. In contrast to the classical transmitters acetylcholine and noradrenaline, the content of peptide transmitters in the terminals are dependent for their replenishment on axonal transport of preformed, packaged peptides from the nerve cell body [52]. Following continuous high-frequency stimulation (40 Hz) of the auriculotemporal nerve for 60 min, the parotid gland of atropinized rats had lost 75% of its content of substance P and VIP, 45% of its content of NPY, and about 20% of its content of CGRP. Already after 20 min of stimulation, the substance P and VIP content was reduced by 25%, whereas the CGRP content was only slightly affected (the NPY content was not analyzed at this time point). Evidently, the depletion was influenced by the presence of atropine, since in the absence of the muscarinic receptor blocker the rate of depletion was somewhat slower for substance P and VIP but higher for CGRP. This loss in peptide content occurred in parallel with the depletion of large dense core vesicles from the parasympathetic nerve terminals in close association with acini [53]. In nonatropinized rats, the number of large dense core vesicles, counted in a fixed number of axon profiles, was reduced by 70%, while the corresponding figure was 90% in the atropinized rats after an 80-min period of stimulation.

In the ferret salivary glands substance P, VIP and CGRP occur in nerve fibres close to the acinar cells, ducts and blood vessels. After cutting the auriculotemporal nerve, aimed to cause a parasympathetic denervation of the parotid gland, virtually all VIP-containing nerve fibres disappeared, whereas the substance P- and CGRP-containing nerve fibres were reduced to a lesser extent. The NPY-containing nerve fibres innervated only the blood vessels, and these nerves disappeared following sympathectomy [54]. The blood flow from the parotid gland is technically difficult to collect. On the other hand, the blood draining the submandibular gland is usually easily accessible, and by using this preparation in the ferret, substance P and VIP were shown to emerge in the venous drainage of the gland in response to prolonged stimulation of the chordalingual nerve at 20 Hz in the presence as well as in the absence of atropine [30]. CGRP appeared, however, in measurable amounts only in the absence of atropine. The outputs of the peptides peaked within 5–10 min, and for substance P and CGRP the outputs fell back to initial values within 20 min, while the output of VIP faded less rapidly. The outputs of substance P and VIP in the presence of atropine exceeded those in its absence, thus showing a general pattern similar to that observed in the rat parotid gland, where the peptide release was measured indirectly. From experiments on the cat submandibular glands, Lundberg et al. [55] have suggested

that the release of VIP upon parasympathetic stimulation is affected by a presynaptic muscarinic inhibition. A similar mechanism may be valid also for the release of substance P. Decreased local degradation of the released neuropeptides has been put forward as an alternative explanation, but objections may be raised to this hypothesis, since in the rat parotid gland peptides remaining in the nerves were measured. Blockade of presynaptic muscarinic receptors of the subtype 2 is known to enhance the cholinergic transmission as shown in various tissues including salivary glands [56], and there is thus the possibilty that the M2 receptors are also involved in the release of peptide transmitters. However, the presynaptic muscarinic inhibition of peptide release is not a general phenomenon as judged by the release of CGRP. With respect to this peptide, a presynaptic muscarinic facilitation might be at work, perhaps involving the M1 subtype.

Secretory Responses to Administration of Neuropeptides

Tachykinins exert their effects, with a certain degree of selectivity, on three categories of receptors: neurokinin (NK)-1 (the substance P-preferring receptor type), NK-2 (the neurokinin A-preferring receptor type) and NK-3 (the neurokinin B-preferring receptor type). Each tachykinin may act as a full agonist at all three receptors but the affinity differs [57, 58]. Radioligand-binding studies as well as functional studies suggest that salivary glands are supplied with the NK-1 type of receptors [59]. The volume of saliva secreted from the three major salivary glands of the rat in response to substance P exceeds that of neurokinin A (previously called substance K), at submaximal doses but not at the maximal dose for secretion [60–62]. The response is not affected by the presence of atropine and adrenoceptor blockers and is also elicited in denervated glands, thus favouring the idea that tachykinins act on specific receptors without engaging the classical ones. Further support for a direct effect is gained from the in vitro observations of Gallacher [63]. Rat glands respond promptly, the submandibular gland in particular, but also the parotid gland secretes large volumes of saliva (calculated both per gland and tissue weight). The nonmammalian tachykinin physalaemin is even more potent [60]. More than one type of neurokinin receptor might be involved in the secretory responses, since the neurokinin A-induced flow of saliva had a higher concentration of amylase than that of the substance P-induced saliva [62]. Despite this difference in amylase concentration, tachykinin-induced saliva is, on the whole, poor in protein and amylase. Similar effects of tachykinins were also seen in the parotid and submandibular glands of the mink and the ferret [23, 24, 64]. However, in ferret parotid glands, intracarotid infusion of substance P also released acinar secretory granules, the percentage loss in number of granules being as large as 46% [36]. The glands mentioned so far

also respond with salivary secretion on parasympathetic nerve stimulation in the presence of both atropine and adrenoceptor antagonists. In the dog, both the parotid and submandibular glands secrete saliva in response to physalaemin [10, 11]; thus, it is possible that substance P would also cause secretion in this species, so it might perhaps be worthwhile to reconsider the old opinion that atropine completely abolishes secretion of saliva upon parasympathetic stimulation in this classical experimental animal. Finally, in the glands of the dog as well as in the submandibular gland of the rat, physalaemin caused contraction of the myoepithelial cells as judged by the rise in intraductal pressure [65, 66].

VIP, injected intravenously, evokes a small flow of saliva from the three major glands of the rat, in the presence of atropine and adrenoceptor antagonists; the submandibular gland secretes most and the sublingual gland least (calculated both per gland and tissue weight) [67]. However, the volume secreted in response to VIP amounts to only a fraction of that to substance P and further, in comparison with substance P, the latency in onset of secretion (e.g. 1.5–2 min versus <10 s in the parotid gland) as well as the duration of the secretion (5–10 min versus 2–5 min) is longer. When the secretory threshold dose of VIP is compared, on a molar basis, with that of other secretagogues it shows a high efficacy. For both parotid and submandibular glands the rank order is: substance P > VIP > isoprenaline ≥ methacholine > phenylephrine. The parotid saliva secreted in response to VIP is very viscous and shows high concentrations of protein and amylase, the amylase concentration being about 30 times that of substance P-induced saliva. The VIP-induced submandibular saliva shows a 5 times higher protein concentration than the substance P-induced saliva [67, 68]. Besides the salivary glands of the rat, the parotid and submandibular glands of the mink are, so far, the only glands where VIP, on its own, has been shown to evoke secretion [24]. In the sheep parotid gland which secretes continuously, VIP accelerates the flow rate and increases the protein concentration [25]. However, even 'silent' glands with respect to fluid secretion are stimulated by VIP administration as shown by an 'occult' release of protein and by the depletion of the acinar content of secretory granules in the ferret and the cat [31, 32, 35, 36]. Further evidence for a role of VIP has been gained in experiments on salivary glands of the cat, pig and calf where VIP has been infused during ongoing secretion elicited by parasympathetic stimulation (in the absence of atropine) or infusion of a parasympathomimetic drug , showing increases in both fluid and protein outputs [32, 69, 70]. The action of VIP has been shown to occur in the presence of the blockade of the classical receptors, in the absence of adrenal glands and further also on denervated glands. Salivary gland tissues from rat, cat and ferret respond to VIP in vitro underlining a direct effect of VIP [31, 32, 71]. Furthermore, although VIP

evoked no secretion of saliva in the dog [72], in vitro observations show that the dog parotid gland has to be added to the list of glands that release proteins in response to the peptide [73]. In the rat submandibular gland VIP released peroxidase from the acinar cells but not kallikrein from the granular tubules, which may suggest that the various secretory cells are not equally affected by the peptide [74].

PACAP has an N-terminal sequence that exhibits 68% homology with VIP. It possesses 1,000 times the potency of VIP in activating adenylate cyclase in rat pituitary cells in culture [75]. Intravenous injection of PACAP (PACAP1-38) in the rat, in the presence of adrenoceptor blockers and atropine, evokes secretion of saliva from the major salivary glands [47]. The response is similar to that of VIP with respect to the long latency in onset of secretion, the high protein and amylase concentration, the long-lasting secretion, and the relative contribution of the three glands. PACAP also released protein and potassium from pieces of rat parotid and submandibular glands in vitro. In the ferret submandibular gland PACAP alone evokes no secretion of saliva but, when injected during an on-going parasympathetic nerve-induced flow of saliva (in the absence of atropine), it enhanced the flow rate and, in particular, the output of protein [76], and also released protein in vitro. PACAP occurs in tissues as PACAP1-38 and PACAP1-27; the biological activity was found to reside in the N-terminal 1-27 sequence, which exhibits homology with VIP. When comparing the secretory responses to VIP with those to PACAP in the rat and the ferret, the effectiveness of PACAP is the same or less than that of VIP. However, when comparing the effect of the peptides on the vascular response of the submandibular glands, PACAP was more effective than VIP in reducing the vascular resistance and in increasing the blood flow in both species. At least two types of PACAP receptors have been postulated. Type I receptors bind PACAP with higher affinity than VIP, whereas type II receptors bind PACAP with similar or lower affinity than VIP [50]. Thus, the results suggest that the vascular responses to PACAP involve type I receptors, while the secretory responses rather involve type II receptors. The difference in relative potencies between PACAP and VIP, the existence of separate nerve fibre populations containing the two peptides, the difference in magnitude of reduction following denervation attempts suggest different physiological roles for them in salivary glands.

Alone, CGRP evokes no fluid secretion from the salivary glands of the rat upon intravenous administration. It does, however, cause a release of amylase in a dose-dependent way from the parotid gland as shown by subsequent wash-out injections of either substance P or metacholine. It can also release amylase from parotid gland fragments in vitro. The release of amylase as well as the fluid secretion was not affected by atropine or adrenoceptor

antagonists [45]. In the ferret submandibular gland i.v. injections of CGRP resulted in salivary secretion. Compared with the effect of substance P, the secretory cells were relatively unresponsive to CGRP [30]. In the calf submandibular gland CGRP has been found to accelerate the salivary flow and to increase the protein output during on-going parasympathetic nerve stimulation [70].

In vitro studies on rat parotid gland fragments have shown NPY to stimulate a release of amylase and protein in the presence of atropine and adrenoceptor blockade and after parasympathetic as well as sympathetic denervation [46, 77]. It was less effective than VIP and adrenaline but in the same range as substance P and the choline ester bethanechol. The same relations were obtained in the rat submandibular gland. In fragments of the rat sublingual gland, however, the NPY-induced protein release exceeded that of substance P and bethanechol [46]. In vitro release of potassium, to indicate fluid secretion, was increased in response to NPY administration from the two studied glands, the parotid and the submandibular glands. The response was larger from the submandibular gland. Compared to the potassium release caused by VIP and substance P, the release in response to NPY was less in both glands. The secretory effect of NPY in vivo has, so far, not been thoroughly assessed. When injected intravenously in the rat a trace secretion of saliva appeared from the parotid and submandibular glands. Since NPY is known to be a very potent vasoconstrictor, the high doses used might have hampered the salivary fluid response. In other parts of the digestive tract NPY is not considered as a secretagogue [78], but it stimulates mucous and not serous secretion in the ferret trachea [79].

Peptide Interactions

Many observations show positive interactions between various peptides on the one hand and between peptides and the classical transmitters on the other as to both fluid secretion and protein output. For example, in the rat parotid gland, a low intravenous secretory dose of substance P combined with doses of VIP, being subthreshold or supratheshold for fluid secretion, caused enhanced volume responses, the enhancement being largest at subthreshold doses of VIP and amounting to about twice that to substance P alone [68]. Although the amylase concentration in the saliva increased, the question whether the amylase response was enhanced was not addressed. In the ferret, where VIP on its own elicits no overt fluid secretion, the substance P-evoked volume of submandibular and parotid saliva also increased in response to the combination of the two drugs [23, 31]. By comparing the protein output in a wash-out by means of substance P 10 min subsequent to the injection of VIP to that in response to the combined injection of the two peptides, an enhanced

protein output was revealed, using substance P, 0.2 μg/kg, and VIP, 1 μg/kg. The volume of submandibular saliva was enhanced 120%, while the protein output was enhanced by 30%, thus resulting in a fall in salivary protein concentration. The opposite occurred in the parotid gland where the volume was increased by 55% and the protein output by 110%, consequently giving rise to an increase in salivary protein concentration. Substance P in combination with CGRP also resulted in enhanced responses from rat parotid glands with an almost 3-fold increase in the amylase output and a 2-fold increase in the fluid secretion [45].

Lundberg et al. [80] showed that VIP enhanced the acetylcholine-induced flow of saliva from the submandibular gland of the cat. When comparing the effect on both the fluid response and the protein output, the protein output was, however, more affected by the combined action of VIP and a choline ester [32]. Thus in both the submandibular and parotid glands of the cat the protein output in response to the combination of VIP and methacholine was about 5 times that of the additive output in response to the two secretagogues separately, while the amount of saliva secreted was doubled.

Positive interactions in the fluid response have also been detected in the rat and ferret salivary glands between substance P and the β-adrenergic receptor agonist isoprenaline [Ekström and Mirfendereski, unpubl. observation], and also between VIP and noradrenaline [81] or the α-adrenergic receptor agonist phenylephrine [82]. Substance P combined with methacholine however, did not enhance the fluid response of the rat parotid gland [29].

Many explanations are offered to account for the enhanced responses, including a VIP-induced increase in the binding of muscarinic agonists, increases in the blood flow, improved distribution of the secretagogues in the gland and decreased degradation of the agonist. However, both in vivo and in vitro studies mainly performed on the rat salivary glands point at intracellular interactions between the cyclic AMP pathway used by VIP, PACAP and CGRP as well by $\beta(\beta_1)$-adrenoceptor agonists, and the calcium/inositoltriphosphate pathway used by tachykinins, muscarinic and α-adrenoceptor agonists as the cause of enhanced fluid and protein responses (see chapter 3) [83, 84].

Involvement of Nitric Oxide

Nitric oxide (NO) may be regarded as a transmitter. However, unlike other transmitters, it exerts its effect independently of cell-surface receptors by diffusing through cell membranes to activate soluble guanylate cyclase to form cyclic GMP [85]. In the rat, a large number of the cell bodies of the parasympathetic otic ganglion as well as the parasympathetic submandibular and sublingual ganglia contain the NO synthesising enzyme NO synthase (NOS). Triple immunolabelling combined with confocal microscopy reveals,

that in the otic ganglion of the rat some nerve cell bodies may simultaneously contain NOS, VIP and the acetylcholine synthesizing enzyme, choline acetyltransferase, while in others either VIP or NOS is co-localized with choline acetyltransferase [86; Ekström, unpubl. observation]. In the periphery, periacinar cholinergic nerve terminals, indicated by the presence of vesicular acetylcholine transporter, also contained VIP and NOS. The sympathetic cervical ganglion lacked NOS-containing cell bodies and they were few in the trigeminal ganglion [86]. Finally, in the parotid gland of the rat and the ferret, denervation experiments, including treatment with the sensory neurotoxin capsaicin, showed NOS to be confined to the parasympathetic innervation [87].

Inhibition of the generation of NO by the NO synthase inhibitor L-NAME, reduced the parasympathetic nerve-evoked salivary flow rate as well as the protein output from the submandibular gland of the cat and the ferret and from the parotid gland of the sheep [88–91]. Furthermore, peptide release upon nerve stimulation may be affected by the NOS inhibitor [88, 92]. In the cat submandibular gland, the release of VIP upon stimulation of the parasympathetic innervation was diminished, suggesting reduced amounts of released VIP as a cause of the reduced protein output and flow of saliva from the cat submandibular gland in the presence of L-NAME [88]. However, a reduction of this dose of L-NAME to one-tenth still reduced the response of the submandibular gland, but now the release of VIP was unaffected, a finding suggesting an action of NO at the postsynaptic level [89]. In the ferret submandibular gland the NOS inhibitor also reduced the flow rate and the protein output in response to parasympathetic stimulation without affecting the release of VIP [90]. Acetylcholine-induced protein output and flow of saliva were, however, unaffected in the ferret gland by the presence of L-NAME. In this gland, the fluid secretion in response to administration of CGRP but not that to substance P was reduced by L-NAME [Ekström, unpubl. observation].

The source of origin of NO implicated in the secretory response to the various agonists injected into the blood stream is presently unknown. In the ferret, the output of protein in response to VIP from the parasympathetically and sympathetically denervated parotid gland is still demonstrable after L-NAME [Ekström, unpubl. observation]. There is the possibility that the secretory cells themselves generate NO, creating a background activity upon which various agonist may act. NOS activity has, in fact, been demonstrated in the cytosolic fractions of parotid and submandibular glands of a number of species, including the rat [93]. In the absence of exogenous agonists, pieces of the rat parotid gland in vitro release amylase by a mechanism partly dependent on the generation of NO, since the release decreases following administration of L-NAME or a specific inhibitor of soluble guanylate cyclase (ODQ) [94; Ekström, unpubl. observation]. Lastly, a minor fraction of the in vitro release

of amylase in response to VIP depended on NO generation, while the amylase release to bethanechol, isoprenaline and substance P was independent of NO generation. Interestingly, in the sheep parotid gland, which secretes continuously, L-NAME lowers the basal rate of protein output without affecting the salivary flow rate [91].

On the Involvement of Sensory Nerve Fibres in Parasympathetic Nonadrenergic, Noncholinergic Secretory Responses upon Electrical Stimulation

The auriculotemporal nerve trunk and the chordalingual nerve trunk carry sensory nerve fibres emanating from the trigeminal ganglion. Thus electrical stimulation of the auriculotemporal or chordalingual nerves presumably activates both afferent and efferent nerve fibres. Retrograde tracing with the fluorescent dye True Blue, injected into the parotid gland parenchyma of the rat, revealed the presence of labelled nerve cell bodies not only in the otic ganglion but also in the trigeminal ganglion and further, a proportion of the labelled cell bodies in these ganglia also showed substance P-like immunoreactivity [95]. Thus there is the possibility that antidromic stimulation of sensory nerves contributes to the secretory NANC responses of the salivary glands. Whether this is the case was addressed by examining the parotid gland of the rat 1–2 weeks following the treatment with the sensory neurotoxin capsaicin [49]. However, no evidence accrued for any involvement of afferent fibres in the secretory response of the parotid gland to stimulation of the auriculotemporal nerve. The fluid response to a whole range of stimulation frequencies was unaffected. The response in the presence of atropine and adrenoceptor antagonists persisted undiminished as to both fluid secretion and amylase output. A tachykinin antagonist reduced the NANC fluid response by the same magnitude as in the noncapsaicin-treated rats (i.e. by about 30%). Furthermore, the secretory cells of the parotid glands were just as sensitive to intravenous injections of substance P and a muscarinic agonist as those of control rats which makes it unlikely that acetylcholine, substance P or other substances released upon nerve stimulation acted on sensitized cells thereby masking a sensory contribution to the response of the gland. Recent experiments also show that stimulation of the auriculotemporal nerve at high frequencies, in the presence of atropine and adrenoceptor antagonists, 2–3 weeks after removal of the otic ganglion, allowing time for degeneration of efferent nerve fibres, evokes no fluid secretion [Ekström, unpubl. observation]. In addition, the NANC-induced fluid responses in the submandibular glands of the rat and the ferret on chordalingual nerve stimulation are completely prevented by the

ganglion-blocker hexamethonium [20, 23], findings which give further support to the idea that the secretory response is an efferent phenomenon.

A minor proportion of substance P (11%) and a larger proportion of CGRP (36%) seemed to be located in sensory nerve fibres in rat parotid glands as judged from capsaicin treatment [49]. For comparisons, the same capsaicin treatment decreased the total amounts of substance P and CGRP in the urinary bladder by about 90%, and the nerve fibres showing co-localization between substance P and CGRP virtually disappeared, while neither the VIP content nor the number of VIP containing nerve fibres were reduced. Periacinar substance P-containing nerve fibres (devoid of CGRP) and periacinar CGRP-containing nerve fibres (devoid of substance P) persisted after the capsaicin treatment. However, the nerve fibres showing co-localization between substance P and CGRP and, which occurred around ducts and blood vessels, had disappeared. Thus nerve fibres that contain substance P together with CGRP are believed to be sensory [96]. It is possible that some of the sensory nerves reached the gland via another route than the auriculotemporal nerve, as some sensory fibres have been found in the facial nerve and cervical dorsal root fibres [45]. Nevertheless, the evidence suggests that the NANC responses to stimulation of the parasympathetic innervation most likely reflect genuine efferent phenomena.

Protein Profile of Nonadrenergic, Noncholinergic Induced Saliva

Although the protein content of rat parotid saliva is markedly influenced by the type of stimulation used to evoke secretion the changes are largely quantitative rather than qualitative so far as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining as well as by two-dimension electrophoresis (2-DE) and silver staining [97, 98]. The protein distribution profile of parasympathetic-nerve-evoked saliva secreted in the presence of atropine and adrenoceptor blockers appears to be the same as that secreted in the absence of muscarinic receptor blockade. Thus, acetylcholine does not seem to add any additional protein bands to those produced by the NANC transmitters. The same conclusion can be drawn from the response to the infusion of bethanechol. In comparison to parasympathetic stimulation the response to infusions of substance P, CGRP, NPY and VIP given separately or in various combinations showed variable levels of protein 1a and the addition of plasma proteins. The result of electrophoretic analysis of cat parotid saliva does also indicate predominantly quantitative differences when comparing the responses to parasympathetic stimulation, infusion of bethanechol alone and bethanechol combined with

VIP [99]. Electrophoretic analysis of the in vitro release of proteins from rat parotid and submandibular acinar cells exposed to carbacholine and neuropeptides does also indicate quantitative rather than qualitative differences in the responses [100, 101]. Thus, the attempt to find specific proteins that would mark the NANC actions has so far met with no success.

Nonadrenergic, Noncholinergic Contribution to the Secretory Response Evoked by Electrical Stimulation of the Parasympathetic Innervation

Effects of Antagonists

The substance P analogue [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}]-SP (0.75–1 mg/kg i.v.) abolishes or markedly reduces the substance P (0.2–0.5 μg/kg i.v.) evoked secretion of parotid saliva in the rat. It also reduces the fluid response to parasympathetic nerve stimulation (at 20 Hz) in the presence of atropine and adrenoceptor antagonists, by 30–50%. However, in the absence of atropine, the substance P analogue did not reduce the flow of saliva in response to the nerve stimulation [102]. In the rat submandibular gland, the VIP antagonist [*N*-Ac-Tyr^{1}, D-Phe^{2}]-GRF-[1–29]-NH_2 reduced the amount of saliva secreted in response to stimulation of the chordalingual nerve by 15% and the protein concentration of the saliva by 35% [103].

In the ferret submandibular and parotid glands, the substance P-analogue [D-Arg^{1}, D-C^{12}Phe^{5}, Asn^{6}, D-Trp^{7,9}, Nle^{11}]-SP (0.75 mg/kg i.v.) completely abolished substance P (0.5 μg/kg i.v.) and neurokinin A (5 μg/kg i.v.) evoked salivation, without affecting the response to a muscarinic agonist. In the presence of atropine and adrenoceptor antagonists, and in contrast to the rat parotid gland, the persisting NANC-evoked nerve-stimulated secretion of saliva was completely or almost completely abolished by the substance P analogue [64]. However, under these circumstances and in response to prolonged nerve stimulation the exocytotic response of the parotid gland was unaffected, the loss in number of secretory granules being 25% compared to 27% in the presence of atropine only [36]. When turning to the nerve-evoked response in the absence of muscarinic receptor blockade, the analogue reduced the amount of saliva secreted over a range of stimulation frequencies. At the maximal response for fluid secretion the reduction was 40% in the submandibular gland (at 20 Hz) and 20% in the parotid gland (at 40 Hz). Upon administration of atropine the persisting response was completely or almost completely abolished [64].

Thus, several transmitters are potential contributors to the parasympathetic secretory NANC responses but, not suprisingly, no single one appears to fit the role as the sole transmitter [104].

Effects of Neuropeptide Depletion

Since many NANC transmitters are likely to contribute to the parasympathetic fluid response, the approach was taken to deplete the rat auriculotemporal nerve of its peptide stores by prolonged nerve stimulation (40 min) at a high frequency (40 Hz) [29]. This was followed by a 40-min rest period (during which there was no recovery in the function of the NANC mechanisms). The initial period of high frequency stimulation was preceded by intravenous injections of standard doses of methacholine and substance P and by stimulation of the auriculotemporal nerve over a wide range of frequencies (0.2–60 Hz). When this protocol was repeated after the rest period, the amount of saliva secreted at the various frequencies was reduced, by 100–50% at the lower frequencies (0.2–2 Hz) and by 20% at the frequencies in the upper range (10–60 Hz). It seems unlikely that the shift to the right in the frequency-response curve was due to a decrease in the secretory capacity of the gland or to a failure in the cholinergic transmission: the responsiveness of the secretory cells to submaximal doses of methacholine and substance P was not decreased but actually increased; the maximal secretory response to substance P was unaffected by the preceding period of prolonged nerve stimulation; the acinar cells had the capacity to secrete saliva at a high and steady rate at 40 Hz for at least twice the time presently used; and the maximal secretory response to nerve stimulation (following prolonged stimulation) was not enhanced by preventing the breakdown of released acetylcholine, while submaximal responses were. Thus, the NANC mechanisms, when acting in concert with acetylcholine, are of greater importance for the magnitude of the fluid response at frequencies lower than those required to elicit secretion of saliva on their own in the presence of muscarinic and adrenoceptor blockade.

Role of Nonadrenergic, Noncholinergic Mechanisms in Reflex Secretion

Exocytosis and Flow of Saliva

In their study on anesthetized and atropinized (but not adrenoceptor antagonist-treated) sheep, Reid and Titchen [25] found the continuous flow of saliva from sympathectomized parotid glands to accelerate and the salivary protein concentration to increase in response to distension of the distal thoracic esophagus. A more definite role of NANC effects in the transmission of secretory impulses emerged from a series of experiments on the parotid gland of the conscious rat in response to feeding, where loss of acinar secretory granules and reduction in glandular amylase activity indicated reflexly elicited

secretory activity [105–108]. The two indices changed, on the whole, in parallel, but re-synthesis of amylase during on-going parasympathetic stimulation may have occurred [109]. However, the formation of new secretory granules takes a much longer time [110, 111]. Therefore, comparisons based on changes in number of granules rather than on changes of glandular amylase activity appears to be more appropriate. It should also be mentioned that, in contrast to many other species, the rat chews on both sides at a time (see Matsuo, chapter 10) [112] and secretes at equal flow rates from the parotid and the submandibular glands of both sides.

Sympathetic activity is usually considered to be responsible for the bulk of acinar cell degranulation in rat parotid glands in response to eating [113]. This view is supported by the marked reduction in the number of secretory granules (65%) in response to prolonged sympathetic nerve stimulation in anesthetized rats, a reduction prevented by the pretreatment with adrenoceptor antagonists [107]. However, in contrast to this general belief, the parotid gland, sympathetically denervated 10–12 days in advance, lost 22% of its granular number in rats pretreated with adrenoceptor blockers and offered hard chow (for 60–90 min) subsequent to a 30-hour period of fasting [105]. Following atropinization, the loss was even greater (50%) and it persisted after the additional pretreatment with adrenal medullectomy or the sensory neurotoxin capsaicin (51 and 45%, respectively). The feeding response required an intact parasympathetic auriculotemporal nerve, since no degranulation occurred when, in addition to the other treatments, this nerve had been cut in advance. In rats exposed to cold (2–4 ºC) and offered hard chow at the same time the parotid glands, sympathectomized in advance, also show an extensive acinar degranulation [106, 114]. Cold stress is known to activate the sympathoadrenal system [115] and the acinar degranulation that occurred under these conditions was initially attributed to the action of circulating catecholamines [114]. However, in that study the parasympathetic nerve supply was intact, and neither the effects of adrenal medullectomy nor adrenoceptor antagonists were assessed. When the sympathectomy was combined with adrenal medullectomy and pretreatment with adrenoceptor antagonists and atropine, the response to feeding in the cold was a 60% decrease in the granular number [106]; and, once again, the persisting degranulation depended on an intact auriculotemporal nerve. Thus, the parasympathetic NANC mechanisms were potentially responsible for the exocytotic response of the sympathetically denervated glands regardless whether the rats were exposed to cold stress or not.

Circulating catecholamines from the adrenals and extra-adrenal sources under cold stress might contribute to acinar degranulation if the secretory cells have been markedly sensitized by combined parasympathetic and sympathetic denervation. In one and the same atropinized rat, the sympathectomized plus

parasympathectomized parotid gland did respond to cold, but the contralateral sympathectomized gland did not; further, medullectomy reduced the response to feeding of glands subjected to the combined denervations but not of the gland subjected to sympathectomy only [106].

It should be noted that chronic sympathectomy is likely to create favorable conditions for demonstrating NANC induced responses in salivary glands. The sympathetically denervated parotid gland of the rat develops a supersensitivity to substance P [60] and VIP [44] injected intravenously. Further, the neuropeptide content (VIP and CGRP) of the parasympathetic salivary innervation tends to increase as a consequence of the sympathetic denervation [44, 45]. It has been reported by Harrop and Garrett [116] that little acinar degranulation (or decrease in glandular amylase activity) occurs in the parotid gland in response to food intake in rats if subjected to unilateral sympathetic decentralization (i.e. cutting the preganglionic nerve) 24 h before feeding. Thus, it may be argued that the observed effects in chronically sympathectomized parotid glands in response to feeding is a long-term effect produced by the sympathetic denervation. However, another experimental approach [107], avoiding surgery and using morphological assessment, gave results that were different from Harrop and Garrett [116], but a role for noradrenaline was supported. The experiments showed that the loss in number of granules from normal glands in response to feeding was 52% (and that of amylase activity 38%), whereas after acute elimination of the catecholamine influence by the intraperitoneal administration of adrenoceptor antagonists there was a fall in granule numbers of 31% (and a decrease in gland amylase activity by 32%) [107]. If the pharmacological treatment included not only the adrenoceptor antagonists but atropine also, a fall of the same magnitude occurred. These NANC responses depended on an intact parasympathetic innervation, since they were not observed after parasympathetic denervation performed well in advance. Thus, in glands supplied with an intact innervation parasympathetic NANC mechanisms are responsible for the acinar responses, in the presence of α- and β-adrenoceptor antagonists, with respect to both degranulation and loss in amylase activity and play an important part in the absence of any receptor blockers.

The NANC-induced acinar degranulation and loss in glandular amylase activity in response to the intake of hard chow depended on masticatory-salivary reflexes (see chapter 11 of Hector and Linden). This conclusion was drawn from experiments where the consistency of the pelleted diet was changed to a liquefied form [108]. However, parotid glands of rats kept on liquid diet over a period of time atrophy [117, 118] and the total amounts of substance P, VIP and CGRP in the glands decrease [119]. Therefore, it might be argued that the NANC mechanisms following liquid regimen would be less efficient.

As a consequence of such an argument, the protocol included not only animals offered liquid diet but also animals then offered pelleted diet in the final test. The glands activated in response to the hard chow (in the presence of atropine and adrenoceptor antagonists) showed a reduction in the number of granules by 50% and in the total amylase activity by 70%, changes that were more marked than those in the rats maintained on the pelleted diet. Most likely, the enlarged responses reflect the development of the phenomenon of supersensitivity, and increased fluid responses to secretagogues, administered at submaximal doses, have previously been demonstrated in rats kept on a liquid diet [120]. Although the glands acquired an increased responsiveness as a result of the liquid regimen, and though the rats offered the liquid diet at the final test consumed twice as much food as those offered the hard chow, there was no decrease in number of granules or in glandular amylase activity. Thus, despite the fact that any sapid components of the hard chow would be more available for stimulation of the taste buds in the liquefied form, gustatory stimuli did not participate to a great extent in the reflexly evoked NANC degranulation and loss in amylase activity under these conditions.

However, taste can elicit a NANC-mediated flow of saliva from the duct-cannulated parotid gland of the concious rat in the presence of muscarinic and adrenoceptor blockade [121]. The mean (as well as the peak) NANC-induced flow rate to ascorbic acid (0.5 *M*), applied on the tongue (every 30 s for 10 min), was 10–30% of the flow rate in the absence of any receptor blockade. This NANC secretion depended on an intact parasympathetic innervation, since it did not occur when the auriculotemporal nerve had been cut acutely. The response showed similarities with the NANC response upon parasympathetic stimulation: there was a high concentration of amylase activity (4–6 times that in the absence of muscarinic receptor blockade with or without adrenoceptor blockade); its onset was slow (20–70 s versus <10 s in the absence of muscarinic receptor blockade with or without adrenoceptor blockade); and it fatigued rapidly. A tachykinin antagonist abolished or almost abolished the NANC-induced flow of saliva, indicating an important role for tachykinins in the response. However, NANC transmitters other than the tachykinins were also most likely participating, and were responsible for the high concentration of amylase in the saliva and probably enhancing the fluid response.

It was more difficult to standardize the experimental protocol with respect to chewing, since the eating periods varied, but the mean and peak flow rates in the presence of atropine and adrenoceptor antagonists were 35 and 47% of those in the absence of any blockers, respectively. Again, the onset of secretion was slow (70 s versus <10 s) and the flow rate decreased with time.

When comparing the relative contribution of the NANC mechanisms to the reflexly elicited flow of rat parotid saliva with that to the reflexly elicited release of secretory granules, the NANC mechanisms seem to play a much greater role in the exocytotic response: they were potentially responsible for the whole of the parasympathetic exocytotic response and for an important part of the response in the absence of any blockers. However, their role in the fluid response may be greater than the present result implies, since they may interact positively with acetylcholine (and possible with noradrenaline also) as seems to be the case upon stimulation of the parasympathetic nerve (see above).

Effects on the Gland Contents of Neuropeptides

As with parasympathetic nerve stimulation, at 40 Hz, feeding resulted in neuropeptide release when muscarinic receptors were blocked [122, 123]. In the rats pretreated with atropine and fed, the parotid gland lost 40% of its total amount of substance P and 25% of its total amount of VIP. The content of the third peptide tested, NPY, did not decrease. The result was almost the same if the pretreatment included adrenoceptor blockade (being 42% for substance P and 23% for VIP). These values may be compared with those obtained upon stimulation of the auriculotemporal nerve at 40 Hz, during which substance P and VIP decreased in parallel, with time and after 60 min the total amounts of substance P and VIP were reduced by 75% and that of NPY by 50% [46, 51]. However, in the absence of atropine or just in the presence of adrenoceptor blockade, the contents of substance P and VIP were unaffected on feeding. Neither an intact sympathetic innervation nor a capsaicin-sensitive(sensory) innervation was a neccessary prerequisite for the depletion to occur under the muscarinic receptor blockade.

On the Participation of Neuropeptides in Vascular Protein Leakage and Edema Formation under Reflex Conditions

Some of the neuropeptides that are present in the salivary glands and likely to transmit parasympathetic secretory impulses are also known to cause microvascular protein leakage and, as a result, tissue swelling. Substance P, neurokinin A and CGRP are important mediators in sensory neurogenic inflammatory responses in a number of tissues such as the airways, eye, skin and the urinary bladder [124–127]. The parotid gland is encapsulated by a fascial layer. In response to feeding or electrical stimulation of the parasympathetic nerve in rats, a swelling of the soft tissue surrounding the gland was often found, usually without morphological support for any intraglandular water accumulation. The periglandular edema, which was of varying size, occurred in the absence as well as in the presence of atropine and α- and β-

adrenoceptor antagonists, and thus implies a contribution by NANC mechanisms in the phenomenon. Evans blue binds to albumin and other plasma proteins, and an accumulation of this dye in a tissue indicates vascular permeability changes [128]. In salivary glands, permeability to macromolecules such as albumin is thought to be particularly restrictive [129]. However, after feeding hard chow over a period of 60 min in the absence of any autonomic receptor blockers, the total amount of extractable Evans blue in the parotid gland tissue (together with its periglandular edema) was increased by 116%, compared with the glands of nonfed animals. This indicates that plasma protein extravasation is a natural event [130]. The increase was 126% in those rats not only given atropine and α- and β-adrenoceptor blockers but also pretreated with the sensory neurotoxin capsaicin 2 weeks in advance, showing that mediators of sensory origin are not a prerequisite for the phenomenon to develop. Evans blue accumulated in response to parasympathetic nerve stimulation applied at a frequency of 40 Hz. In the presence of the three autonomic receptor blockers, the total extractable amount of the dye increased by 50 and 53% (compared with the contralateral gland) after 10 and 20 min of stimulation, respectively, while in the absence of any blockers, the corresponding increases were 81 and 123%. When the parenchyma and the periglandular fluid were analysed separately, the increase in extractable dye was 56 and 177%, respectively, after the 20-min period of stimulation in the absence of blockers.

The effects of a number of peptides of parasympathetic origin were tested. Upon the separate intravenous administration of CGRP, VIP, PACAP, substance P and neurokinin A, only the latter induced protein extravasation increasing the accumulation of Evans blue in the parotid gland by 75%. However, combinations of substance P with either VIP, PACAP or CGRP also increased the vascular permeability. The parasympathomimetic drug pilocarpine which by itself had no effect on the accumulation of Evans blue, enhanced the neurokinin A induced response, causing an increase of 236%. The effect of pilocarpine was evidently specific, since this drug in combination with CGRP or VIP lacked effect. Neither a profuse secretion, such as that in response to substance P, or a high gland blood flow, such as that in response to VIP and PACAP, was enough to cause plasma extravasation. In some other parts of the digestive tract of the rat neurokinin A, and not substance P, has also been found to be the more effective tachykinin in inducing protein extravasation on its own.

Extravasation of macromolecules usually takes place at postcapillary venules through endothelial gaps [131]. However, the distance over which peptides must diffuse to exert their effect on the vascular permeability may be long if originating from parasympathetic periacinar and periductule nerve fibres, as might be the case for the tachykinins. Furthermore, the tachykinins may have

to be resorbed into the capillary circulation before acting on appropriate receptors on the endothelium of the venules [132]. The magnitude of the microvascular leakage in the parotid gland, showing 1.5- to 3-fold increases in the accumulation of Evans blue, is in the same range as that observed in the gastrointestinal tract and pancreas in the rat and mouse [133–135]. This is in contrast to the urinary bladder where the tachykinins alone or in various combinations caused 22- to 34-fold increases in the accumulation of Evans blue. Interestingly, bradykinin which is thought to release neuropeptides from sensory nerve fibres induced a 24-fold increase in the bladder but no effect on the parotid gland.

Nonadrenergic, Noncholinergic Mechanisms and Trophic Effects

Gland Weights

Salivary gland atrophy following denervation was originally reported by Claude Bernard in 1864 [136] and has since been found in many studies. The gland size diminishes more profoundly in response to parasympathetic denervation than to sympathetic denervation. Some glands may even increase slightly in weight following extirpation of the superior cervical ganglion as the submandibular gland of the cat [137]. Surprisingly, prolonged treatment with atropine, or an atropine-like drug, does not mimic the effect of parasympathetic denervation on gland size; atropinization causes just a slight atrophy or none at all [17, 138–142]. The parotid gland of the rat looses about 30–40% in weight in response to section of the auriculotemporal nerve, whereas that to the removal of the superior cervical ganglion is about 10% [143, 144]. Pilocarpine administered for 10 days is either without effect on rat parotid gland weight [145] or causes a weight gain of about 20% at a very high dose (10 mg/day) [146]. However, at this dose level, the effect of pilocarpine on the parenchyma may be indirect, through a β-adrenergic pathway evoked by the action of pilocarpine on the superior cervical ganglion [147]. Isoprenaline induced gland enlargement is a well-known phenomenon [148], and when given over a period of time the rat parotid gland weight may increase 10-fold [149, 150], the effect being mediated via β_1-adrenoceptors [151, 152] as for secretion [153, 154]. The atropine-like drug Hoechst 9980 (piperidino-ethyl-diphenyl acetamide hydrochloride) administered over some weeks increased the parotid gland weight by about 15%, both with and without an intact sympathetic innervation [17]. The atropinization was accompanied by an increase in the activity of the acetylcholine synthesising enzyme, choline acetyltransferase, and was thought to reflect increased traffic of parasympathetic secretory nerve impulses as a consequence of the dry mouth [155]. In the light

of present knowledge, it seems reasonable to attribute the gain in weight of the rat parotid gland to the action of parasympathetic NANC mechanisms.

Circumstantial evidence also associates neuropeptides with gland weights. During periods when the salivary gland development in the rat is characterized by rapid growth and differentiation, the total amounts of the peptides VIP, substance P and CGRP increase markedly and in surges. There is an initial rise within the first 2–4 weeks of life and then another rise 1 or 2 weeks later [156]. Further, disuse caused by a change in dietary regimen from pellets to a liquid diet, induces a gradual decrease in parotid gland weight, while the weights of the two other major glands are affected only slightly or not at all [117, 118, 157]. In the parotid gland, showing a weight loss of about 40%, not only the total activity of choline acetyltransferase but also the total amounts of substance P, VIP and CGRP decreased (by 62, 57 and 45%, respectively) [119].

More direct evidence for the involvement of neuropeptides in the regulation of the gland weights is gained from experiments where rats were exposed to long-continued peptide treatments. In early studies by Bertaccini et al. [158] in 1966 and Cantalamessa et al. [159] in 1975 intraperitoneal injections of the nonmammalian tachykinin, physalaemin, for about 2 weeks increased the weights of both parotid and submandibular glands, while another nonmammalian tachykinin, eledoisin, did not. In another type of experiment, the approach was taken to attempt to prevent the fall in parotid gland weight following parasympathetic denervation or change to a liquid regimen [144]. In conscious rats, intravenous infusions, twice daily, with substance P and VIP diminished or largely prevented the expected fall in gland weight after 6 days. Infusions of bethanechol and pentagastrin, the latter trophic to the pancreatic gland, were without effect as was saline. To exclude any catecholamine influence arising from the infusions, these were performed in the presence of adrenoceptor antagonists. As judged by the levels of RNA and DNA, the effects of substance P and VIP on parotid gland weight seemed to be related to cell size rather than to cell number.

Stimulation of the parasympathetic innervation increases the incorporation of radiolabelled thymidine in sublingual and parotid glands of the rat, indicating an increase in mitogenic activity [160,161]. Ongoing studies show that this parasympathetic effect on rat parotid glands involves the action of NANC mechanisms [Ekström, unpubl. observation]. The NANC mechanisms are, in fact, potentially responsible for the whole mitogenic response to the parasympathetic nerve stimulation in this gland in the absence of atropine (but in the presence of α- and β-adrenoceptor antagonists).

Polyamine Metabolism

The polyamines putrescine, spermidine and spermine are low molecular aliphatic amines that seem to occur in all living tissues. They are usually associ-

ated with cell replication, differentiation and growth rate and affected by growth-promoting hormones or factors in tissues such as the prostate gland, regenerating liver, ovary, mammary gland, kidney and hepatomas [162, 163]. The enzyme ornithine decarboxylase catalyzes the formation of putrescine from ornithine, a reaction considered as rate-limiting in the production of polyamines. In all three major salivary glands of the rat, the formation of polyamines was influenced by both sympathetic and parasympathetic nervous activity, and a role for parasympathetic NANC mechanisms was discovered [160, 164–169].

The nerve-evoked NANC responses were most conspicuous in the sublingual glands. Upon continuous parasympathetic stimulation at 20 Hz (over 3 h) the ornithine decarboxylase activity increased 30-fold in these glands and 10-fold in the parotid and submandibular glands (versus 25- and 2-fold increases, respectively, in the absence of the autonomic blockers). The putrescine concentration increased 90-fo1d (versus 130-fold) in the sublingual glands, while it was unaffected in the two other glands (versus a 2-fold increase in the absence of the blockers). A further focus on the sublingual glands showed enhanced responses when the stimulation was changed to an intermittent mode, and already at 2 Hz applied intermittently, the enzyme activity increased 2-fold in the presence of blockers.

Infusion of substance P and VIP (over 3 h in the presence of atropine and the adrenoceptor antagonists) induced dose-dependent changes in all three types of glands, and once again the most marked effects were observed in the sublingual glands. Furthermore, VIP was much more effective than substance P. In sublingual glands, at a dose hundred times less than that of substance P, VIP induced a 155-fold increase in ornithine decarboxylase activity, while the increase in response to substance P was only 10-fold. The putrescine concentrations increased 10-fold in response to VIP and 2-fold in response to substance P.

Putrescine may also be formed from the higher polyamines spermine and spermidine. However, neither substance P nor VIP was found to induce such an interconversion. The fact that VIP does not mobilize the inversed pathway deserves comments. Both VIP and isoprenaline are thought to use cyclic AMP as intracellular messenger, but evidently the chain of events initiated by VIP and isoprenaline differs, since the β-adrenoceptor agonist showed a high selectivity for the inversed pathway.

Observations on Human Glands

NANC mechanisms are likely to be of importance for both the major and the minor salivary glands in humans, since the acinar cells are innervated

by peptide-containing nerve fibres (see chapter 1) [170–173]. The presence of VIP-containing nerve fibres is particularly frequent. The acinar supply of NPY-containing fibres is less abundant, and any supply of substance P- and CGRP-containing nerve fibres is rare or absent. The blood vessels are not only well supplied with both VIP- and NPY-containing fibres, but also by substance P- and CGRP-containing fibres and the two latter peptides may be co-localized, suggesting a sensory origin.

Functional studies on human glands are few. The in vitro release of potassium, indicating fluid secretion, from fragments of the submandibular gland is not affected by administration of substance P and VIP. However, VIP elevates the tissue content of cyclic AMP, known to be associated with protein secretion [174]. Interestingly, a number of peptides, i.e. substance P, neurokinin A, CGRP, NPY and VIP, occurred in resting parotid saliva and increased in response to chewing [175]. In humans, quantitative data on the effectiveness of atropine or atropine-like drugs to inhibit the secretion of saliva seem to be sparse. The parotid and submandibular flow rates evoked by 2% of citric acid were reported to be unaffected by the oral intake of atropine at a dose of 1 mg, while the flow of pilocarpine was reduced by 75% [176]. In another study [177], the parotid flow rate in response to chewing decreased by 64% following the oral intake of the atropine-like drug oxyphencyclimin at a dose of 10 mg, while the flow rate elicited by citric acid was reduced by 76 and 54%, respectively, to acid concentrations of 0.5 and 5.0%. Whether a complete blockade of the muscarinic receptors was achieved is not known, and the combined effect of a muscarinic receptor blocker and adrenoceptor antagonists was not tested.

In the clinic, swelling of the parotid gland with or without pain, is one of the more common conditions affecting the gland and, while thought to be due to the formation of edema and stretching of the glandular capsule, the fundamental cause is often unknown. A number of case reports concern transient swelling of the parotid gland associated with general anesthesia and the use of belladonna alkaloids as premedication [178]. Neuropeptides may be involved in these conditions, as judged from the fact that some of them increase the vascular permeability. In addition, release of neuropeptides have been put forward as one of the hypotheses on the etiology to postsympathectomy pain in the parotid gland appearing on eating [179, 180].

Epilogue

In some glands the parasympathetic NANC mechanisms evoke an overt secretion of saliva. The relatively high threshold frequency required for the

response to appear, the slow onset and the fading over time may cause the phenomenon to go unnoticed. In other glands the NANC mechanisms just cause the release of proteins and acinar secretory granules without any overt secretion of fluid. The parasympathetic secretory NANC mechanisms are reflexly mobilized in conscious animals by mastication and taste, underlying their physiological significance under natural conditions. The NANC mechanisms also exert long-term influences on the salivary gland systems. They were of importance for both gland cell size and gland cell number, and they stimulate the synthesis of polyamines involved in growth and cell differentiation, and further, they may play a role in gland growth during development. A number of neuropeptides are likely to transmit the parasympathetic NANC effects, and the action of some may to some extent depend on the generation of NO. The role of NO in secretion as well as the origin of NO are presently unclear. Lack of effective peptide antagonists has hampered the progress in the research field of regulatory peptides. However, powerful tachykinin antagonists are available, and they interfere with the parasympathetic secretory nerve effects.

A striking feature of the NANC-evoked flow of saliva is its long latency in onset upon parasympathetic nerve stimulation and upon reflex activation. Apart from the fact that the effector response to nerve stimulation may vary in latency due to such things as to which type of postsynaptic receptor is being activated, the viscosity of the saliva and whether myoepithelial contractions are elicited or not, the speed of transmitter release and transmitter diffusion are likely to be of importance. Peptide neurotransmitters are stored in large dense cored vesicles and these vesicles are more slowly released from the nerve terminals than those small vesicles storing the classical transmitters. Furthermore, the large dense cored vesicles commonly appear at a distance from the presumed 'synaptic' membranes, whereas the small vesicles are more concentrated there, but such sites are variably close to the adjacent postsynaptic membrane (chapter 1).

Conditions of prolonged electrical stimulation of the whole parasympathetic nerve trunk at a high frequency such as 40 Hz applied continuously or infusion of secretagogues into the blood stream continuously over a period of time are no doubt unphysiological. Nevertheless these protocols provide reproducible findings about distinctions in effector responses accruing from the action of different agonists. Under normal reflex conditions a wide range of impulses is likely to occur intermittently, in a variable number of nerve fibres at any one time and allow various agonists to interact synergistically. It is usually emphazised that the greatest release of NANC transmitters in response to electrical nerve stimulation requires high frequency stimulation, often applied in bursts [181, 182]. However, the NANC mechanisms presently under study were found to exert actions on the secretory cells at low frequencies

applied continuosly. Protein was released from atropinized glands at 0.2–2 Hz [31]. A seemingly ineffective parasympathetic stimulation in the range of 0.5–2 Hz in atropinized animals, when combined with exogenous substance P in subsecretory or low secretory doses, gave rise to secretion of saliva or enhanced secretion of saliva, respectively, revealing a release of NANC transmitters at these low frequencies [23, 68]. In the absence of atropine, the relative contribution of the NANC mechanisms to the parasympathetic nerve evoked fluid response was found to be greatest at 0.2–2 Hz [29], presumably due to synergistic effects. Compared to the corresponding continuous mode of stimulation, a burst pattern, of high-frequency stimulation, did not improve the parasympathetic NANC-elicited volume of saliva secreted nor the outputs of protein and amylase in rats and ferrets [26, 183]. The activity of ornithine decarboxylase, was affected by low frequencies applied to the parasympathetic nerve in the presence of atropine but here, on the other hand, the intermittent mode of stimulation seemed to be the most effective [160]. Emmelin and Holmberg [184] compared, in the same dogs, the reflexly evoked submandibular flow rate with that to electrical stimulation of the parasympathetic nerve, applied in a continuous mode, and found that the fastest flow rate to feeding and to lemon juice, respectively, corresponded to 4–8 Hz and 10–30 Hz. Direct recordings from the parasympathetic salivary nerves in the sheep revealed instantaneous frequencies up to 120 Hz [185], and in the rat two types of patterns, a tonic discharge of 5–30 Hz and a transient burst discharge of 50–80 Hz followed by a prolonged discharge at 5–40 Hz were found [186]. So impulse rates are likely to vary greatly under different natural conditions.

The results presented in the reflex studies on the rat parotid gland seem to imply a larger role for the NANC mechanisms in exocytoses and protein secretion than in fluid secretion. So far, a NANC induced flow of saliva has only been demonstrated in a few species. In contrast, a general feature in the glands of a large number of species is the NANC- and neuropeptide-induced protein secretion. The relative contributions of adrenergic, cholinergic and nonadrenergic, noncholinergic transmitters in the reflex control of salivary secretion are not easily defined. Transmitters of the various pathways interact. Pharmacological or surgical interruption of one pathway to glandular activation may create increasing demands on those remaining and, as a consequence, induce short-term compensatory mechanisms. Atropinization, in itself, influences the release of neuropeptides from the nerve terminals [51]. Furthermore, long-term compensatory mechanisms following chronic denervations may involve increases in transmitter levels [44, 45, 155] in the persisting pathways and the development of supersensitivity in the secretory cells [143, 187] (chapter 9). Under natural conditions, the various nervous mechanisms supported by the action of circulating catecholamines, when liberated, are likely to work in

concert to achieve the most purposeful reflex response, and in this case the neuropeptide content of the nerve terminals are probably less reduced.

A number of puzzling observations made in the past on cat submandibular glands, seems to be explained by the action of NANC transmitters, like VIP, which exert prolonged actions on the secretory cells without evoking secretion of saliva on their own. Thus, in the presence of a dose of atropine that completely prevented the fluid response to stimulation of the parasympathetic innervation: Macintosh and Rawlinson [188] found the sympathetic fluid response to be markedly enhanced by a preceding period of parasympathetic stimulation, illustrating Langley's phenomenon of 'augmented secretion' [189, 190]; Barcroft [191] and Strömblad [192] found an increased oxygen consumption in response to parasympathetic stimulation that was not correlated with the increase in blood flow; Anrep and Cannan found the blood-sugar consumption to increase in response to the parasympathetic stimulation [193]; and, more recently, membrane potential changes in the secretory cells were recorded in response to the parasympathetic stimulation [194].

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Chapter 7

Effects of Autonomic Denervations on Parenchymal Structure and Nerves in Salivary Glands

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Historical Introduction

Changes that occur in salivary glands after sectioning one or other division of their autonomic nerve supply, thereby removing the normal reflex impulse traffic, help to shed further light on the roles of these nerves in salivary gland function. In this way, Bernard [1] showed that the reflex secretion from dog submandibular glands, in response to placing sapid substances on the tongue, was inhibited by acute section of the chorda-lingual nerve. In 1864 [2], he studied the long-term effects of 'section and degeneration of the nerves' to the submandibular gland of dogs and found that 'the gland shrank and showed marked structural change', but no details were given about the latter.

Some confusion developed with subsequent studies on the microscopical effects of denervations on salivary glands because of the inadequacies of the methods. In the 1930s, Rawlinson [3, 4] attempted a systematic study of the microscopic effects of chronic denervations on cat submandibular glands, but he was also hampered by inadequate methods. Nevertheless, he clearly showed that the striated duct cells undergo atrophy with loss of their striations after lingual nerve section [3] and eventually look like 'simple conducting tubes'. He also found that the central acinar ('alveolar') cells soon showed atrophy after sectioning the lingual nerve, and the demilunes became very small, but this occurred more slowly. After longer times, when the demilunes were small, they showed more conspicuous vacuolation on sympathetic nerve stimulation [4] than in normal glands. He was equivocal about the effects

of sympathectomy but thought atrophy occurred occasionally in some striated duct cells.

In 1951, Emmelin et al. [5] found similar morphological changes as Rawlinson [3, 4] in cat submandibular glands after sectioning the chorda where it leaves the chorda lingual nerve. They described the light-microscopic events [5] as varying in degree, with reduction in the size of all parenchymal structures, particularly the demilunes which were often vacuolated, and the striated ducts showed clear areas. Similar changes were also caused by chronic antimuscarinic treatment, using repeated large doses of atropine. The morphological changes could be reversed by chronic pilocarpine treatment after nerve section, or by cessation of atropine treatment. Gland weights were reduced after chorda section, compared with control glands from normal untreated animals. However, the glands contralateral to those receiving chorda section showed an increase in weight compared to untreated control animals, suggesting that some compensation was occurring from an increased nerve impulse traffic on that side from reflex stimulation. In the atropine-treated animals, however, the gland weights remained the same as in the control animals. Post-ganglionic sympathectomy did not appear to cause any obvious morphological changes.

Schneyer and Hall [6] showed that 'severance of the post-ganglionic parasympathetic innervation to the parotid gland of the rat resulted, at 4 weeks, in appreciable reduction in the size of the whole gland and of the individual acinar cells'. Amylase was also greatly depleted in the glands and it was concluded that 'The parasympathetic innervation is involved in maintenance of normal concentrations of amylase in the acinar cells.' Sympathetic ganglionectomy [7], on the other hand, led to only a small decrease in gland size after 2 weeks but there was a slight increase in amylase concentration in the gland, and auriculotemporal nerve stimulation caused a greater secretion of amylase into the saliva from the denervated gland. It was concluded that the sympathetic innervation exerts only a small influence on gland structure. Nevertheless, combined post-ganglionic parasympathetic and sympathetic denervation caused greater atrophic effects than parasympathetic denervation alone.

Emmelin devoted much time to studying the functional changes caused by denervating salivary glands [8, 9]. He discovered that a 'paroxysmal secretion' of saliva occurred intermittently from the parotid duct of anaesthetised cats 1–3 days after post-ganglionic parasympathectomy [10]. Subsequently, after confirming that it was attributable to leakage of transmitter from the degenerating nerve, it became known as 'degeneration secretion' [11]. Such secretion occurs over different time scales in different animals and is influenced by the length of nerve that has to degenerate [11]. Post-ganglionic sympathec-

tomy also induced a degeneration secretion from rat salivary glands during anaesthesia that was plentiful from submandibular glands but sparse from parotid glands [11].

Emmelin and co-workers [8, 9] initiated systematic studies on the increases in glandular sensitivities, to transmitters and other agonists, after denervations had deprived the glands of normal neurotransmitter release, and this aspect is given special attention in chapter 9.

Changes in the Nerves within Salivary Glands following Post-Ganglionic Axotomy

Extensive post-ganglionic parasympathectomy can be done on parotid glands but parasympathetic denervation of submandibular glands is more limited because they contain many of their ganglion cells within the substance of the gland. However, extensive post-ganglionic sympathetic denervations are posible in both parotid and submandibular glands.

Surgical denervations have been useful for identifying (1) the course of different nerves to the glands; (2) the sources of different neurotransmitters in the glands; (3) the existence of uncharted nerves from unconventional sources; (4) any regrowth of nerves in the long term; (5) neuro-effector sites served by the cut nerves, in short-term experiments, and (6) axons remaining in neuro-effector relationship after degeneration of the cut nerve (the last two require electron-microscopic assessment).

Light-Microscopic Changes

Post-Ganglionic Parasympathectomy of Parotid Glands

These studies show that the routes of the nerves are somewhat variable, so the denervations are always incomplete to greater or lesser degrees and, as a consequence, some regeneration occurs with time.

In cats, avulsion of the auriculotemporal nerve caused a progressive depletion of acetylcholinesterase (AChE)-positive nerves from the parotid glands between 2 and 6 days later [12]. Although extensively depleted at 6 days onwards, some nerves always persisted and their numbers showed variations between animals. Even after combined auriculotemporal nerve avulsion and post-ganglionic sympathectomy some nerves still persisted. So the remaining nerves were unlikely to be sympathetic and were seemingly parasympathetic nerves from uncharted sources. From 16 days after axotomy there was a gradual increase of AChE-positive nerves in the parotid glands, but their

distribution was patchy and by 64 days they were considered to represent only about 60% of the nerves in the contralateral control glands.

Similar results occurred with dog parotid glands [13], no change was detectable in the AChE-positive nerves 48 h after surgery, but thereafter there was a progressive variable loss of these nerves. Their depletion was least after simple section of the auriculotemporal nerve. It became greater when this was combined with stripping nerves from the internal maxillary artery in the vicinity of the auriculotemporal nerve. This procedure was undertaken because Holmberg [14] had found functionally that many of the parasympathetic nerves to dog parotid glands course with the artery rather than in the auriculotemporal nerve. Nevertheless, this denervation was always incomplete within the gland. However, there was a further, but still incomplete, depletion of AChE-positive nerves in the parotid gland when the above combined manoeuvre was undertaken together with section of the facial nerve, as it left the stylomastoid foramen. Even so, small numbers of AChE-positive nerves always persisted in the gland, and those around the main ducts seemed unaltered, so must have come from a further source. However, most, if not all, of the pre-ganglionic parasympathetic input to the dog parotid gland had been found functionally, with respect to secretion, to occur via the classical tympanic nerve source [15], so the neuronal relay for the post-ganglionic nerves was likely to be in the otic ganglion, with somewhat variable pathways from there to the parotid gland. In long-term recovery animals, more nerves were always present than in the short term [13], suggesting that there had been sprouting from intact nerves. However, the process was always patchy and never complete.

Similar denervation studies on rats [16] and rabbits [17], combining functional assessments and AChE histochemistry, indicated that their parotid glands also receive some post-ganglionic parasympathetic nerves from uncharted sources in addition to those that travel in the auriculotemporal nerve.

Post-Ganglionic Sympathectomy

Parotid Glands. In some species sympathetic ganglionectomy does not remove all the adrenergic nerves in parotid glands.

Removal of the superior cervical ganglion in dogs caused an extensive depletion of parotid adrenergic nerves [13], but it was never complete and a few adrenergic nerves always persisted in association with parenchymal cells, indicating that these nerves must have arisen from another ganglionic source. However, all the vascular sympathetic nerves appeared to have been destroyed. Stripping the external carotid artery caused a big decrease in adrenergic nerves in the lower half of the gland, but the innervation of the upper half appeared to be normal, which suggests that its adrenergic innervation may course with the internal carotid artery.

In rats also, excision of the superior cervical ganglion did not remove all of the adrenergic nerves from the parotid gland [18] but, in contrast to the dog, some adrenergic nerves persisted around blood vessels. The source of residual nerves is likely to include the contralateral superior cervical ganglion [19]. Nevertheless, even after bilateral ganglionectomy occasional adrenergic nerves still persisted in rat parotid glands [20], so their ganglionic relays must have been closer to the glands and were possibly intracranial.

Contrasting with dogs and rats, unilateral excision of the superior cervical ganglion in rabbits caused a complete disappearance of adrenergic nerves from the parotid gland on the same side [17, 21]. So, in this species, all the adrenergic nerves to the parotid gland appear to arise conventionally from the ipsilateral superior cervical ganglion. It has also been reported that superior cervical ganglionectomy causes total loss of adrenergic nerves from the parotid gland of the cat [13]. Section of the intracranial post-ganglionic extension from the superior cervical ganglion in cat was found to cause a considerable loss of adrenergic nerves from the parotid gland [22]. This may help to explain Nordenfelt's [23] functional observation that many of the sympathetic nerves to cat parotid glands pass via the tympanic cavity.

Submandibular Glands. Removal of the superior cervical ganglion caused total loss of adrenergic nerves from the submandibular gland on the same side, in rabbits [21], rats [18] and cats [22]. In the latter, the loss of catecholamine from the glandular nerves after ganglionectomy occurred mainly between 24 and 48 h. Section of the post-ganglionic sympathetic nerve trunks on the external carotid artery caused extensive but incomplete loss of adrenergic nerves in cat submandibular glands [22]. The denervation was more complete if the nerve trunks and external carotid artery were divided together between ligatures, but a few residual nerves persisted in the gland and their course there, after leaving the superior cervical ganglion, is not known. In these experiments, with incomplete loss of adrenergic nerves in the short term, some reappearance of adrenergic nerves tended to occur in the glands with time, but its extent varied between animals.

Effects of Denervations on Neuropeptides

Surgical denervations have been very useful for determining whether a particular neuropeptide present histochemically in nerves in a gland arises from parasympathetic or sympathetic neurones, or afferent nerves. This has also been corroborated by changes in the glandular content of the transmitter assessed immunochemically [24]. More detailed information is provided in chapter 1 and by Ekström (chapter 6) so no further account will be given in this chapter.

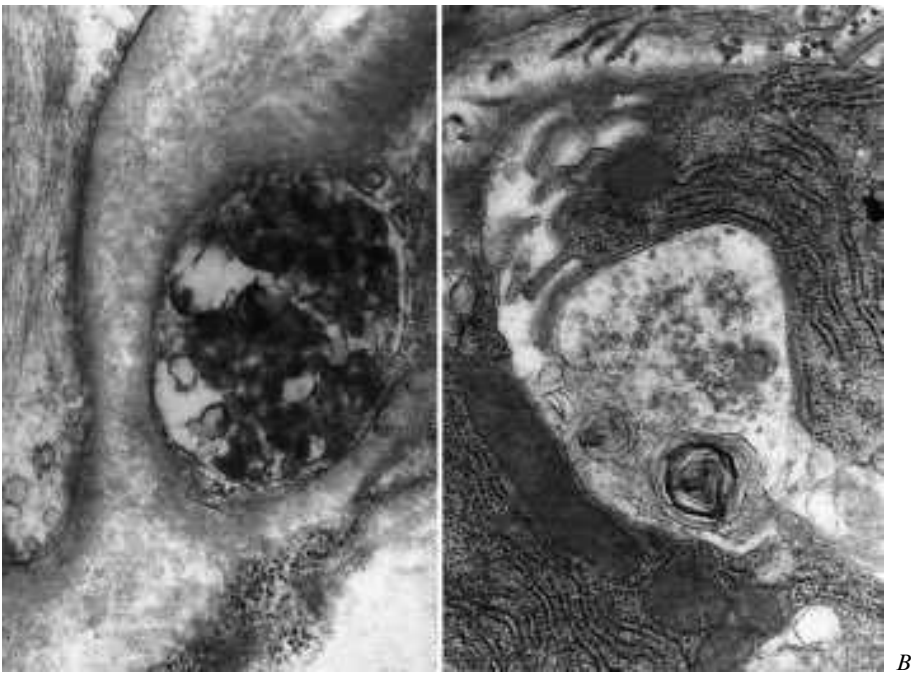

Fig. 1. Electron micrographs of degenerating intraglandular axons following surgical section of the post-ganglionic sympathetic nerve supply outside the gland. *A* Cat submandibular gland 48 h after axotomy showing an osmiophilic degenerative axon in epilemmal association with an arteriolar smooth muscle cell. × 45,000. Reproduced from Garrett and Kemplay [22]. *B* Rat parotid gland 24 h after axotomy showing a degenerative hypolemmal axon (below) in association with an acinar cell and adjacent to a normal parasympathetic axon (above). × 24,000. Reproduced from Garrett and Thulin [26].

Ultrastructural Changes

In cats, early electron-microscopic studies [25] showed that, at 2–4 days after section of post-ganglionic nerves, characteristic osmiophilic degenerative changes appeared in the terminal axons within the glands (fig. 1A). These dark degenerative appearances were very conspicuous using primary fixation with osmium tetroxide, as was the fashion in those days. Degenerative axons were detected in neuro-effector relationships with parenchymal cells, blood vessels and myoepithelial cells in cat parotid and submandibular glands after sympathetic ganglionectomy, and in parotid glands after post-ganglionic parasympathectomy. Furthermore, when degeneration of the post-ganglionic sympathetic axons was complete at 8–12 days after surgery, and before any regeneration had occurred, residual axons considered parasympathetic were

found in similar neuro-effector locations. From these results it was deduced that, in cats, the salivary parenchymal cells, blood vessels and myoepithelial cells each receive dual innervations from sympathetic and parasympathetic efferent nerves.

In rat parotid glands degenerative osmiophilic changes occurred in terminal axons between 12 and 24 h after post-ganglionic sympathectomy [26], and were detected in hypolemmal situations between acinar cells (fig. 1B). Similar osmiophilic degenerative changes were also found in terminal axons of rat parotid glands in the early stages after chemical sympathectomy by 6-hydroxydopamine [27].

Parenchymal Changes in Salivary Glands following Denervations

This aspect of denervations has not been studied as extensively by modern methods as is desirable. However, even with electron microscopy, the changes are not always consistent and often not readily describable, so concise impressions do not always emerge. Nevertheless, the work helps to throw light on the influences impulses from one or other type of the autonomic nerve supply have on maintaining the structural integrity of the cells in salivary glands.

Long-Term Effects of Denervations

Cat Submandibular Glands

As mentioned in the introduction, Rawlinson [3, 4] studied the histology of these glands after surgical denervations and found little change after post-ganglionic sympathectomy, but cutting the chorda affected each of the 3 main types of parenchymal cells (in central acini, demilunes and striated ducts). The changes were, however, ill-defined by the methods used. The subject was reinvestigated by Kidd and co-workers [28–31] using more modern methods, including histochemistry and electron microscopy, in the expectation that they would provide greater clarification. Nevertheless, the results were still not always as clear cut as hoped for. Throughout, post-ganglionic sympathectomy caused little structural effect on the parenchymal cells, though at 4 days after surgery there was a depletion of secretory granules from the striated ducts. This was attributed to 'degeneration secretion' that had been shown to occur episodically from cat submandibular glands round about 2 days after sympathetic ganglionectomy [32]. So it was considered that the granules had not yet reformed by 4 days [29], although they were seen at later times after sympathetic ganglionectomy.

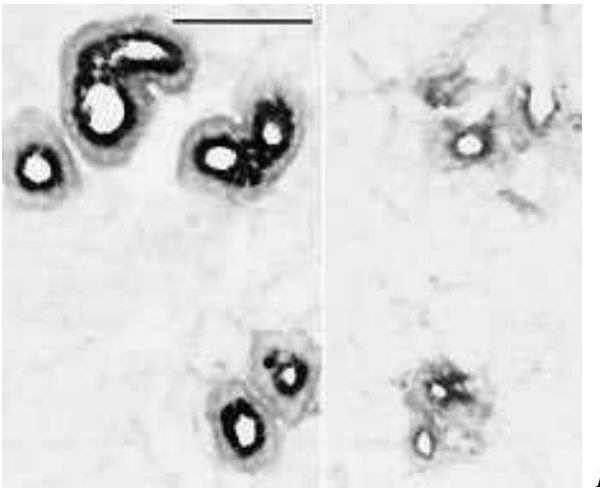

Fig. 2. Light micrographs of sections of both submandibular glands from the same cat stained enzyme histochemically for tissue kallikrein on the same slide. Bar = 100 μm. *A* Normal gland showing dense periluminal granule staining in striated ducts. *B* Contralateral gland 3 weeks after chorda excision showing atrophy of the striated ducts and extensive reduction in staining for kallikrein. Modified from Garrett et al. [30].

Parasympathetic denervations of cat submandibular glands, on the other hand, always caused atrophic changes, but the effects on acini tended to be variable in extent and to have a patchy distribution [28], probably due to the variable amount of any post-ganglionic denervation that is achieable. Accordingly, the changes were greatest when there had been an extensive removal of the chorda from the duct, up to the hilum of the gland; a procedure that creates a partial post-ganglionic denervation. The less atrophic areas in the glands after parasympathectomy may have reflected parts still possessing their own intraglandular ganglion cells, from which there may have been small axonal leakages of neurotransmitter, even though their neurones received no pre-ganglionic impulses.

The most conspicuous change in the glands after partial postganglionic parasympathectomy was a progressive atrophy of the striated ducts, as Rawlinson [3] had found. Mucosubstance histochemistry and electron microscopy showed that this was accompanied by a loss of secretory granules from these cells [29]. Staining for kallikrein, by means of a chromogenic oligopeptide substrate, showed that the atrophy of striated ducts after parasympathectomy was accompanied by a severe depletion of glandular kallikrein from their cells (fig. 2) [30]. However, the cells could still secrete residual amounts of kallikrein

on sympathetic nerve stimulation. Thus, although the secretory drive to degranulate the cells remained, feline striated ducts must depend on parasympathetic impulses for normal synthesis, even though parasympathetic drive itself causes only minimal secretion of kallikrein. Emmelin and Henriksson [33] showed that not only parasympathectomy but also chronic antimuscarinic treatment would greatly reduce the amount of submandibular kallikrein secreted in sympathetically induced saliva. Thus, it would appear that acetylcholine, normally released from parasympathetic terminals in cat glands, has an important function in maintaining the structure of striated ducts and their capacity to synthesise kallikrein.

The acinar changes in cat submandibular glands after parasympathectomy were less well defined, apart from a reduction in cell size [28, 31]. This was accompanied by an increased prominence of myoepithelial cells with protuberances into the interstitium together with loose pleating of associated redundant basal lamina. The secretory granules in demilunes and central acinar cells showed ultrastructural alterations that made it difficult to distinguish between the two types of cell. Enzyme cytochemistry for peroxidase, normally found only in demilune cells, stained granules in some cells that were difficult to classify after parasympathectomy, suggesting that these modified cells were of demilunar origin [31].

It is concluded from these studies that structural well being and normal protein synthesis plus formation of secretory granules in central acinar, demilunar and striated ductal cells of cat submandibular glands are dependent on reflex stimulation by transmitters from parasympathetic nerves.

Rabbit Submandibular Glands

Parasympathetic denervations of rabbit submandibular glands [34] caused them to lose weight and show atrophy of the acinar and granular tubule (neck) cells, but the changes were not uniform throughout any gland. As with the cat, the effects were greater after partial post-ganglionic denervation (chorda excision along the duct) than with pre-ganglionic denervation (section of the chorda lingual nerve). A depletion of secretory granules occurred from both types of cell at 2 days after chorda excision and this was attributed to 'degeneration secretion' found 1–3 days after similar surgery by Ohlin [35]. Thereafter, the secretory granules did not reform to a normal extent, tended to develop unusual appearances and in granular tubule cells often showed intracellular fusions, a feature not seen in normal glands. In contrast, the striated duct cells looked healthy and tended to accumulate glycogen, suggesting that there was a reduced metabolic demand on these cells after removal of parasympathetic drive. Functionally, at 3 weeks after pre-ganglionic parasympathectomy, a 'paralytic secretion' of saliva from the submandibular gland

was discovered in urethane anaesthetised rabbits [36], in addition to the usual slow 'spontaneous' secretion from these glands. The extra secretion was totally blocked by atropine but only partially blocked by large doses of α-adrenoceptor blockade and unaffected by β-adrenoceptor blockade. Thus, acetylcholine leakage from terminal parasympathetic nerves still connected to their neurones appears to be an essential factor in this 'paralytic secretion' of saliva, and depends on the activation of cells made supersensitive by the discontinuation of reflex transmitter release. Catecholamines made only a secondary contribution under these circumstances. This contrasts with parasympathetic 'paralytic secretion' from dog submandibular glands first described by Bernard [2] in morphine-anaesthetised animals, which was eventually found by Emmelin [37] to be due to adrenal catecholamine release, under these conditions, acting on supersensitive cells. Perhaps the 'paralytic secretion' after pre-ganglionic parasympathectomy of rabbit submandibular glands helps to explain why the reduction of gland weight lessens after 3 weeks and the cells then look less abnormal [34].

Chronic post-ganglionic sympathectomy, although causing total loss of adrenergic nerves from the gland, induced no detectable changes in the secretory cells, and there was no change in the weight of the glands [34].

Rat Parotid Glands

As mentioned in the introduction, Schneyer and Hall [6, 7] found that post-ganglionic parasympathectomy of rat parotid glands caused reductions in gland weight, acinar cell size and also of glandular amylase. Ekström [38] found that 2–3 weeks after post-ganglionic parasympathectomy (avulsion of the auriculotemporal nerve), both the wet and dry weights the glands on the operated side were about 40% less than on the control side. Chronic post-ganglionic sympathectomy (excision of the superior cervical ganglion) caused a reduction in gland weight on the operated side of about 13% compared to the intact side. Morphologically, after chronic post-ganglionic sympathectomy, resting parotid glands showed no ultrastructural differences in the acini and their granule content was similar to normal glands [20, 39]. Even after combined post-ganglionic parasympathectomy plus sympathectomy, although causing extensive atrophy, normal-looking acinar granules were seen light microscopically [40]. This must mean that, although the synthetic capacity to form amylase was severely reduced, an ability to form and fill secretory granules continued to exist, despite the extensive removal of normal reflex neurotransmitter stimulation of the cells. So an innate ability for secretory granules to be formed must persist in these cells once they have reached maturation and full genetic expression. Thus, rat parotid acinar cells are not so dependent on continuing stimulation from neurotransmitters as is the synthesis and pack-

aging of kallikrein by striated duct cells in cat submandibular glands (see previously).

Despite the seemingly normal morphology of resting parotid acini after chronic post-ganglionic sympathectomy [20, 39], the glands showed a functional defect in their capacity to secrete fluid maximally in response to high frequency parasympathetic stimulation. Saliva output was drastically reduced to only about one third of that from normal animals, and a greater tendency for watery vacuolation was detected in sympathectomised glands after the parasympathetic stimulation. Nevertheless, the total output of amylase secreted was the same as from normal animals and the extent of the degranulation appeared similar. These unexpected results serve to emphasise that the secretion of pre-packaged secretory protein from the cells does not necessarily run in tandem with the mobilisation of fluid by the same cells. The results also show that a normal input of sympathetic impulses are required in some way for rat parotid cells to maintain their capacity for maximal secretion of fluid.

The foregoing parts of this section point out that the deprivation of sympathetic impulses has only small effects on the resting morphology and granule contents of parotid acini in adult rats. However, neonatal avulsion of the superior cervical ganglion in rats caused a significant decrease in parotid acinar cell size and granule content [41]. This indicates that normal maturation of rat parotid acinar cells does depend on trophic influences from sympathetic nerves in the gland but, once mature, the cells are not so dependent on such influences for their continuing structural well-being. The developmental trophic influences did not require β-adrenoceptor activation [41] (although it induces degranulation and hypertrophy), suggesting that non-adrenergic transmitter release from the sympathetic nerves may be involved.

Rat Submandibular Glands

Ohlin [42] found that pre-ganglionic sympathectomy induced a 13% reduction in the wet and dry weights of rat submandibular glands after 3 weeks. Ekström and Malmberg [43] confirmed this and showed that removal of the superior cervical ganglion caused a 10% reduction in submandibular gland weight after 3–4 weeks. Pre-ganglionic parasympathectomy (sectioning the chorda-lingual nerve) caused a reduction in submandibular gland weight of 25% at this time.

Unilateral post-ganglionic sympathectomy, for 4–47 days' duration, was found to have no effect on the overall light-microscopic features in rat submandibular glands [44]. However, the tendency for watery vacuoles to form in acinar cells increased on the operated side, and the authors considered that this may have been a functional response due to an increased sensitivity to neurotransmitter released from parasympathetic nerves.

After chorda lingual nerve section in rats, Peronace et al. [45] indicated that the most pronounced atrophic changes occurred in the submandibular acinar cells, which became very small. The tubule cells were said to show less change. We can confirm that after excision of chorda fibres from along the duct the acinar cells become small and dark staining with haematoxylin and eosin, whereas the granular tubules seem unchanged [Garrett and Proctor, unpubl. obs.]. Uddin [46] found that pre-ganglionic parasympathectomy caused no obvious change in rat submandibular striated ducts, but he did not consider the granular tubules. He also described an increase of the kallikrein activity in the glands, on a unit/mg protein basis. However, he did not take into account any loss of weight in the glands associated with the acinar atrophy, so it is possible that the total amount of kallikrein approximated that in the control glands and was not actually increased. We have found that neither sympathectomy nor parasympathectomy caused any changes in the levels of kallikrein in glandular homogenates [Proctor and Garrett, unpubl. obs.].

The absence of overt change in the granular tubules of rat submandibular glands [45], or of any reduction in the kallikrein content of the glands [46], after parasympathectomy, contrasts with the dramatic atrophic effects of similar denervation on the striated ducts in cat submandibular glands and their kallikrein content [29, 30, 33], so warrants special comment. It would seem that, in the cat, release of parasympathetic transmitters is essential in mature submandibular glands both for maintaining the structural integrity of the striated duct cells and for their capacity to synthesise kallikrein. On the other hand, with mature granular tubules in rat submandibular glands, the maintenance of genetic expression for kallikrein synthesis depends on a continuing presence of the hormonal influences, initially responsible for their maturation, and not on receiving parasympathetic nerve impulses.

Although lack of space precludes general attention to rat sublingual glands, a special finding from parasympathectomy warrants special mention. Murakami et al. [47] found that resection of the chorda within 48 h after birth inhibited the normal formation of actin in the myoepithelial cells and they did not mature properly. This effect of denervation decreased progressively with the time at which surgery was undertaken and no effects were detected in the myoepithelial cells with denervations at 30 days after birth.

Morphological Changes Accompanying 'Degeneration Secretion'

It has already been mentioned that some of the morphological changes in the early stages after post-ganglionic denervations were possibly due to the secretory effects of transmitter release during degeneration of the terminal axons. For example, the loss of secretory granules in striated ducts of cat

submandibular glands 4 days after avulsion of the superior cervical ganglion was attributed to such 'degeneration secretion' [30]. Similarly, a depletion of secretory granules from acini and granular tubules in rabbit submandibular glands 2 days after chorda excision, from the duct, was also thought to be due to 'degeneration secretion' [34].

However, these interpretations were circumstantial and the subject was not studied objectively in these species. In rat parotid glands, time sequenced morphological events after unilateral avulsion of the superior cervical ganglion have been studied to reveal any 'degeneration activation' of the acinar cells and the results were correlated with changes in the nerves [26]. The animals had been fasted overnight prior to surgery under short-acting anaesthesia and food continued to be withheld from these conscious animals. Parotid tissues were removed from the operated and non-operated sides under terminal anaesthesia at 12, 24 and 48 h after sympathectomy for morphological assessment. Macroscopially, at 12 and 48 h, both parotid glands appeared white and opaque, whereas at 24 h the sympathectomized glands were pinkish, translucent and watery [26], features seen after sympathetic stimulation of the gland [48]. Catecholamine fluorescence showed no obvious loss of adrenergic nerves in the glands after 12 h, extensive loss of their noradrenaline content at 24 h and the depletion was maximal within 48 h [26]. This corresponds with biochemical results [49] showing that a small reduction of noradrenaline began after 8 h and the process was complete within 24 h. Ultrastructural degenerative osmiophilic changes were occasionally seen in intraglandular axons at 12 h, they were common at 24 h (fig. 1B) and no longer seen 48 h after surgery [26]. Parotid acini from both glands appeared similar by light and electron microscopy at 12 and 48 h after sympathectomy and were packed with secretory granules. However, at 24 h after surgery there had been an extensive exocytosis of secretory granules on the operated side (fig. 3). This loss of granules is attributed to 'degeneration secretion' caused by release of transmitter(s) from the terminal sympathetic axons between 12 and 24 h after ganglionectomy, and it corresponds with the timing of 'degeneration secretion' of saliva from rat parotid glands after post-ganglionic sympathectomy [50]. However, the amount of the degranulation was very extensive [26], whereas the fluid formed in anaesthetised animals was sparse [50]. It therefore seems possible that the exocytosis had been facilitated in the conscious animals by coincidental reflex parasympathetic stimulation, from swallowing, etc., and reflects a synergism between different transmitters. Whatever the case, the results suggest that the release of sympathetic transmitter(s) normally has the potential for causing exocytosis of parotid acinar granules in rats.

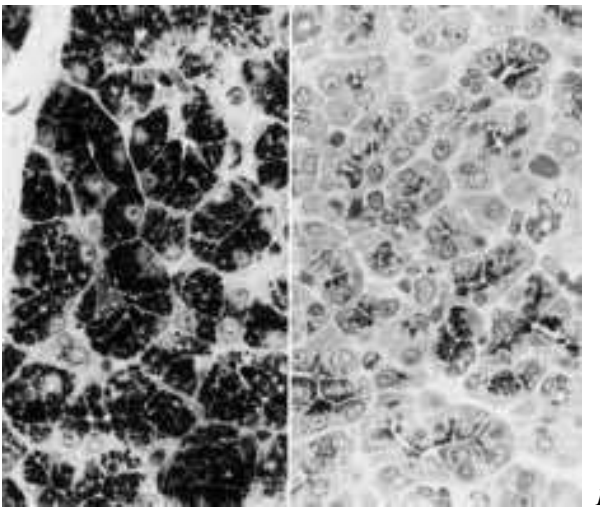

Fig. 3. Light micrographs of plastic sections of both parotid glands from the same rat stained with toluidine blue. × 500. *A* Left control gland showing acini packed with secretory granules. *B* Right gland 24 h after postganglionic sympathectomy showing extensive depletion of secretory granules from 'degeneration activation'. Reproduced from Garrett and Thulin [26].

Effects of Selective Denervations on Reflex Changes

Rat Parotid Glands

Hodgson and Speirs [51] found that pre-ganglionic sympathectomy did not prevent a flow of saliva from rat parotid glands during gustatory reflex stimulation, but it did reduce the normal depletion of parotid amylase on chewing hard chow. So we undertook similar experiments to detect if any morphological differences occurred in the glands [52]. This study showed that in normal fasted rats the parotid acini were packed with granules. Animals which had eaten a meal of hard chow showed extensive exocytosis of the secretory granules, with a corresponding decrease in glandular amylase. Twenty-four hours after unilateral pre-ganglionic sympathectomy, eating produced the customary reduction of acinar granules on the intact side, but exocytosis had been greatly inhibited on the sympathectomised side and the glandular amylase content was greater. These results indicate that sympathetic impulses normally make an important contribution to the reflex secretion of parotid acinar granules in rats.

A study by Ekström and co-workers [40], using special manoeuvres including post-ganglionic denervations and blocking drugs, has shown that non-

adrenergic, non-cholinergic transmitters from parasympathetic nerves, as well as adrenoceptor stimulation, can also be involved in reflex degranulation.

It is concluded from these studies that, under natural reflex conditions, interactions between different transmitters released from both sympathetic and parasympathetic nerve impulses are likely to contribute normally to the secretion of rat parotid acinar granules. This provides another example indicating that the autonomic nerves in the glands act in concert and not in conflict (as is still commonly believed [53]).

Rat Submandibular Glands

Unilateral pre-ganglionic sympathectomy was performed on rats fasted overnight [54]. Food was withheld for a further 24 h, then some animals were given a meal of hard chow and others remained unfed. Submandibular glands were then removed for microscopic examination. Results were very similar in the fed and unfed groups. Eating therefore appears not to be associated with any extra secretion of pre-formed protein from the submandibular acini. On the denervated side the glands were larger. Light and electron microscopy showed that the acinar cells were much larger in the denervated gland and packed with mucosubstance-containing granules. On the normally innervated side the acinar cells were smaller, contained fewer smaller granules and appeared as if there had been recent secretory activity which, in the absence of eating, is attributable to reflex stimulation from grooming, swallowing and thermal regulation.

It is concluded from the finding of an accumulation of mucins in submandibular acini deprived of sympathetic impulses in conscious animals, that it is necessary to have an intact sympathetic pathway for normal reflex secretion of submandibular acinar mucosubstance to occur in rats. This is given support from nerve stimulation experiments which showed that sympathetic stimulation induced exocytosis of granules from normal acinar cells but prolonged strong parasympathetic stimulation did not [55].

Rabbit Submandibular Glands

Unilateral pre-ganglionic sympathectomy was performed under short acting anaesthesia on rabbits, that had been fasted overnight and copraphagia prevented [34]. Then after about 3 h of recovery the animals were fed hay for 1 h, which they ate vigorously. Submandibular glands were then removed for morphological assessment. A similarly denervated but unfed animal acted as control. No differences were detected between sections from the denervated and the control gland in the unfed animal, as was also found after long-term post-ganglionic sympathectomy. In the fed animals an extensive depletion of acinar mucosubstance had occurred in the normally innervated gland, but on the sympathetically decentralized side there was far less secretion of any

mucosubstance from the acinar cells. No differences were detected between the granular tubule cells in either gland.

It is concluded that, in rabbit submandibular glands, there is a reflex secretion of acinar mucosubstance on chewing. This contrasts with the absence of any special changes in the corresponding cells of rat submandibular glands on eating chow [54]. The results indicate that the reflex acinar secretion from rabbit submandibular acini, on chewing, normally involves an intact sympathetic nerve supply, but any role these nerves may have on the granular cells under these conditions was not apparent. This is in accord with nerve stimulation experiments which showed that sympathetic impulses caused acinar degranulation but no changes were evident in the granular cells [56]. However, parasympathetic nerve stimulation per se can also cause an acinar degranulation [57]. Therefore, the extent of any reflex involvement of parasympathetic impulses acting on their own under the eating conditions used is questionable, since any acinar degranulation was very small after pre-ganglionic sympathectomy. Nevertheless, it is likely that synergistic interactions normally occur between the effects of the sympathetic and parasympathetic transmitters released reflexly and this may have been responsible for the extensive acinar degranulation seen in response to eating in the submandibular glands with intact nerve supplies.

Concluding Remarks

Assessment of early degenerative changes in intraglandular axons and of those that persist, after sectioning the main postganglionic nerve trunks outside the gland, confirm that the cells in cat salivary glands receive a dual innervation by parasympathetic and sympathetic nerves. However, the routes by which the nerves travel to the glands are not confined to conventional anatomical pathways in cats or other species.

In acute experiments, pre-ganglionic sympathectomy causes abnormal retention of secretory granules in certain acinar cells during reflex stimulation, indicating an importance of these nerves for normal responses. Nevertheless, the evidence indicates that such nerves are unlikely to operate in isolation and, under natural conditions, harmonious interactions between the various transmitters from both divisions of the autonomic nerve supply occur, and lead to a greater effectiveness in the secretory changes being induced.

During an early phase after post-ganglionic denervations morphological changes are detectable in some parenchymal cells that can be attributed to ‘degeneration activation’, due to the release of transmitters from the degenerating axons.

Long-term denervation studies show that a normal reflex impulse traffic in autonomic nerves is commonly required to maintain the structural normality of parenchymal cells in salivary glands. As with all other aspects of salivary function, wide variations exist between species, gland types and cell types in their individual requirements. In general terms, chronic deprivation of parasympathetic impulses tends to produce greater atrophic effects. However, subtle effects from loss of sympathetic impulses are being revealed. For example, postganglionic sympathectomy, in some obscure way, reduces the fluid secretory capacity of rat parotid glands in response to parasympathetic stimulation. The effects of denervations on protein synthesis and secretion are considered in chapter 8. Paradoxical differences exist between the extent of the changes induced by denervation on certain cells and the effects that the same nerves have on exocytosis from them. Thus, whereas parasympathetic impulses cause little or no structural changes in cat submandibular striated ducts or rat submandibular acini, depriving the same cells of parasympathetic impulses causes them to undergo severe atrophic changes.

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Chapter 8

Effects of Automatic Denervations on Protein Secretion and Synthesis by Salivary Glands

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Introduction

Saliva performs a number of functions which are crucial to the maintenance of oral homeostasis. Moistening of food before swallowing or the removal of food residues and debris from the mouth could in theory be fulfilled by the presence of water or any other fluid in the mouth. However, saliva has special physical and biochemical properties which result from its composition and enable it to interact with microorganisms, coat tissue surfaces and maintain oral calcium homeostasis. Most of these functions are dependent to a large extent upon the protein components of saliva. Clearly, if oral health is dependent upon salivary proteins then it is also dependent upon the mechanisms which control the synthesis and secretion of salivary proteins. Autonomic nerves not only exert profound acute control over salivary gland secretion but also have long-term influences on the gland and are required for the maintenance of normal parenchymal cell metabolism. These short- and long-term influences of autonomic nerves on salivary glands can be examined through the delivery of autonomimetics or receptor-blocking drugs.

However, as the parasympathetic and sympathetic nerves supplying sali vary glands contain combinations of neurotransmitters, an effective way of examining their influences is to remove them by surgical denervation. In this chapter the influence of denervation on the synthesis and secretion of salivary proteins will be considered.

Salivary Protein Secretion from Normal Glands

The main protein-secreting cells in salivary and other exocrine glands are the acinar cells which contain prominent protein storage granules. Acinar cells have been the focus of research into salivary protein secretion and the cellular mechanisms of protein secretion have been described in the previous volume of this series [1, 2]. In many salivary glands, particularly the rat parotid and submandibular glands, in which protein secretion has been most extensively studied, the sympathetic nerves provide an important impetus for secretion of stored protein. Stimulation of the sympathetic nerves leads to a profound exocytosis of storage granules from the protein-storing acinar cells which is accompanied by a decrease in the secretory protein (e.g. parotid amylase) content of a gland and a secretion of saliva rich in protein (see chapter 4). The effects of sympathetic stimuli on rat acinar cells are mediated by β_1-adrenoceptors and the intracellular coupling of stimulus to secretion involves rises in cAMP and the activity of protein kinase A [3] (see chapter 3). Stimulation of the parasympathetic nerves in general leads to secretion of a copious saliva containing lower concentrations of protein which nevertheless may lead to substantial secretion of proteins. These parasympathetic effects are mediated through muscarinic cholinergic receptors and receptors for neuropeptides such as VIP and substance P (see chapter 6).

However, during feeding both sympathetic and parasympathetic nerves are reflexly activated by taste and chewing reflexes but the saliva formed may not exhibit such contrasting features as occurs upon stimulation of individual nerve supplies. When both the parasympathetic and sympathetic nerves are electrically stimulated suboptimally under experimental conditions, there tends to be an augmented secretion of protein, that is, protein output is greater than on individual nerve stimulation [4]. Thus, the sympathetic and parasympathetic nerves tend to co-operate rather than antagonize each other's secretory effects.

Ductal cells, particularly those in striated ducts, have a well-recognized role in modulating the ionic composition of saliva but they also secrete some proteins. For example, in man, cat and other species the proteolytic enzyme kallikrein has been localized in small apical secretory granules of ductal cells [5] whilst in rats and mice there exists a population of cells, in granular ducts, which are major protein-storing cells [6]. In all of these cells sympathetic nerve stimuli again provide the main impetus for protein secretion but this time the effects are mediated mainly through α-adrenoceptors. Parasympathetic nerves appear to have much less of a protein-mobilizing effect on these cells [7].

Effects of Denervation on Salivary Protein Secretion

Following an initial 'degeneration secretion' that accompanies section of both autonomic nerves supplying salivary glands, fluid secretion ceases almost entirely [8]. Given that nerves are responsible for protein as well as fluid secretion it might seem that denervation of both the sympathetic and parasympathetic nerve supplies to salivary glands should be accompanied by an absence of protein synthesis and secretion. However, other protein secretory pathways exist which do not involve neurotransmitter-stimulated exocytosis of protein storage granules [1]. Thus the constitutive and constitutive-like protein secretory pathways operate under anaesthesia in the absence of nerve-mediated stimuli and most likely continue to operate following denervation (see 'IgA secretion' later). It is uncertain whether such pathways provide an effective secretion to the oral cavity in the absence of fluid secretion which in general terms is dependent upon nerve-mediated stimuli, but they probably help to ensure that some of the cellular synthetic processes continue in the absence of nerve impulses.

'Degeneration Secretion' and Supersensitivity of Protein Secretion

In the hours after post-ganglionic parasympathetic nerve section there is a period of 'degeneration secretion' which results from neurotransmitter being released from the degenerating nerves and acting on salivary secretory cells [9]. This phenomenom is not observed following decentralization where the post-ganglionic nerve fibres remain intact. During post-ganglionic sympathetic denervation, 'degeneration secretion' of fluid is barely perceptible from rat parotid glands under anaesthesia although it is more obvious from rat submandibular glands [10]. However, following sympathetic denervation of the rat parotid gland a large depletion of amylase does occur as a result of the 'degeneration secretion' of protein under these conditions [11]. This secretion can be abolished by β-adrenoceptor blocking drugs and so is due to the release of noradrenaline from degenerating nerves acting through β-adrenoceptors to cause exocytosis. These results were consistent with those of an earlier morphological study which demonstrated that a massive degranulation of protein storage granules occurs from the parotid gland as a result of 'degeneration secretion' [12]. Studies on 'degeneration secretion' provided evidence for the existence of some sympathetic fibres originating from the contralateral superior cervical ganglion, since stimulation of the contralateral sympathetic chain led to secretion from the denervated rat gland [13].

Parasympathetic and/or sympathetic denervation leads to a change in the responsiveness of salivary cells to nerve-mediated stimuli. Thus supersensitivity of salivary glands in response to autonomimetics develops over the course of

some weeks (see chapter 9). Emmelin [14] conducted extensive studies of the effects of parasympathetic denervation and the development of supersensitivity in salivary secretion. However, these and other studies which followed were mainly concerned with fluid secretion and protein secretion was seldom examined.

As amylase is a prominent secretory protein in the rat parotid gland it has been assayed in studies of the effects of denervation on protein secretion by salivary cells. Asking and Emmelin [15] stimulated sympathetic fibres arising from the contralateral superior cervical ganglion following ipsilateral sympathetic denervation and examined parotid amylase secretion. The development of supersensitivity followed a similar timescale to that found previously in studies of fluid secretion. Thus, after 3 days there was a threefold increase in amylase secretion which was attributed to the development of pre-junctional supersensitivity, that is reduced re-uptake of noradrenaline by sympathetic nerves. Amylase secretion in response to contralateral nerve stimulation at 10 weeks after denervation was increased eightfold and this later effect of denervation was attributed to the development of post-junctional supersensitivity. The latter had previously been shown in vitro to develop by 3 weeks following sympathectomy [16] but apparently not by 7 days [17] and was attributable to β_1-adrenoceptor-mediated events [16]. Following post-ganglionic parasympathectomy and degeneration of the auriculo-temporal nerve a supersensitivity for amylase secretion developed in rat parotid glands in response to methacholine (and substance P) and the magnitude and time course of this supersensitivity was similar to that of the fluid secretory response [18].

The post-junctional supersensitivity of salivary secretory cells which develops following parasympathetic or sympathetic denervations cannot be explained simply by changes in receptor numbers on salivary cells. For example, β-adrenoceptor density was increased in the absence of supersensitivity following reserpine treatment [e.g. 19]. As knowledge of the intra-cellular coupling mechanisms operating in salivary cells increased it became apparent that the non-specific supersensitivity to both cholinergic and α-adrenergic agonists, which develops following parasympathetic denervation, might be related to disuse of the calcium-dependent intra-cellular coupling pathway which evokes fluid secretion and is shared by these agonists [20, 21].

Pharmacological and Nerve-Stimulated Protein Secretion following Sympathectomy

In early experiments on salivary protein secretion it was observed that 2 weeks following sympathetic ganglionectomy, there was an increased pilocarpine-induced secretion of protein and amylase from parotid glands of anaesthetized rats [22]. One possible explanation for this observation, later

considered by Anderson et al. [23], was an increased contribution by circulating catecholamines to secretion from the sensitized gland. Following adrenalectomy there was some reduction in protein secretion evoked by auriculo-temporal nerve stimulation in pentobarbitone anaesthetized rats but it remained elevated at twice that of the control gland. In a subsequent study [24], using chloralose anaesthetized rats an increased protein secretion was also observed in response to methacholine 4 days after sympathetic decentralization, when classical post-junctional supersensitivity is only just beginning. However, no reduction in protein secretion occurred when both α- and β-adrenoceptor blocking drugs were given, suggesting that chloralose anaesthesia abolished the release of circulating catecholamines observed previously [23] under pentobarbitone anaesthesia. An increased amylase secretion was also observed in response to substance P, physalaemin and phenylephrine [24] and a similar pattern of increased protein release on parasympathetic nerve stimulation was observed in later studies [25–27]. Parasympathetic stimulation and the agonists used in these studies engage the inositol triphosphate/calcium intracellular coupling pathway and thus it may be that sympathectomy influences this signalling pathway. This would represent another example of the 'cross-talk' which takes place between the stimulation-secretion coupling pathways operating during parasympathetic and sympathetic stimulation and may be responsible for the phenomenon of augmented secretion [28]. Recent experiments in which the parasympathetic chorda lingual nerve, supplying the rat submandibular gland, was stimulated one week following pre-ganglionic sympathectomy again revealed increases in the protein content of saliva. Assays of salivary peroxidase and tissue kallikrein suggested that the increased secretory protein was derived from acinar cells and not ductal cells [Proctor et al. unpubl. observ].

An alternative possibility in trying to explain the above phenomenon is that the increased levels of salivary protein reflect an increased glandular content of secretory protein following sympathectomy as seen with the amylase content of parotid glands which increases after sympathetic denervation [26]. However, there is evidence to indicate that the levels of secretory protein in parasympathetic saliva are not necessarily dependent upon glandular levels of stored protein. Asking and Proctor [27] found that, despite a greater output of amylase into saliva on parasympathetic activation 1 week after sympathectomy this did not lead to depletion of the glandular content of amylase (fig. 1). Under the stimulation conditions used (5 Hz for a period of 120 min) the output of amylase was steadily maintained throughout the stimulation and an amount of amylase corresponding to 36% of the initial gland content was secreted on the control side and 53% of the content was secreted on the sympathectomized side. Since the equivalent secretion of amylase on sympa-

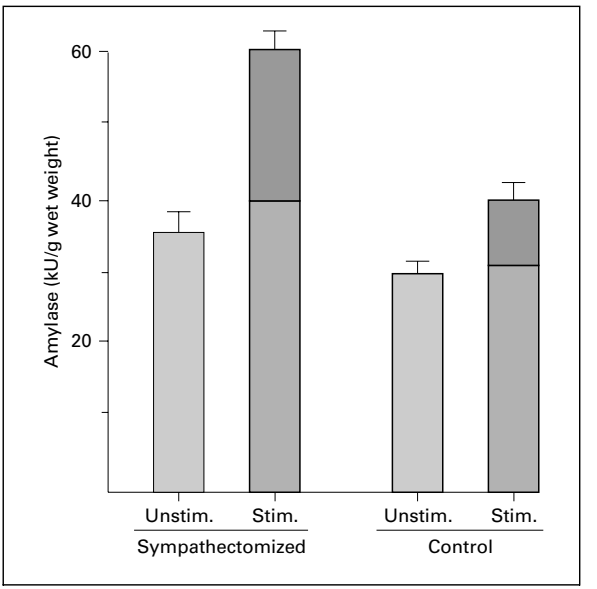


Fig. 1. Amylase secretion from sympathectomized and intact parotid glands. The amylase content (expressed as kU per gram gland wet weight; mean $\pm$SEM; n = 5) of unstimulated intact and 1 week sympathectomized glands (dotted columns) is unchanged following stimulation of the auriculo-temporal nerve at 5Hz (4–5 V, 2 mS duration) for 120 min (Stim-hatched columns) compared to unstimulated glands. If the output of amylase into saliva (dark columns) is added to the remaining glandular content in the stimulated glands the sum is significantly greater than the content of the unstimulated glands ($p < 0.001$ for sympathectomized; $p < 0.01$ for intact). These results indicate that amylase is synthesized during the period of stimulation and that the glandular content is not depleted in contrast to the depletion which takes place with the equivalent stimulation of the sympathetic nerve.

thetic activation leads to a decrease in stored glandular content of amylase [29] these results suggested that the protein secretory mechanism operating on parasympathetic activation was not conventional storage granule exocytosis as occurs on sympathetic activation [12]. Qualitative morphology suggested that there was no decrease in the numbers of protein storage granules within acinar cells of sympathectomized or intact glands. Furthermore, the lack of depletion of glandular content of amylase in either sympathectomized or intact gland suggests that resynthesis was ongoing (see later). However, it should be stressed that during high frequency parasympathetic nerve stimulation some degranulation of the parotid gland is seen (see chapters 4 and 6). Other evidence for the existence of stimulated secretion from a non-granule pool has been presented previously [1].

Reflex-Stimulated Protein Secretion following Denervation

Studies of the effects of sympathetic denervation on parotid amylase secretion under reflex feeding conditions have been conducted in the rat and rabbit. The amylase concentration of rat parotid saliva secreted upon feeding was significantly reduced on the pre-ganglionically sympathectomized side compared to the intact contralateral side, although by only 14% [30]. In the rabbit, output of amylase in parotid saliva following pre-ganglionic sympathectomy decreased by 30–70% depending on the feeding stimulus [31]. This phenomenon appears to be specific to stimuli mediated through β-adrenoceptor mechanisms, since administration of the β-adrenoceptor blocker propranolol reduced the amylase and protein content of reflexly evoked parotid saliva in rabbits by 40–60% [32]. Gjörstrup [31] found that β-adrenoceptor blockade produced a further reduction in protein output compared to sympathectomy alone and the difference was attributed to the effects of circulating catecholamines. Recent studies on rat submandibular glands have extended observations of reflex secretion and assays of protein concentrations in the saliva, before and after acute sympathetic decentralization, and indicate that fluid secretion is largely unaffected whilst salivary protein concentration is reduced by approximately 40% [Matsuo et al. unpubl.].

From all of these investigations it appears that protein secretion under reflex conditions is not as dependent upon β-adrenoceptor-mediated responses from sympathetic nerves as perhaps might have been concluded from pharmacological or nerve stimulation studies or in vitro studies which established the link between protein secretion and such stimuli (see chapters 3 and 4). The contribution of parasympathetic nerves to protein secretion during reflex stimulation is difficult to assess as fluid secretion is largely reduced or abolished by nerve section or muscarinic blockade. However, Ekström and co-workers [33, 34] assessed the involvement of the parasympthetic nerve in reflexly induced protein secretion from rat parotid glands during feeding by examining the influences of muscarinic, α-adrenoceptor and β-adrenoceptor blockades with or without previous sympathectomy and/or parasympathectomy. These studies established that under certain reflex conditions neuropeptides, probably including VIP and substance P from parasympathetic nerves, can account for the degranulation and reduction in amylase content of parotid acinar cells. However, it is clear from the foregoing discussions (see 'Pharmacological and nerve-stimulated protein secretion following sympathectomy') that lower frequency electrical stimulation of parasympathetic nerves, or stimulation with cholinergic agonists, does not necessarily lead to a depletion of glandular stores of protein. It is likely therefore that assessments of glandular content of protein or protein storage granules provide underestimates of protein secretion during reflex stimulation.

In conclusion, denervation studies have established that both sympathetic and parasympathetic nerve impulses play important roles in evoking protein secretion under reflex conditions and that both conventional and peptidergic neurotransmitters are involved in a collaborative manner.

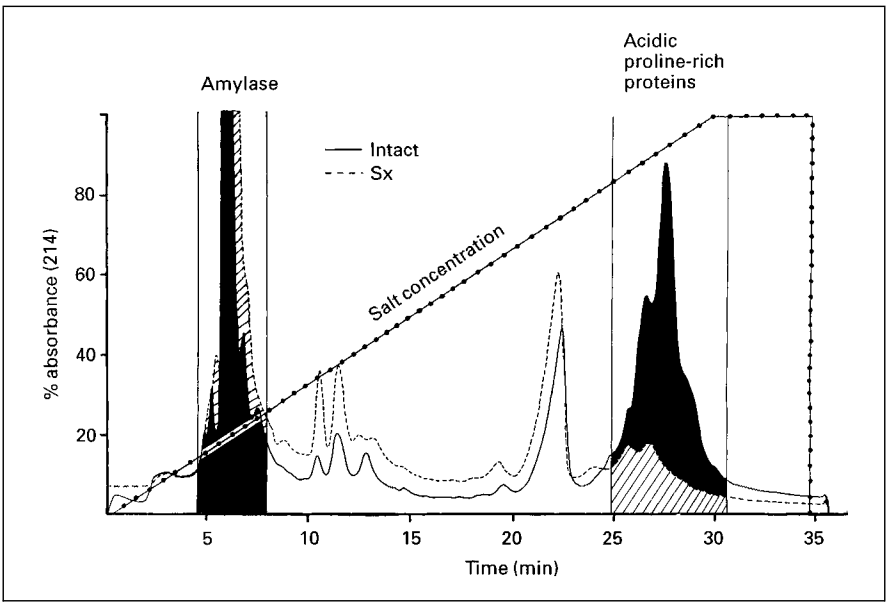
Immunoglobulin A Secretion following Denervation

Immunoglobulin A (IgA) is the predominant immunoglobulin in the mouth and is secreted by the major and minor salivary glands [35]. The movement of IgA was demonstrated to be via the epithelial cell transporting protein, polymeric IgA receptor (pIgR), since the form of IgA in all salivas was secretory IgA, that is IgA complexed with secretory component (the cleaved product of pIgR). Recent studies have focussed on the role of autonomic nerves in regulating IgA secretion in rats and have shown that electrical stimulation of both parasympathetic and particularly sympathetic nerves can up-regulate IgA secretion into saliva above the basal rate of secretion that occurs in the absence of nerve stimulation [36]. In follow-up studies [unpubl.] unilateral pre-ganglionic sympathetic denervation was performed, then 1 week later the levels of IgA in parasympathetically evoked salivas from the sympathectomized and contralateral control glands were compared. This revealed that the unstimulated, basal rate of secretion was unaffected by the denervation but the parasympathetically stimulated rate was reduced by 80% after preganglionic sympathectomy. This reduction was not due to an acute absence of β-adrenoceptor-mediated responses since rats given propranolol do not shown such a decrease; nor was the decrease due to decreases in the glandular levels of IgA or pIgR. Movement of IgA across salivary and other glandular epithelial cell types is dependent upon vesicular transcellular transport [37], so it would appear that this mechanism requires an on-going influence from sympathetic nerve impulses to remain at a normal level in the rat.

Effects of Denervation on Salivary Protein Synthesis

Parasympathetic and sympathetic nerve impulses have been found to increase protein synthesis in the rat parotid gland and, as with secretion, synthesis was augmented when both nerves were electrically stimulated simultaneously [29]. These responses appeared to be mediated by the same receptor plus intracellular coupling events as seen with protein secretion, although the distal intracellular events responsible for activating transcription and translation are less well understood [1].

Since the feeding cycle in the laboratory rat occurs at regular intervals, the depletion and resynthesis of stored secretory protein from the parotid



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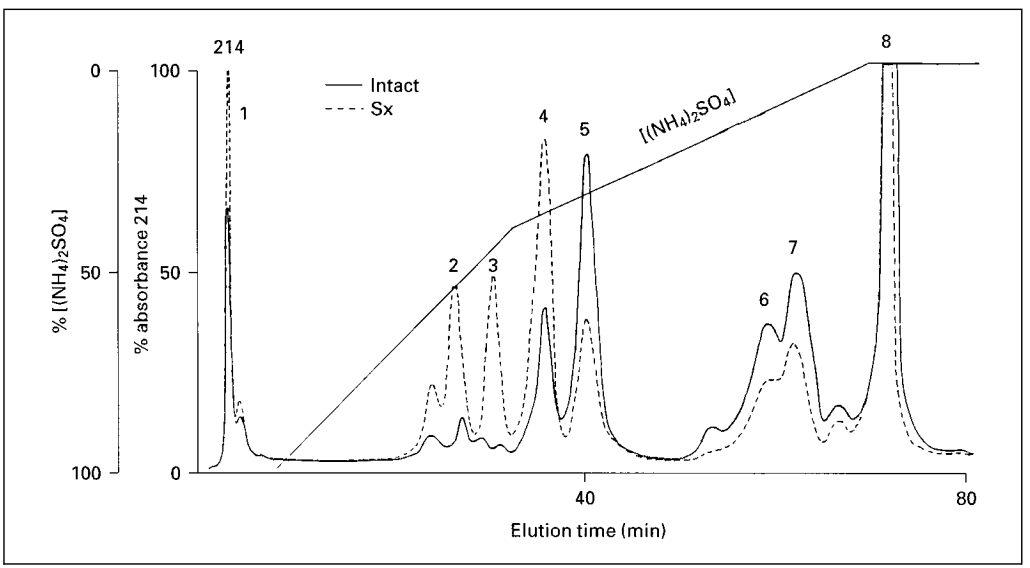

2b

Fig. 2. Assessment of the composition of salivary proteins following sympathetic denervation of parotid glands. Chromatographic analysis of the composition of salivary proteins, with detection at 214 nm, proved to be useful for assessing changes in amounts of proteins, particularly proline-rich proteins, which form a large proportion of parotid salivary protein but are generally underestimated by other protein detection techniques. *a* Anion-exchange chromatography was used to compare parasympathetically evoked saliva (5 Hz)

gland shows a distinct circadian pattern. The importance of autonomic nerve mediated chewing stimuli to protein synthesis as well as protein secretion from the rat parotid gland is demonstrated by the loss of this pattern when feeding on a liquid diet [38]. Maintenance of rats on a liquid diet for longer periods of time (1–2 weeks) causes an atrophy of the parotid glands which is associated with general reduction in protein secretory and synthetic capacity [39, 40].

Changes in Secretory Protein Composition following Denervation

Analysis of the protein composition of autonomimetically evoked parotid saliva from rats maintained on a liquid diet indicated that syntheses of different salivary proteins have varying dependencies on neurally mediated stimuli [40, 41]. Thus the proportions of proline-rich proteins and amylase were reduced whilst those of other proteins remained unchanged. The influence of individual branches of autonomic innervation on salivary protein synthesis has more recently been investigated through the use of selective denervations followed by analysis of salivary protein composition. Proctor et al. [25] performed unilateral sympathetic ganglionectomies on adult rats and one week later obtained salivas from denervated and control contralateral glands by parasympathetic nerve stimulation. During such short-term sympathectomy no significant glandular atrophy had occurred. Nevertheless, there was a profound change in the protein compostion of saliva, indicative of a reduced synthesis of secretory proteins (fig. 2). In particular there were marked reductions in the content of proline-rich proteins as a proportion of total protein and increases in the proportion of amylase as shown by protein chromatography (FPLC) (fig. 2a) and by sodium dodecyl sulphate (SDS) gel electrophoresis [25]. Similar changes in the composition of secretory proteins were observed subsequently in glandular homogenates [26] indicating that the compostion of the proteins secreted reflected the composition of proteins synthesized and stored within the gland. Saliva and glandular homogenates from

from intact and 1-week post-ganglionically sympathectomized parotid glands. The increase in amylase and decrease in acidic proline-rich proteins on sympathectomy is clearly demonstrated. Modified from Proctor et al. [25]. *b* Hydrophobic-interaction chromatography was used subsequently to analyze protein compositions and proved to be more resolving. Peak identities: (1) basic proline-rich glycoprotein; (2, 3) basic proline-rich proteins; (4) acidic proline-rich protein; (5) deoxyribonuclease; (6, 7) cysteine-rich proteins; (8) amylase. In particular the levels of different proline-rich proteins could be assessed. This typical chromatogram compares protein composition in parasympathetically evoked saliva (40 Hz) from an intact parotid gland and 6 weeks following pre- or post-ganglionic sympathectomy. The acidic and basic proline-rich proteins are still seen to be reduced as with anion-exchange chromatography 1 week after sympathectomy (*a*), but the amylase peak was not increased. Modified from Ekström et al. [42].

chronically sympathectomized (6 and 12 weeks) rat parotid glands also showed reductions in proportions of proline-rich proteins but amylase levels appeared to have returned to normal values [26, 42]. The better resolution of salivary proteins by hydrophobic interaction chromatography (fig. 2b) demonstrated that most other secretory proteins remained in constant ratios despite the sympathectomy. Overall, the results indicate that the rates of synthesis for different parotid secretory proteins have differing dependencies on impulses arriving from sympathetic nerves. In particular, the synthesis of proline-rich proteins appears to be heavily dependent on such impulses and this has been confirmed by a reduction in the incorporation of radiolabelled proline into parotid proline-rich proteins after sympathectomy [43]. Similar reductions in proline-rich proteins in parotid saliva were observed when rats were treated for 10 days with the β-adrenoceptor blockers metaprolol or propranolol [44]. These data are consistent with the well-characterized dependence of proline-rich protein synthesis upon β-adrenoceptor mediated stimuli and the cAMP intracellular coupling pathway [1]. However, unlike parotid saliva from sympathectomized rats, there was no elevation in the amylase content of parotid saliva obtained from the rats treated for 1 week with β-blockers [44]. It might be that elevated levels of amylase in parasympathetic saliva and in glandular homogenates 1 week after sympathectomy are not dependent upon a chronic absence of β-adrenoceptor responses, but on some other sympathetic response. The latter is unlikely to be due to a greater influence of circulating catecholamines on sympathectomized glands during the reflex stimulation from feeding, for supersensitivity reaches a maximum after 2 weeks and is maintained, whilst the increased amylase was present at 1 week but was absent 12 weeks after sympathectomy.

Parasympathetic denervation also caused changes in the composition of proteins secreted into rat parotid saliva [45]. One week after postganglionic parasympathectomy the protein composition of sympathetically evoked saliva was analysed by hydrophobic interaction chromatography and scanning densitometry of electrophoresed proteins in SDS gels. The amylase content of saliva was reduced whilst other secretory proteins, for example cysteine-rich protein and deoxyribonuclease, remained unchanged. Unlike the effect of sympathectomy, only two out of four resolved proline-rich proteins showed a decrease. Another protein, the common salivary protein 1 (CSP-1) [46] showed an increase in proportion following parasympathectomy. The roles of different neurotransmitters in the effects observed following parasympathectomy were not established as specific adrenoceptor blockade experiments were not performed.

Comparing the results from sympathectomy and parasympathectomy it can be concluded that amylase synthesis shows a greater dependence upon parasympathetic than on sympathetic stimuli. The synthesis of two of the

proline-rich proteins also shows a dependence on parasympathetic stimuli (whether cholinergic or peptidergic or both) in addition to the well-characterized dependence on sympathetic stimuli. The composition of salivary protein following complete denervation of the rat parotid gland has not been assessed, but given the great dependence of secretion from this gland on chewing, one might expect the salivary compostion following double denervation to resemble that of rats fed a liquid diet. The latter did show reductions in both proline-rich proteins and amylase [41].

Ductal cells in salivary glands not only modify the ionic content of saliva but also can store and secrete proteins. The proteolytic enzyme, tissue kallikrein, is a prominent ductal cell secretory protein of uncertain function that is found in storage granules in the apical cytoplasm of submandibular gland striated duct cells in cat, man and other species. Studies on feline submandibular glands have demonstrated that tissue kallikrein secretion is evoked principally by stimuli from sympathetic nerves but that resynthesis is dependent upon stimuli from parasympathetic nerves. Thus section of the chorda lingual nerve led to a reduction of stored tissue kallikrein in striated ductal cells [5] which was accompanied by massive reductions in the tissue kallikrein content of sympathetically evoked saliva [47]. A reduction in the salivary content of tissue kallikrein also occurred with chronic muscarinic receptor blockade [48], so it would appear that synthesis of the enzyme is dependent particularly on acetylcholine-mediated stimuli. The granular duct cells of rat submandibular glands contain large amounts of tissue kallikrein and other kallikrein-related secretory proteinases stored in prominent protein storage granules. Unlike in cat submandibular ductal cells, neither parasympathectomy nor sympathectomy altered the levels of these proteinases in rat glandular homogenates [49; Garrett and Proctor, unpubl. results]. These specialized cells, seen in rat and mouse submandibular glands, show a dependence on sex steroid hormones and it would appear that such hormonal influences override any form of autonomic nerves on the resynthesis of the secretory kallikreins.

Epilogue

It is well established that salivary fluid secretion is largely dependent upon acetylcholine acting via muscarinic receptors. Pharmacological studies on rat parotid salivary glands and isolated acinar cells have indicated that salivary protein secretion occurs with β_1-adrenoceptor-mediated stimuli from sympathetic nerves. Thus the roles of autonomic nerves in salivary secretion tend to be conveniently presented as a dichotomy; parasympathetic nerve, fluid secretion; sympathetic nerve, protein storage granule exocytosis and secretion.

Clearly not all glands conform to this dichotomy since parasympathetic stimulation can cause extensive degranulation in some glands (e.g. the cat parotid gland [50]). However, the use of denervation in combination with receptor blockers in studies of reflex secretion also suggests that such a simplification may obscure the reality even in the rat parotid gland, the paradigm for such studies. Although sympathetic nerve-mediated stimulation has been shown to make a contribution to protein secretion in such studies, this may not represent the main impetus for protein secretion under reflex conditions since either sympathectomy or β-adrenoceptor blockade reduce the protein content of saliva by only 50% at most. In addition the degranulation and decrease in amylase content of glands following a feed can be made to be similar with or without an intact sympathetic innervation. Denervation studies have helped delineate the roles of parasympathetic and sympathetic nerves but it must again be emphasized that under reflex conditions it is likely that both nerves contribute to the activation of a variety of different receptors and intracellular pathways linked with secretion.

Studies of amylase secretion from rat parotid glands have demonstrated that protein secretory responses of salivary cells change as a result of parasympathetic or sympathetic denervations. Following sympathectomy there is an approximate doubling of protein secretion in response to stimuli which activate the inositol triphosphate/calcium pathway in salivary cells; this is a separate phenomenon from β_1-adrenoceptor-mediated supersensitivity of protein secretion following sympathectomy. Recent studies of IgA secretion suggest that transcytosis can be stimulated by nerve-mediated stimuli. However, following sympathectomy IgA secretion, unlike general protein secretion, is profoundly reduced. Further studies will be required in order to establish the short- and long-term influences of nerves on secretion of IgA into saliva. Studies of the effects of denervation on other vesicular (non-storage granule) pathways operating in salivary glands may indicate whether nerves influence the distribution of membrane proteins in salivary cells. Nerve impulses influence the synthesis of different secretory proteins and denervations alter the protein composition of rat parotid saliva. It is likely that similar influences operate on submandibular protein composition since in vitro studies indicate that autonomimetics, particularly β-adrenoceptor agonists, can increase the synthesis of proteins in submandibular cells in a similar way to that seen in parotid cells [51].

In cross-sectional studies of man it quickly becomes apparent that there is a high degree of variation between individuals in the amounts of different proteins secreted [52, 53]. Despite such variation, the saliva appears to fulfill all of its functional requirements and this is probably due to the overlapping functions of many salivary proteins [54]. Thus, deficits in one protein might

be compensated for by other proteins which can fulfill similar function. Given such normal variation it is likely that relatively large changes in nerve-mediated stimulation of protein synthesis would be required before salivary function was affected adversely. Nevertheless, the importance of nerve-mediated regulation of the transcription and/or translation of salivary proteins is that it enables salivary glands to meet the need for different functionally important salivary proteins under normal conditions.

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Chapter 9

Degeneration Secretion and Supersensitivity in Salivary Glands following Denervations, and the Effects on Choline Acetyltransferase Activity

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Introduction

Salivary glands may serve as model organs for studies of various neurobiological phenomena. 'Degeneration activity', a transient activity in a denervated organ elicited by a temporary increase in the transmitter release from degenerating nerves, was first discovered in salivary glands [1]. Salivary glands have also been in focus in studies on supersensitivity and Cannon's 'law of denervation' [2], which states that surgical postganglionic denervation of an autonomic effector produces a more pronounced sensitivity to chemical agents than does preganglionic denervation (i.e. decentralization), and, as a consequence of these studies on the glands, the 'law' was extended to include pharmacological denervation and decentralization as well [3, 4]. Salivary glands turned out to be valuable tools in studies on transmitter metabolism, and particular attention has been paid to the activity of the acetylcholine synthesizing enzyme choline acetyltransferase [5, 6].

I Degeneration Activity

Parasympathetic Degeneration Secretion

About a day after section of the postganglionic parasympathetic nerve, the silent denervated gland starts to secrete fluid, at a rate which increases gradually and then decreases and stops after having lasted for a period of 1

or 2 days. This phenomenon was first observed by Emmelin and Strömblad [7, 8] in the parotid gland of the cat after section of the auriculotemporal nerve. In this preparation, bursts of secretory activity occurred, and the response was named 'paroxysmal secretion'. However, in the (partially) denervated submandibular gland the flow rate was much more regular and therefore the term 'degeneration secretion' was introduced [9]. Parasympathetic degeneration secretion has also been found in salivary glands of dogs, rabbits and rats [10–14]. Emmelin concluded that the degeneration secretion is a response to local events at the degenerating nerve terminals, which results in transient release of acetylcholine in concentrations high enough to stimulate the denervated gland cells [1]. In the search for explanations of the paroxysmal flow of saliva, several possibilities have been considered such as an acquired property of rhythmicity of the secretory cells or of the myoepithelial cells, extruding the saliva, as a consequence of the denervation or a release of acetylcholine in bursts, but the paroxysm still appear enigmatic. Furthermore, the synchronization of the intermittent activity appears puzzling. Electrical changes such as higher resting membrane potential, short-lasting potential fluctuations and resistance decreases may be observed in the gland cells during ongoing degenerating secretion, but simultaneous recordings from different cells revealed no signs of electrical synchronization [15, 16].

The time of onset of the degeneration secretion occurred earlier the shorter the stump of nerve left in connection with the gland [17], and has been associated with a proximodistal axonal transport in the peripheral nerve stump [18, 19]. A conspicuous finding is the possibility to provoke the degeneration secretion with injections of acetylcholine before it has started and to restart it during a period after it has stopped [17] and, further, to cause the phenomenon to diminish or to cease for a short period of time by injections of noradrenaline [Ekström and Emmelin, unpubl. observations], effects likely to reflect facilitation and inhibition of the acetylcholine release.

Sympathetic Degeneration Secretion

In cat submandibular glands, secretion of saliva occurred 1–2 days after superior cervical ganglionectomy but the response was not seen in all preparations despite the fact that the glands had been sensitized to noradrenaline by chronic treatment with parasympatholytic agent or by partial parasympathetic denervation [20, 21]. In the rat parotid gland, the secretory response was small and erratic. Using this preparation intracellular recordings revealed 'degeneration potential changes' similar to those occurring in response to administration of noradrenaline [15]. A more pronounced secretory response was found in submandibular glands of the rat [22, 23]; it commenced about 15 h after the ganglionectomy and ceased 7–9 h later. The onset of the secretion

started earlier when adrenergic axons of the perihilar tissue were crushed [24]. A fall in body temperature delayed the start of the degeneration secretion [22], and a number of drugs influence its time of onset, most likely by lowering the metabolic activity [25]. A progressive deterioration of the neuronal reuptake mechanism for noradrenaline leading to deviation (prejunctional) supersensitivity and a reduction of the breakdown of intraneuronal noradrenaline during nerve degeneration play important roles for the magnitude of the secretory response [23].

The uptake of the drug 6-hydroxydopamine induces events that lead to the destruction of adrenergic nerve terminals and to the release of noradrenaline [26]. The degeneration is accompanied with hyperactivity of the effector organs similar to those following surgical denervation. However, the time of onset, duration and magnitude of the response differ. In the submandibular gland of the rat, secretion started a few seconds after the beginning of the injection of the drug, the flow rate accelerated to reach a maximum within 10–15 min, then it gradually slowed down and had ceased about 2 h later, the total volume secreted being severalfold that after sympathetic ganglionectomy [27, 28].

Action of Nonadrenergic, Noncholinergic Mechanisms

Atropine abolished the parasympathetic degeneration secretion and adrenoceptor blockers abolished the sympathetic degeneration secretion, so the classical transmitters acetylcholine and noradrenaline are of primary importance for the secretory responses. Nevertheless, it is worth considering possible contributions of nonadrenergic, noncholinergic (NANC) transmitters (see chapter 6) to the secretory response upon nerve degeneration. It might, for instance, be wondered whether a release of vasoactive intestinal peptide (VIP) influences the magnitude of the acetylcholine-evoked secretion from the salivary glands of the cat during parasympathetic degeneration secretion. Perhaps the positive interaction between VIP and acetylcholine contributes to the paroxysmal flow of parotid saliva (chapter 6). In the rabbit eye, the degeneration mydriasis was partly dependent, and the degeneration hyperemia of the iris entirely dependent on some other agent or agents than noradrenaline released from the degenerating sympathetic nerve terminals following removal of the superior cervical ganglion [29, 30].

Concluding Remarks

Degeneration secretion of fluid has so far only been studied in anesthetized animals. The secretion is affected by anesthetics so that when additional doses are given during the experiments the flow of saliva may decrease or even cease for a period of time, the likely cause being an effect of the anesthetics

prejunctionally decreasing the transmitter release [9]. Thus, the time sequence as well as the magnitude of the secretory response may be different in conscious animals. So far no secretion of saliva has been reported upon preganglionic denervation.

II Supersensitivity

When the amount of a drug required to elicit a certain (submaximal) biological response diminishes the tissue is referred to as being supersensitive [31, 32]. One type of supersensitivity ('deviation', 'prejunctional', 'presynaptic') depends on a greater fraction of the administered drug reaching the receptors. It results from the inhibition of the neuronal and extraneuronal uptake or the enzymatic breakdown of the drug. It develops rapidly and shows a high degree of specificity. The majority of examples on deviation supersensitivity are from sympathetic (adrenergic) systems. In parasympathetic (cholinergic) systems enhanced responses to various choline esters, sensitive to the activity of cholinesterase, are found in the presence of cholinesterase inhibitors. The contribution of deviation supersensitivity, as a consequence of less cholinesterase activity, to the total increase in sensitivity to acetylcholine after denervation seems small [33, 34]. Another type of supersensitivity ('nondeviation', 'postjunctional', 'postsynaptic', 'true') is due to the alteration in the physiology of the responding tissue. It develops slowly and is nonspecific, i.e. the response is also increased by agonists which are structurally or pharmacologically unrelated.

The Effect Exerted by the Transmitter

Emmelin and coworkers performed extensive studies on the phenomenon of supersensitivity of the nondeviation type after surgical parasympathetic denervation or decentralization, or chronic treatments with drugs that impair the cholinergic transmission at the ganglionic or the neuroglandular level [3, 4, 34, 35]. The degree of sensitization in response to various drugs, injected into the bloodstream, is usually determined by a lowered threshold dose for secretion and an increased total volume of saliva secreted in response to some low standard doses of the drug. The phenomenon is discernible after 2–3 days, increases gradually and reaches its maximum within 2–3 weeks; it then declines 1–2 months later as the reinnervation proceeds [36].

The results of these studies, mainly performed on the cat submandibular gland, led Emmelin to the conclusion that contact between acetylcholine and the effector cells was of primary importance for the level of sensitivity of a tissue, and that development of the nondeviation supersensitivity was

due to the loss of some action of the transmitter [4]. He showed that two fractions of acetylcholine release regulate the responsiveness of the glandular cells. One is released from the postganglionic nerve terminals upon the arrival of secretory impulses, and lost after surgical decentralization or treatment with a ganglion blocker or diminished after reducing the inflow to the salivary centers by deafferentation. The other is continuously released from the nerve terminals of postganglionic nerves (with or without connection with the central nervous system) in amounts subliminal for eliciting secretion. Postganglionic denervation or treatment with an antimuscarinic agent or an agent such as botulinum toxin, which prevents the release of acetylcholine, would then deprive the gland cells of the bombardment of both fractions of acetylcholine and thus cause the degree of sensitivity to rise above that occurring after surgical or pharmacological decentralization, in agreement with Cannon's law of denervation. If, on the other hand, the gland cells are exposed to excessive amounts of the transmitter, as after chronic treatment with a cholinesterase inhibitor, a subsensitivity develops. Supersensitivity and subsensitivity are likely to be the opposite expressions of the same phenomenon.

Surgical or pharmacological interventions create extreme situations. In an extension of previous studies a difference in the sensitivity of parotid glands, with functionally intact reflex arcs, was also caused by 'disuse' and 'overuse' of them. This was done by keeping one group of rats on a liquid diet and another group of rats on a pelleted bulk diet. After 3–4 weeks the glands of the rats maintained on the liquid diet showed a greater degree of sensitivity to parasympathomimetics than those of the rats maintained on the pelleted bulk diet, illustrating that the sensitivity of a tissue may vary under physiological circumstances and that a state of 'normal sensitivity' is indeed a relative condition [37].

Supersensitivity to Neuropeptides

Neuropeptides also serve as autonomic transmitters in salivary glands (see chapters 1 and 6). The parasympathitic postganglionic nerves of the parotid and submandibular glands of the rat contain the neuropeptides substance P and vasoactive intestinal peptide (VIP), and upon intravenous injection of these peptides the glands secrete saliva. Parasympathetic denervation (parotid gland) and decentralization (submandibular gland) caused a marked sensitization to substance P [38], whereas a low degree of sensitization developed after sympathetic denervation. In submandibular glands, the degree of sensitization to substance P after sympathetic decentralization was the same as after sympathetic denervation, whereas in parotid glands no sensitization to this peptide could be demonstrated after sympathetic decentralization.

Since afferent nerves containing substance P also reach the glands via the parasympathetic trunks (see chapter 6), it might be wondered whether their loss contributes to the sensitization of the secretory cells. This seems, however, not to be the case. Some weeks following treatment with the sensory-neurotoxin capsaicin the parotid glands showed no change in the sensitivity to substance P (or muscarinic agents) [39].

The parotid and submandibular glands of the rat are also sensitized to VIP after parasympathetic denervation or decentralization, whereas after sympathetic denervation sensitization to VIP can only be demonstrated in submandibular glands [40]. In agreement with these in vivo studies, the in vitro release of amylase from pieces of rat parotid tissue in response to VIP is enhanced following parasympathetic denervation [41]. As in the rat, parasympathetic postganglionic nerve fibers contain VIP in salivary glands of cats and ferrets. However, in these species, VIP evokes no flow of saliva. There is an in vitro release of proteins from pieces of parotid and submandibular tissues in response to VIP, and this release is increased after parasympathetic denervation and decentralization [42, 43]. The enhanced responses of the gland cells to the two peptides were demonstrated in the presence of blockers of adrenoceptors and muscarinic antagonists. The fact that substance P and VIP are not inactivated by neuronal uptake mechanisms, but by enzymatic degradation [44], and that the glands become sensitized to these agonists after sympathetic denervation seem to suggest that the supersensitivity that develops to these agonists is of the nondeviation type.

The present knowledge of the existence of postganglionic peptide-containing nerve fibers innervating the secretory cells and the development of supersensitivity to these peptides makes the picture more complex to interpret than if only the actions of acetylcholine are considered. For instance, can the development of supersensitivity to substance P and VIP after parasympathetic decentralization be taken as evidence for their release during normal nerve impulse traffic or is the sensitization merely a nonspecific consequence of the reduced amounts of acetylcholine acting on the cells? Conversely does the loss of the action of neuropeptides contribute to the degree of sensitivity to acetylcholine after parasympathetic denervation?

Sympathetic Decentralization and Denervation

Studies on the effects of sympathetic decentralization and denervation on the sensitivity of salivary glands are few, one obvious reason being that there is a great variability in the density of the sympathetic secretory innervation between various glands (chapter 1). However, in the cat submandibular gland stimulation of the sympathetic nerve trunk and administration of adrenaline evoke secretion of saliva but section of the preganglionic sympathetic nerve

was not followed by any increase in the sensitivity to adrenaline. It was therefore concluded that the flow of sympathetic secretory impulses to the gland under natural conditions was low [45].

The submandibular and parotid glands of the rat are supplied with a relatively rich sympathetic secretory innervation which has been shown to take part in digestive reflexes [46–48]. By using these preparations, it was possible to demonstrate an effect of decentralization on the sensitivity of the gland cells, which suggests that the flow of secretory sympathetic impulses may also play a part towards the level of sensitivity [49, 50].

It seems as if any continuous release of transmitter from adrenergic nerve terminals, in the absence of impulse traffic, is of minor importance for the level of sensitivity of the secretory cells [49, 50]. A much more conspicuous sensitization to adrenaline, noradrenaline and the α-adrenoceptor agonist phenylephrine developed in the submandibular glands after sympathetic ganglionectomy than after decentralization. Similarly, in the parotid gland the degree of sensitivity was further increased after denervation. However, analytical pharmacology showed that the major part of the supersensitivity after sympathetic denervation depended on the inhibition of the amine pump (deviation supersensitivity). In fact, the nondeviation part of the response after sympathetic denervation was the same as after the sympathetic decentralization; and with respect to phenylephrine the supersensitivity that emerged in the parotid gland was entirely of the deviation form. The β-adrenoceptor agonist isoprenaline is not inactivated by the amine pump. In line with the previous observations, the (nondeviation) supersensitivity to this drug after decentralization was not further increased by denervation. Too small a release of the transmitter and an effective amine uptake pump at the nerve terminals probably limit the concentrations of noradrenaline at the receptors. The fact that a certain degree of sensitization to a muscarinic agonist and substance P was found after sympathetic denervation but not after sympathetic decentralization in the parotid gland, may nevertheless indicate that the adrenoceptors are under some influence of noradrenaline released for the nerves in the absence of impulse flow [38, 49].

On the Mechanisms of Supersensitivity

There are probably a number of cellular changes that contribute to the sensitization and they may vary in importance depending on which tissue and which agonist are under study.

Changes in Receptors

In denervated skeletal muscles, the appearance of extrajunctional nicotinic receptors appears to be a major factor in the development of supersensitivity

[51, 52]. Available data seem, however, to suggest that the development of supersensitivity in the glands reflects changes beyond the receptor level. Changes in the binding affinity of the receptors for various ligands seem not to occur in sensitized salivary glands. Furthermore, in rat parotid glands the number of muscarinic receptors was decreased after parasympathetic denervation, by 28–45% 3–16 days postoperatively [53], and in a subsequent study by 40–47% 1–3 weeks postoperatively [54], while at the same time the number of muscarinic receptors in the parasympathetically decentralized submandibular glands was unaffected, although both glands were sensitized to muscarinic agonists. After sympathetic denervation for 3 weeks, the muscarinic receptor density in the parotid gland was either unchanged [54] or decreased [55], while in the submandibular gland it was increased [54, 56]; both glands displayed a modest sensitization to muscarinic agonists. After sympathetic decentralization the receptor number was unaffected, while the submandibular gland was slightly sensitized to muscarinic agonists [54]. Chronic treatment with atropine or a cholinesterase inhibitor, resulted in supersensitivity and subsensitivity, respectively, of the rat submandibular gland to parasympathomimetics (when tested 1–2 days after the last administration), while the muscarinic receptor number was increased after atropinization but unchanged after inhibition of the cholinesterase activity [57]. Observations made in smooth muscles also demonstrate a lack of correlation between muscarinic receptor density and supersensitivity to muscarinic agonists [58].

Parasympathtic denervation was reported to increase the number of β-adrenoceptors in the rat parotid gland [59] but neither the fluid response [49] nor the amylase secretion [56] were increased to isoprenaline. After parasympathetic denervation of the rat parotid gland the number of sites of VIP binding was increased by as much as 3-fold [41], so here the sensitivity to VIP and the receptor number changed in the same direction.

Following sympathetic denervation, there were no changes in number of β-adrenoceptors in the parotid gland of the (adult) rat 1–4 weeks postoperatively [55], while in the submandibular glands an increase was reported [60], although both glands became sensitized to β-adrenoceptor agonists. With respect to α-adrenoceptors, the receptor number of the submandibular gland either increased or remained unaltered [60, 61], while a supersensitivity to α-adrenoceptors develops [56].

Specific Patterns of Sensitization

Emmelin usually assessed the secretory flow responses to adrenaline following various procedures directed towards the cholinergic system, illustrating the nonspecific nature of the phenomenon. Nevertheless, some more recent observations point to the development of specific aspects in the sensitization

(of the nondeviation type) of the glands: (1) Despite the fact that both α- and β-adrenoceptors lost their bombardment of noradrenaline after sympathetic denervation, the rat parotid gland became sensitized to the β-adrenoceptor agonist isoprenaline but not to the α-adrenoceptor agonist phenylephrine. Though the rat submandibular gland was sensitized to both types of agonists there was a clear preponderance for the β-adrenoceptor-mediated response as also observed in vitro [50, 62]. (2) Despite the fact that both α- and β-adrenoceptors remained under the influence of the sympathetic nerve after parasympathetic denervation, the parotid gland developed a marked sensitization to the α-adrenoceptor agonist but not to the β-adrenoceptor agonist. Though the parasympathetically decentralized submandibular gland was once again sensitized to both types of adrenoceptor agonists, this time there was a clear preponderance for the α-adrenoceptor-mediated response [49, 50].

Muscarinic receptors, tachykinin receptors (substance P) and α-adrenoceptors are known to use the intracellular Ca^{2+}/inositoltriphosphate pathway, while β-adrenoceptors are known to use the cyclic AMP pathway (chapters 3 and 5). It was hypothesized that the development of preferentially α- or β-adrenoceptor-mediated pattern of sensitization was related to the activity of the intracellular pathways [38, 49, 50, 63]. After sympathetic denervation the Ca^{2+}/inositoltriphosphate pathway would still be mobilized by parasympathetic nerve activity (acetylcholine and substance P), whereas after parasympathetic denervation or decentralization the activity would be reduced. By analogy, it was thought that the pattern of sensitization that would develop to VIP would be similar to that to a β-adrenoceptor agonist, since VIP also acts via the cyclic AMP pathway, rather than to those agonists which share the Ca^{2+}/inositoltriphosphate pathway. Indeed, the VIP-evoked secretion resembles that induced by isoprenaline: it is small, protein-rich and viscous from the parotid gland [40]. However, experiments showed that the parasympathetically denervated parotid gland and the parasympathetically decentralized submandibular gland became supersensitive to VIP, while after sympathetic denervation the sensitivity of the glands to VIP was unchanged (parotid gland) or only slightly increased (submandibular gland) [40]. In vitro observations on parotid acini of the rat after parasympathetic denervation revealed further signs of differences in the responses to VIP and isoprenaline: the adenylate cyclase activity, the accumulation of cyclic AMP and the release of amylase increased to VIP but not to isoprenaline [41]. VIP, in contrast to a β-adrenoceptor agonist, seems to share intracellular effector characteristics also with muscarinic agonists in some tissues including the rat parotid gland [64–66]. To conclude, changes in the activity of the intracellular pathways may be of importance for the patterns of sensitization but the picture emerging is presently far from clear.

Electrophysiological Changes

The resting membrane potentials of rat parotid acinar cells remain unaffected by parasympathetic or sympathetic denervation [67]. However, lower doses of muscarinic and adrenoceptor agonists were needed to evoke changes in membrane potentials in the glands after either type of denervation but it was not investigated whether the response to some of the sympathomimetics was, in some part, dependent on the elimination of the amine uptake pump [68, 69]. An increase in sensitivity of the membrane changes after parasympathetic denervation was also observed in response to substance P and VIP but only to substance P after sympathetic denervation, a pattern similar to that found when estimating the secretion of saliva (see above). The number of acinar cells responding to VIP increased from 7 to about 25% after sympathetic or parasympathetic denervation, whereas the responding number of cells to substance P increased from 40 to 57% (sympathetic denervation) and 82% (parasympathetic denervation) [67]. Whatever the underlying mechanism may be, change in the promotion of responding cells was not necessarily associated with the development of supersensitivity: after sympathetic denervation, neither the flow of saliva [40] nor the in vitro release of amylase [41] were increased to VIP despite the increase in the number of responding cells to this peptide.

In salivary gland acini cell-to-cell communication through gap junctions is well developed. Gap junctions between the acinar cells allow both the spread of electric current and the passage of small molecules in rat parotid and submandibular glands [70–72]. Muscarinic and α-adrenoceptor agonists as well as substance P, but not β-adrenoceptor agonists, suppress the coupling ratio between the cells. If of importance for the development of supersensitivity, an increase in cell coupling would be expected after denervation. However, 2 weeks after (partial) parasympathetic denervation the cell coupling was decreased as shown in the rat submandibular gland, whereas sympathetic denervation did not affect the coupling ratio [72, 73].

Concluding Remarks

We lack a number of pieces of information to understand the cellular processes behind the development of supersensitivity in salivary glands.

III Choline Acetyltransferase Activity

In rats, virtually all nerve cell bodies of the parasympathetic otic ganglion display immunoreactivity for choline acetyltransferase [Ekström, unpubl. observations], and section of the parasympathetic auriculotemporal nerve results

in an almost complete disappearance of the total activity of the acetylcholine synthesizing enzyme choline acetyltransferase in the parotid gland. However, some residual enzyme activity is still demonstrable in the denervated gland of the various species studied, being in the rat and the rabbit 1–3%, in the cat 10% and in the dog 30% of the normal activity per gland [74–79]; the activity is not further reduced by sympathetic denervation. The residual activity reflects an incomplete denervation procedure [76, 77, 80–83]. In the cat and the dog, reflex secretion blockable by atropine can still be elicited, albeit at a reduced rate; injection of a cholinesterase inhibitor into the salivary duct towards the denervated gland evokes a small flow of saliva indicating release of acetylcholine from the persisting nerve fibers and further, cholinesterase-positive nerve fibers are still present although at a markedly reduced number. By extending the denervation procedure to include nerve fibers on the internal maxillary artery the activity of choline acetyltransferase fell to 10% in the dog parotid gland [76]. Twigs of the facial nerve traverse the parotid gland, and when section of the facial nerve was also included in the denervations, the enzyme activity reached values below the limit of detection in the dog [76]. The fall in choline acetyltransferase activity reached its lowest value within 3–7 days depending on the level of nerve section [75, 77].

In submandibular glands, where ganglia are located within the gland, dissection of the parasympathetic innervation along the duct deep into the hilus causes a fall in choline acetyltransferase activity to 14% in the cat [74] and to 12% in the rat [Ekström, unpubl. observation].

Dependence on Nerve Impulse Traffic

Impaired Ganglionic Transmision

Section of the preganglionic parasympathetic nerve induces a fall in the total activity of the choline acetyltransferase in both the submandibular (by 30–40%) and parotid glands (by 25–30%) as originally observed in the cat by Nordenfelt [84], and later confirmed in studies on the dog [85] and the rat [86]. In the submandibular glands the decrease in the enzyme activity might in part be attributed to degeneration of some preganglionic nerve fibers within the gland. Prolonged blockade of ganglionic transmission by chlorisondamine reduced the choline acetyltransferase activity in the rat parotid gland (by about 25%), showing that the activity of the enzyme in the postganglionic nerves is dependent on ganglionic nicotinic receptor stimulation [87]. Impaired ganglionic activation of the nicotinic receptors is also a likely cause of the fall in choline acetyltransferase in the cat submandibular gland after ductal injection of botulinum toxin [88].

Variations in Reflex Activity

The hypothesis of a dependence of the acetylcholine-synthesizing capacity on the neuronal impulse-propagating activity has gained support from a number of experiments. Over a period of time (usually 2–3 weeks) the intensity of the impulse traffic in the cholinergic pathway, reflexly elicited, was decreased or increased above average conditions. By changing the consistency of the food offered to rats, from a pelleted diet to a liquefied diet, the total choline acetyltransferase activity of the parotid gland decreased by about 25% [89]. Conversely, a change from the standard pelleted diet to a particularly bulk-rich pelleted diet increased the choline acetyltransferase activity by about 20% [78]. Increased demands on individual parotid glands were also achieved by ligating the contralateral parotid duct as well as the ducts of the submandibular and sublingual glands on both sides, and the enzyme activity of the not duct-ligated parotid gland increased by about 30% [90]. After ligating the ducts of all major salivary glands but those of the submandibular and sublingual glands on one side, the choline acetyltransferase activity in these nonligated glands increased by 30 and 10%, respectively [90]. Furthermore, repeated teeth amputations of the incisors aiming at enhanced glandular stimulation by irritation of the pulpal receptors, increased the enzyme activity by 15–20% in the submandibular and sublingual glands [91].

The capacity of the cholinergic neurons to adapt to altered functional requirements is also illustrated by prolonged treatments with pilocarpine or the atropine-like drug Hoechst 9980 aiming at extreme situations: pilocarpine causing a rich flow of saliva by stimulating the muscarinic receptors and Hoechst 9980 causing mouth dryness by blocking these receptors; and were consequently considered to decrease and increase the salivary reflexes, respectively. In line with the general pattern, the choline acetyltransferase activity in the parotid gland of the pilocarpine-treated rats decreased by about 10% and increased by 20–30% upon atropinization [78, 92].

The weights of the glands usually changed in the same direction as the total activity of choline acetyltransferase. Nevertheless, as judged from a number of experiments [79, 91–93], variations in gland mass appeared a less likely alternative cause of the changes in the enzyme activity, for instance prolonged treatment with isoprenaline increased the parotid gland weight 10-fold but the enzyme activity remained unaffected.

Concluding Remarks

Changes in the synthesis of the enzyme in the soma and in the slow rate of its axonal transport are likely to occur when the nerve adapts to long-term changes in nervous activity [90, 94]. There seems to be a high safety margin for the synthesis of acetylcholine in the parasympathetic nerves

in salivary glands. The in vitro formation of acetylcholine is 15–50 times higher than the maximal release of acetylcholine from nerve stimulation in vivo [95, 96]. This may be one explanation for the fact that the percentage changes in the activity of choline acetyltransferase to variations in the impulse flow are relatively small. Due to the continuous activation of the salivary glands reflexly from various oral and pharyngeal stimuli, the cholinergic tone can be expected to be relatively high under 'normal' conditions. In the rat urinary bladder, which is only activated intermittently, at micturition, the decrease in the choline acetyltransferase activity after decentralization or disuse is less, if any [97, 98].

Interaction with the Sympathetic Innervation

Sympathectomy

After ganglionectomy, the total activity of choline acetyltransferase may increase over some weeks in some salivary glands but not in others. The phenomenon was initially thought to be species dependent, since the enzyme activity increased in the parotid and submandibular glands of the cat, but not in these glands of the rabbit. However, when the investigation was extended to include the dog and the rat, the activity was found to increase in the dog parotid gland but not in the dog submandibular gland and, further, to increase in the rat submandibular gland but not in the rat parotid and sublingual glands [99–101]. The submandibular glands of the mouse and the golden hamster also show an increase, but not those of the guinea pig [101]. The rat submandibular gland has a rich sympathetic secretory innervation and the increase in choline acetyltransferase activity might be thought of as a compensatory response to the loss of the secretory sympathetic impulses. Loss of sympathetic nerve impulse traffic by cutting the preganglionic sympathetic nerves to the gland caused, however, no increase in the activity of choline acetyltransferase [86]. So the increase in choline acetyltransferase activity following sympathectomy is evidently not due to loss of sympathetic impulse flow.

A low concentration of choline acetyltransferase and a high density of adrenergic nerve fibres seemed to favor the increase in choline acetyltransferase activity. The largest increase, by 40%, occurred in the rat submandibular gland. An increase of the same magnitude was observed in response to 6-hydroxydopamine which destroys the adrenergic nerve terminals [101]. Depletion of the neuronal noradrenaline stores (without concomitant nerve degeneration) was not enough to initiate an increase, as judged by the outcome of prolonged reserpine treatment [102]. However, if at the end of the treatment,

the superior cervical ganglion was removed, the enzyme activity increased once again.

Local Events

Surprisingly, an increase in the activity of choline acetyltransferase in the postganglionic nerves of the rat submandibular gland can be brought about despite the fact that these nerves have lost their connection with the central nervous system [86]. One and 4 weeks after surgically isolating the postganglionic parasympathetic nerves, the activity of choline acetyltransferase was reduced by about 15 and 30%, respectively. If the parasympathetic decentralization was combined with removal of the superior cervical ganglion at the same time, no reduction in enzyme activity was observed. By first performing parasympathetic decentralization allowing the fall in the enzyme activity to occur and then removing the superior cervical ganglion, the choline acetyltransferase activity increased from the reduced level. Support for the idea that the transmitter synthesizing capacity may increase in isolated cholinergic neurones is gained from experiments on the rat urinary bladder, parasympathetically decentralized on one side and parasympathetically denervated on the other [98], in which also decentralized acetylcholinesterase-positive nerves were found to sprout [103] and to functionally influence newly acquired cells [104].

Collateral sprouting was suggested by Nordenfelt [100] as the cause of the increased choline acetyltransferase activity following sympathectomy and in cat submandibular glands acetylcholinesterase activity emerges in the sympathetic trunk, suggesting that a retrograde downgrowth of parasympathetic nerves was occurring [105]. Signs indicating functional effects of the increase in enzyme activity following sympathectomy are presently lacking [106].

Concluding Remarks

In the search for underlying mechanisms for increased choline acetyltransferase activity induced by sympathectomy attention should be paid to the loss of inhibiting factors and to the release of stimulating factors either originating from the degenerating sympathetic nerves or the denervated structures. In the rat iris, administration of nerve growth factor (NGF) increases the activity of choline acetyltransferase as does the removal of either the sensory innervation or the sympathetic innervation, while anti-nerve growth factor antagonizes the response [107].

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Chapter 10

Interrelation of Taste and Saliva

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Neuroanatomical Considerations

Taste is one of the important sensory inputs for evoking salivation. This chapter describes the interrelation of taste and saliva, in view of (1) the neuroanatomical taste afferent pathway and the efferent pathway to the salivary center; (2) the fundamental mechanism of reflex salivation situated in the lower brainstem, and (3) effects on the reflex salivation from the higher brain structures.

The most complete picture of the central gustatory pathway has been provided by histochemical studies in rats and hamsters, with the use of anterograde and retrograde axonal transport tracers. The gustatory system is associated with other visceral sensory systems, and the efferent system is linked with various autonomic functions [1–3]. Therefore, the focus of this section is on the gustatory system, especially concerning salivation. Figure 1A shows a scheme of the taste afferent pathways of rats. Taste receptor cells (taste buds) are innervated by afferent fibres belonging to three cranial nerves, i.e. facial, glossopharyngeal, and vagus nerves. The facial nerve (chorda tympani) innervates taste buds located on the dorsal surface of the fungiform papillae distributed in the anterior part of the tongue. The glossopharyngeal nerve innervates taste receptors located on the epithelial foldings of the foliate and circumvallate papillae in the posterior part of the tongue. Taste buds scattered in the soft palate and pharynx, which are not associated with papillae of any form, are innervated by the vagus nerve. The taste information arising from the cranial nerves is sent to the first order of relay neurones in the rostral portion of the solitary nucleus. This information is transmitted to the second-order taste relay neurones in the posteromedial part of the parabrachial nucleus. This nucleus projects both dorsally to the thalamus and ventrally to the limbic system in the forebrain. In the dorsal route, the taste

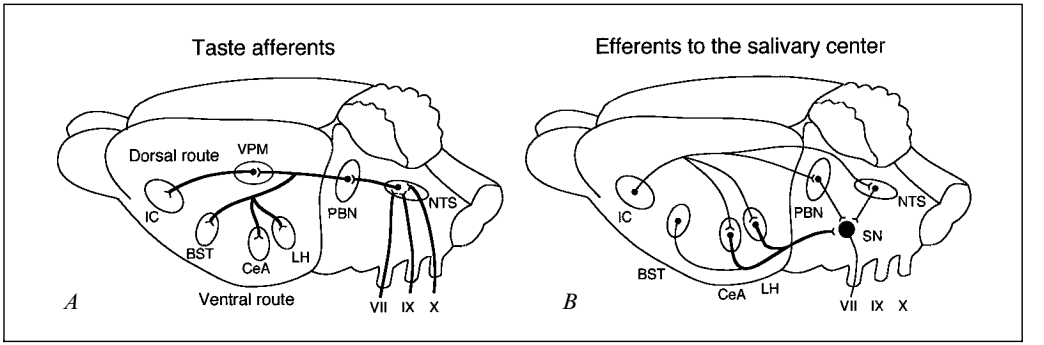

Fig. 1. Taste afferent system (*A*) and efferent system to the superior salivatory nucleus (*B*). BST = Bed nucleus of the stria terminalis; CeA = central nucleus of the amygdala; IC = insular cortex; LH = lateral hypothalamic area; NTS = solitary nucleus; PBN = parabrachial nucleus; SN = superior salivatory nucleus; VPM = thalamic ventral posteromedial nucleus; VII = facial nerve; IX = glossopharyngeal nerve; X = vagus nerve.

information relayed at the ventral posteromedial subnucleus of the thalamus terminates in the insular cortex, which is thought to be involved in the discrimination of taste intensity and quality. The ventral route consists of the parabrachial projections to the lateral hypothalamic area, the central nucleus of the amygdala, and the bed nucleus of the stria terminalis. The functions of the ventral projections may be related to feeding and drinking behavior and/or emotion, as described later.

Figure 1B shows a scheme of the taste efferent pathways to the superior salivatory nucleus, i.e. the parasympathetic preganglionic neurones supplying the submandibular and sublingual salivary glands. The efferent pathways to the parasympathetic primary centre of the parotid gland (the inferior salivatory nucleus) or of the minor salivary glands has not yet been identified. The terminations of the ventral route overlap with the higher centres of the superior salivatory nucleus except for the paraventricular nucleus. The lateral hypothalamic area and amygdala were confirmed to send direct descending projections to the superior salivatory nucleus (thick line in fig. 1B, see chapter 2). The insular cortex provides indirect descending projections to the superior salivatory nucleus via the amygdala, lateral hypothalamus, parabrachial nucleus, and solitary nucleus. These gustatory ascending and descending projections are mainly ipsilateral, although as many as half of the parabrachial taste relay neurones have collaterals that terminate on the opposite side of the thalamus.

As described in chapter 2, synaptic terminals in contact with the superior salivatory neurones may contain various kinds of neurotransmitters; mainly glu-

tamate, γ-aminobutyric acid (GABA), and glycine. All of the brain areas on the taste efferent limb (fig. 1B) may be sources of glutamatergic excitatory inputs. GABAergic and glycinergic inhibitory neurones are thought to exist in the insular cortex, bed nucleus of the stria terminalis, central nucleus of amygdala, and solitary nucleus.

Gustatory-Salivary Reflex in the Lower Brainstem

It is well recognized that salivary secretion is reflexly induced by taste stimulation not only in conscious animals but also in anaesthetized decerebrate animals. Therefore, the taste neurones in the lower brainstem (the solitary nucleus and/or parabrachial nucleus) are involved in the gustatory-salivary reflex arc. Electrical stimulation of these nuclei in decerebrate rats [4] and electrical and chemical stimulation of the parabrachial nucleus in decerebrate cats [5] elicited salivary secretion. Anatomically, the injection of viruses into the submandibular gland and the injection of horseradish peroxidase into the superior salivatory nucleus have revealed the connection between the taste relay nuclei and superior salivatory nucleus in rats (see fig. 2 in chapter 2). A closer connection has been proposed in a study employing intracellular staining with Lucifer yellow, in which the dendrites of rat inferior salivatory neurones innervating below the circumvallate papilla (where von Ebner's gland exists) travel long distances and terminate in the solitary nucleus [6, 7]. The functional properties of the gustatory-salivary reflex arc have been investigated by electrophysiological studies in which responses of the superior salivatory neurones were tested by electrical stimulation of the surface of the rat [8] and rabbit [9] tongue or the cat chorda tympani, glossopharyngeal, and vagus nerves [10, 11]. It was found that a single or train (3–5) electrical stimulation produced a few impulses with unstable latencies, and the latencies of the first impulses of the sampled neurones were widely distributed (i.e. from 10 to 85 ms); this value was obtained from the rat preganglionic fibres (axons of the superior salivatory neurones) innervating the submandibular and sublingual glands when the anterior tongue was stimulated [8]. These response properties suggest that the gustatory-salivary reflex arc is based on oligo- or polysynaptic connections consisting of multiple pathways.

The above-mentioned reflex in the lower brainstem is differentially activated depending on taste quality. For example, both decerebrate and nondecerebrate rats in the awake condition refused bitter solution, with accompanying vigorous salivation, whereas they licked other taste solutions with rhythmical tongue and jaw movements and secreted a far smaller amount of saliva [12]. It is well known that a sour taste such as that of citric acid is the most powerful

sialogogue of all taste qualities in humans. A sweet taste such as that of sucrose, although it induces only a small amount of saliva, results in a significant production of amylase from the parotid gland in humans [13] and awake rabbits [14]. However, Dawes [15] has shown that salt stimuli elicit parotid saliva with a much higher protein concentration than other stimuli in humans. Since high flow rates of saliva usually result from parasympathetic stimulation and amylase or protein secretion usually results from sympathetic nerve activity, it is suspected that the activities of both sympathetic and parasympathetic nerves are quality-specific. Electrophysiological studies have analysed the relationship between taste afferents and autonomic efferents to the submandibular and sublingual glands, and have succeeded in detecting the quality specificity in the sympathetic nerves of hamsters [16], but not in the parasympathetic nerves of rabbits [9] or hamsters [16]. As shown in figure 2, the response magnitude of multiunit parasympathetic nerves in hamsters was largely correlated with that of the taste afferents in the chorda tympani and glossopharyngeal nerves, whereas that of multiunit sympathetic nerves showed much higher responses to high concentrations of NaCl and HCl than to sucrose and quinine.

However, it is conceivable that the quality specificity is susceptible to anaesthesia, because vigorous salivation or brisk parasympathetic discharge was not detected when a rejectable bitter taste stimulus was administered to anaesthetized rats [4, 8]. According to behavioral studies in rats, the destruction of the parabrachial taste area (the second order of taste relay), which does not render the animals ageustic, alters their quality-specific ingestion behaviors of taste solutions [17, 18]. This evidence indicates the importance of the parabrachial nucleus for the quality-specific taste-related reactions. However, taste-elicited salivation persisted even after the destruction of the parabrachial nucleus [4], suggesting the existence of a reflex pathway via the solitary nucleus (the first order of taste relay). Taken together, these findings indicate that the reflex arc for the quality specificity may involve the parabrachial nucleus, which is more polysynaptic than the solitary nucleus pathway, with the result that the quality specificity is susceptible to anaesthesia.

Effects from the Ventral Route of the Taste Pathway

Among the gustatory terminations in the ventral route, the lateral hypothalamic area is apparently involved in salivation, as is the feeding centre. When electrical stimulation was applied to this area in anaesthetized rats, neural responses were evoked in both the sympathetic and parasympathetic nerves supplying the submandibular gland, with an ipsilateral predominance (fig. 3). The magnitude of the response was smaller than that evoked by

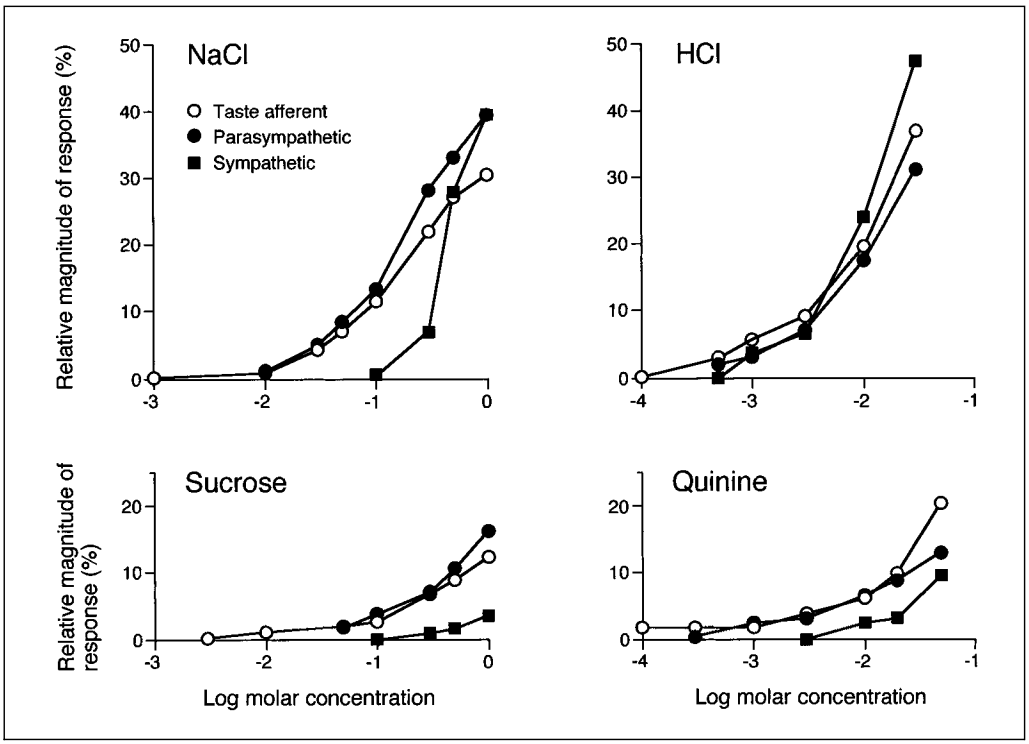

Fig. 2. Taste concentration-response functions of the taste afferents and the parasympathetic and sympathetic efferents. The relative magnitude of response to each stimulus is expressed as the percent of the sum of net responses to the four kinds of stimuli at their highest concentration. The taste afferent values are the mean values of magnitudes obtained from the chorda tympani and glossopharyngeal nerves. Data from Matsuo and Yamamoto [16].

electrical stimulation applied to the anterior part of the tongue. More precisely, a functional single fibre analysis [8] revealed that about half of the rat preganglionic parasympathetic fibres were activated by ipsilateral hypothalamic stimulation. The response latency to the hypothalamic stimulation ranged from 20 to 140 ms, and the maximal discharge rate of impulses was obtained at a relatively low frequency of electrical stimulation (at around 5 Hz). This electrophysiological characteristic implies that the descending inputs from the hypothalamus reach the superior salivatory nucleus by not only a direct pathway but also by an indirect pathway via the parabrachial and/or solitary nuclei; about 60% of the solitary neurones in the so-called taste area are also activated by lateral hypothalamic stimulation [19]. The single-fibre analyses also showed that lateral hypothalamic stimulation activates the parasympathetic fibres ex-

clusively responsive to taste as well as those responsive to mechanical stimulation applied to the oral region [8]. This finding raises the possibility that the feeding centre facilitates various kinds of salivary reflexes induced by taste, mechanical, and thermal stimulation. This non-modality-specific activation has also been shown in the reflex salivation from the rat submandibular gland evoked when an animal's body temperature was elevated in the anaesthetized condition [20].

Gustatory responses in the ventral forebrain areas have been recorded from neurones in the rat lateral hypothalamic area [21, 22], the rat and rabbit amygdala [23, 24], and the rabbit substantia innominata [24]. These neurones are neither purely taste-responsive nor plentiful. Less than 10% of the neurones responded to taste stimulation, and most of them responded to other modalities of sensory stimulation as well. In experiments regarding the feeding behavior of rats [25, 26], some of the lateral hypothalamic neurones increased their impulse discharges during the animal's eating behavior, whereas most of the neurones produced less or no impulse discharges during chewing itself, even when activated during the approach to food. The former type of neurones, the taste responsivity of which remains to be explored, may facilitate the salivary reflex, since vigorous salivation occurs during the period of chewing. These cells were suggested to be the glucose-sensitive neurones [27], which decrease their firing rate in response to the electrophoretic application of glucose [28]. Thus, it may be postulated that the decrease of salivation in the satiety condition is in part attributable to a suppression of the glucose-sensitive neurones as a result of the increasing blood glucose level.

Animal experiments in primates, cats, and dogs undertaken more than 40 years ago showed that the stimulation of the amygdaloid nuclear complex can cause the flow of saliva often associated with licking, sniffing, chewing, and swallowing, similar to that of feeding reactions [29]. Because of the close anatomical relation of the amygdaloid nuclear complex and the hypothalamus, the amygdala-evoked reactions were thought to be the result of hypothalamic activation. In fact, recent studies in rats have shown that lesions of the amygdala produce aphagia and adipsia, which were more transient and of smaller magnitude than those following hypothalamic destruction [30, 31]. In addition, behavioral [32, 33] and electrophysiological [34] findings indicated a modulatory effect of the amygdala on the lateral hypothalamic neurones. However, the results of behavioral studies on the gustatory function in rats have indicated a much more complex action of the amygdala than the classic experiments would suggest. For example, lesions within the amygdala depressed a preference for sweet [30, 35, 36] and salt [37] solutions, and also failed to increase the animal's intake of salt solution after systemic sodium depletion [37, 38]. The amygdala is also thought to be the crucial site involved in the formation

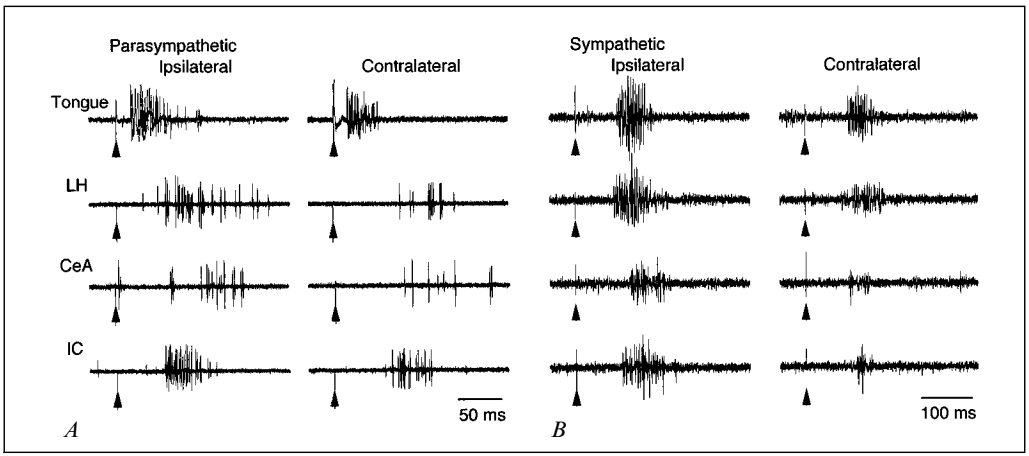

Fig. 3. Multiunit neural activities recorded from the parasympathetic preganglionic fibres (*A*) and sympathetic postganglionic fibres (*B*) innervating the submandibular and sublingual glands of rats. Electrical stimulation (arrowheads) was applied to the ipsilateral and contralateral sides of the anterior part of the tongue, lateral hypothalamic area (LH), central nucleus of the amygdala (CeA), and insular cortex (IC). Unpublished data.

of a conditioned taste aversion [37–39]; once animals ingest a novel food (conditioned stimulus) followed by malaise (unconditioned stimulus), they reject ingesting it thereafter. These observations, taken as a whole, show that the amygdala has various functions, and its effect on salivation is one of these functions. As shown in figure 3, electrical stimulation of the central nucleus of the amygdala induced a relatively small response in the sympathetic and parasympathetic nerves of the submandibular gland of anaesthetized rats. Thus, it has been difficult even to design well-controlled experiments to evaluate such a delicate effect on salivation.

Effects from the Dorsal Route of the Taste Pathway

Robust and pure taste responses were recorded from neurones in the so-called cortical gustatory area, so that the dorsal gustatory afferent route is considered to be responsible for the perception and discrimination of taste intensity and quality [40, 41]. The gustatory cortex sends its corticofugal projections to the terminal nuclei of the ventral forebrain route and the taste relay areas in the lower brainstem (fig. 1). Although the functional aspect of this efferent limb from the cortex is not yet clear, it may act as a pathway for

salivation induced by electrical stimulation of the cortex and for the so-called cephalic phase of salivation, on the basis of the following evidence. First, the cortical area for gustatory perception seems to be identical to, or overlapping with, that for salivation as well as mastication. That is, salivation induced by electrical stimulation of the cortex accompanied rhythmical jaw and tongue movements and swallowing in cats, dogs, primates, and humans [42], and such cortical stimulation also evoked oral sensations including taste and tactile sensations in humans [43]. Second, as previously mentioned, the projection sites from the cortical taste area include the same nuclei of the higher centres for salivation (especially the lateral hypothalamus, central nucleus of amygdala, parabrachial nucleus, and solitary nucleus). In fact, as shown in figure 3, electrical stimulation of the cortical taste area of rats induced impulse discharges in the sympathetic and parasympathetic nerves supplying the submandibular gland of rats.

The efferents from the gustatory cortex project mainly to the ipsilateral subcortical structures. This anatomical characteristic is largely reflected in the physiological results of salivation. As shown in figure 3, electrical stimulation at the frequency of 1 Hz applied to the ipsilateral cortex induced larger responses in the autonomic nerves for rat submandibular gland than did stimulation of the contralateral cortex. Although repetitive cortical stimulation evoked profuse salivation from both sides of the submandibular glands [44], it evoked more vigorous salivation from the ipsilateral submandibular and parotid glands than from the contralateral glands, and chronic hemidecortication of the stimulating area decreased the ipsilateral salivary response for a few days in dogs [45]. The corticofugal fibres for jaw and tongue movements run in the pyramidal tract and affect the activity of the contralateral motoneurones. For example, electrical stimulation of the so-called masticatory area at 50 Hz produced chewing on the contralateral side in rabbits [46]. In contrast, during natural chewing, the flow rate of saliva is higher on the chewing side than on the contralateral side in humans [47, 48] and animals including rabbits [49], sheep [50], horses [51], and mules [51]; humans and these animals chew on one side at a time, but rats can chew on both sides at once and may secrete saliva equally from both sides of the submandibular and parotid glands [12]. Thus, as in the case of rabbits, there is a disparity between the results from cortical stimulation and natural feeding in terms of chewing side versus salivary response.

From the above-mentioned disparity, one can speculate that, at least during chewing on one side, the subcortical structures rather than the cortex dominantly participate in evoking salivation itself. This speculation is largely consistent with the concepts, based mainly on classical stimulation experiments [29], that the relevant function of the cotex may be as an integration of orofacial

reactions including salivation, and that the main reflex arc for salivation is situated in the lower brainstem. This function of 'integration' seems to involve the command signals that start and stop feeding behavior [52], rather than those that maintain ongoing chewing and salivation. For a better understanding of such a function, it is necessary to analyse the activity of cortical neurones in behaving animals, with the monitoring of jaw movements and salivation.

Concluding Remarks

Salivary secretion normally occurs when sapid foods are placed in the mouth, which is well known as the gustatory-salivary reflex. This interrelation of taste and saliva has been studied by means of analyses of secreted saliva (flow rate and composition), neuroanatomical analyses of the reflex pathway, and electrophysiological analyses of neural activity. Many of these studies addressed the reflex arc in the brainstem, and their results can be summarized as showing that the fundamental reflex arc is located in the lower brainstem, and its activity, on flow rate and composition of saliva, depend on the quality of taste stimulation. It is also accepted that higher centers modulate the activity of the reflex arc. Recent neuroanatomical studies have shown the taste efferent pathway from the higher centres to the reflex arc in the lower brainstem. The taste efferent pathway may have many functions, including the control of taste afferent information, jaw and tongue movements, and secretion from digestive glands and endocrine glands (e.g. release of insulin). These various functions may be involved in the modulation of the gustatory-salivary reflex in the lower brainstem.

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Chapter 11

Reflexes of Salivary Secretion

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Introduction

Reflexes are automatic, predictable, reproducible and goal-directed responses to stimuli. Most are innate (some, however, can be learned), and almost all involve the central nervous system. Since the early works of Ludwig [1] in 1850 and Bernard [2] in 1856, it has been known that salivary secretion is dependent on reflex activity. As stated by Emmelin [3] in 1972 'The function of the salivary gland system is characterised by the continuous resting secretion upon which intermittently an enormously increased activity is superimposed.' This 'resting flow' is present throughout the day and night and keeps the mouth and oro-pharynx moist, lubricated and protected. Large increases in secretion are seen during eating and in some species occur during panting as part of a thermoregulatory process.

During eating there are massive increases in secretion over very short periods of time and these increases are attributed, in varying degrees, to the stimulation of a number of sensory receptors, including gustatory receptors, mechanoreceptors, olfactory receptors and nociceptors.

Saliva is produced principally from the three pairs of major glands; parotid, submandibular and sublingual, with contributions from a large number of minor glands distributed all around the mouth. Whole mouth saliva is made up of the mixed secretions from all of these glands, each producing variable volumes and contents, plus debris and other oral fluids such as gingival crevicular fluid. Not only does the secretion from the different types of glands vary in composition and volume but the saliva produced by a single gland is also variable. Therefore, the final mixed saliva can vary considerably in its volume and composition depending on the type and duration of the stimuli applied.

Spontaneous vs. Resting Secretion

Spontaneous secretion has been observed in some salivary glands and involves the continuous production and discharge of small amounts of saliva in the absence of extraneous stimuli [4]. In a deeply anaesthetised animal, including human, most glands are silent but some glands continue to secrete, even in the presence of pharmacological blockers of salivary secretion. Even when totally isolated, some glands will secrete saliva spontaneously. The glands that demonstrate this type of secretion vary between species (e.g. parotid gland in ungulates, submandibular gland in rabbits, the sublingual gland in cats, dogs and rats and minor glands in many species including humans).

Spontaneous secretion should not be confused with resting secretion [3]. Resting secretion may have a component of spontaneous secretion, but also has superimposed upon it a secretion of reflex origin. The stimuli that evoke this reflex resting secretion can be, for example, dryness of the oral mucosa and low-grade mechanical stimulation to the tongue.

Stimuli that Evoke Salivary Secretion

The control of salivation depends on reflex nerve impulses [5]. These reflexes involve afferent limbs, salivary nuclei within the medulla and an efferent limb comprising of both sympathetic and parasympathetic secretomotor nerves. Detailed reviews of the actions of the salivary nuclei and the efferent limb of these reflexes have been covered elsewhere [5] (chapters 2, 10). This chapter will concentrate on the receptors and the adequate stimuli that are known to evoke salivary secretion.

Eating is the main cause of an increase in salivary flow above resting levels. A number of receptors are stimulated before, during and following ingestion of food and drink. Colin [6] in 1854 first described a parotid salivary reflex during feeding in the horse and mule. Stoney [7] in 1873 cannulated the parotid duct of a patient with a parotid fistula and established that masticatory and gustatory stimuli evoked a considerable increase in the rate of parotid salivary secretion. He also reported that a variable salivary response was evoked when food was placed in front of the subject, which presumably provided olfactory and visual (psychic) stimuli. Since these early studies there have been a substantial number of papers that have defined the adequate stimuli involved, and the sites and types of receptors that are stimulated during eating. These can be conveniently classified as gustatory, masticatory, olfactory, psychic, visual and thermoreceptive and possibly nociceptive. Inevitably, the afferent input during normal eating will comprise combinations of the above

stimuli leading to complex reflex responses. However, in order to understand the contributions that each of these afferent inputs make to salivary reflexes they have been studied independently and there are few papers in which a combination of inputs have been used. Furthermore, there are a number of situations not involved with normal eating in which salivary secretion increases such as during nausea, vomiting and pain.

The Gustatory-Salivary Reflex

Stimulation of gustatory receptors, found mainly in the taste buds, leads to a reflex secretion of saliva. This observation has been documented many times since the early work of Stoney [7] but was presumably common knowledge before this. The volume and the chemical composition of saliva induced by gustatory stimulation depend upon the quality of the stimulus [8–16]. There is common agreement that a strong sour stimulus (such as 5% citric acid in man [17], or 0.5 *M* tartaric acid in animals [16]) will evoke a maximal secretory response from most salivary glands. Lower concentrations of acid along with the other basic gustatory stimuli (salt, sweet and bitter) give variable degrees of salivary responses, but all considerably smaller than the maximum (see fig. 1). Kawamura and Yamamoto [16] recorded salivary flow and chorda tympani nerve responses to various concentrations of the basic gustatory stimuli. They concluded that the volume of saliva produced was proportional to the peak summated response of the gustatory nerve when moderate concentrations of gustatory solutions were applied, whilst for the higher concentrations of gustatory solutions, the volume of saliva was proportional to the integrated response. Very few foods contain a hydrogen ion concentration as high as 5% citric acid or 0.5 *M* tartaric acid. It therefore follows that concentrations as high as these cannot be regarded as normal (physiological) gustatory stimuli.

A number of studies provide good evidence that individual gustatory stimuli not only produce different volumes of saliva but can also produce saliva with different overall compositions unrelated to the well-known fact that rate of salivary flow through the ducts affects the concentrations of some electrolytes. For instance a sweet stimulus in the rabbit produces a low flow of parotid saliva with a high protein content [18].

Furthermore, in humans, Dawes and Jenkins [19] and Dawes [20] have shown that salt produces a parotid secretion higher in protein than do other basic stimuli at the same flows. The adequate stimuli for the gustatory-salivary reflex may not be a simple combination of the basic tastes but a common transduction mechanism between different sapid substances. There are hun-

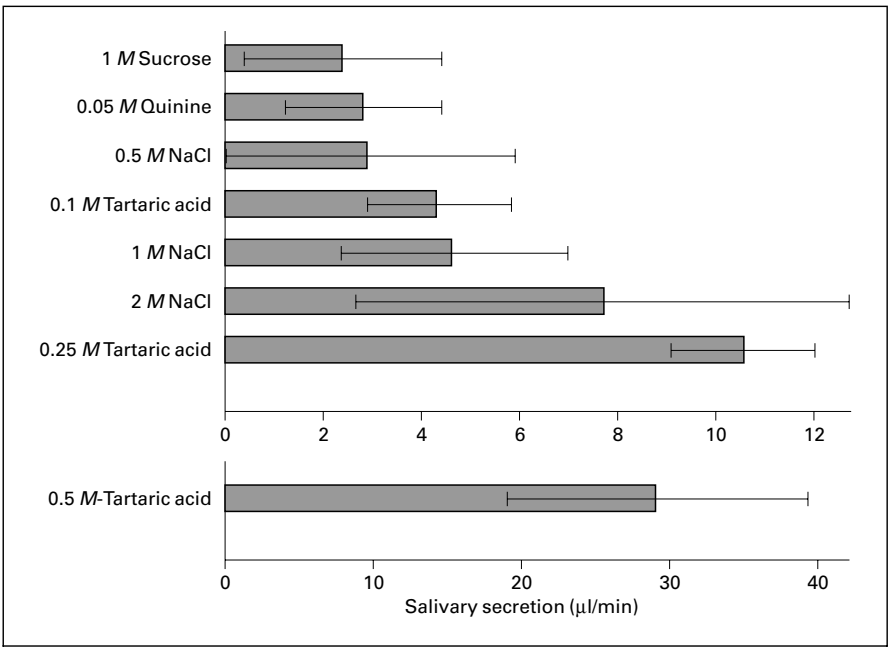

Fig. 1. Gustatory-salivary reflex. Reflex submandibular salivary secretion evoked by different chemical stimuli applied to the anterior region of the tongue of rabbits. Each bar is the mean ± SD for recordings in 4 animals. Note change in abscissa values in 0.5 *M* tartaric acid record. Adapted from Kawamura and Yamamoto [16].

dreds of chemicals that elicit activity from taste receptor cells, and they are generally grouped into the rather broad categories of salty, sour, sweet and bitter. In his recent review, Gilbertson [21] focussed on the mechanisms by which taste receptor cells transduce chemical stimuli from each of the basic taste categories and demonstrated that there are no unique transduction mechanisms for each of the classes of basic taste stimuli. He showed that the individual classes of basic tastants may use one or more different transduction mechanisms and that the mechanisms activated by different classes of tastants may overlap. He suggests that this multiplicity of transduction mechanisms contributes to the many subtle tastes perceived in food and allows us to distinguish different compounds within a single taste class.

The process of transduction of a chemical stimulus to an electrical event within the taste bud receptor cell inevitably leads to the initiation of action potentials. These action potentials (impulses) are transmitted by the afferent limb of the reflex to the salivary nuclei. It must be the pattern of these impulses

and the type and location of the gustatory receptors and not the perception of the taste that leads to a reflex salivary flow. The perception of taste must be a parallel response to these stimuli. Although studies that have used the commonly perceived taste stimuli (salty, sour, sweet and bitter) have added to our basic understanding of this complex gustatory-salivary reflex, future studies must focus on the use of stimuli that use common transduction mechanisms rather than those that use the commonly perceived taste classes.

The Masticatory-Salivary Reflex

Until recently there have been very few studies on the relation between chewing and salivary secretion. Evidence for the involvement of chewing movements and forces has so far come from rather few animal studies. Since the salivary reflexes are greatly affected by general anaesthesia most of the significant contributions to the understanding of the masticatory-salivary reflex have been made on conscious animals including humans.

Colin [6] first described, in the horse and mule, that following bilateral parotid cannulation there was a greater salivary flow from the duct on the chewing side (ipsilateral) than the nonchewing side (contralateral). Gjörstrup [18] reported that, in conscious rabbits, the output of parotid saliva was approximately three times greater when the animal chewed a standard laboratory pellet (range 45–1,260 μl/min) than when they chewed carrots (11–425 μl/min). He suggested, in a subsequent paper [22], that the large ranges seen in parotid flow might be accounted for by secretion alternating from side to side associated with the change in chewing sides. Since Colin [6], Patterson et al. [23] have shown in sheep that parotid flow in response to chewing was greater on the chewing side than on the contralateral side. Anderson et al. [24] confirmed the earlier work of Gjörstrup [18, 22] that rabbits produce greater amounts of saliva whilst feeding on pellets than carrots. Furthermore, they went on to demonstrate that the flow in response to chewing was always greater on the chewing side than contralaterally. By recording strain and presumably masticatory forces, they provided evidence that pointed to a direct relation between chewing force (and presumably intraoral mechanoreceptor input) and parotid flow, and therefore the existence of a masticatory-salivary reflex. They based this conclusion on the following evidence: (1) Flow and mandibular strain were greater with pellets than with carrot, and were greater on the chewing than on the nonchewing side with both types of food. (2) Flow and mandibular strain were both greater with dry pellets than with pellets moistened and softened with water. (3) Injections of water into the mouth during feeding reduced the salivary response to pellets and not to carrots. (4) Flow recordings

superimposed on recordings of mandibular strain showed a remarkable similarity of pattern (fig. 2).

Stoney [7] was one of the first researchers to record salivary secretion in man using a patient with a parotid fistula and he established that masticatory stimuli evoked an increase in the rate of parotid salivary secretion. Lashley [25] in 1916 was the first to investigate the masticatory-salivary reflex systematically using the human parotid gland, albeit on an unstated number of subjects. He recorded parotid flow bilaterally and showed that unilateral chewing on a piece of rubber between the molar teeth resulted in an increase in flow on both sides, but the ipsilateral response was greater than the contralateral. He went on to show that the output of saliva increased with the biting force and from these and other observations be concluded that 'each gland seems to be most intimately associated with the receptors of its own side'.

There was very little work of significance concerning the masticatory-salivary reflex in the 45 years following the work of Lashley. In 1961 a monograph was published, posthumously, which described the work of Kerr [10]. Amongst other studies, the monograph described a series of observations concerning the masticatory-salivary reflex. These observations were mostly on one subject and we presume they were made on Kerr himself. Kerr [10] confirmed Lashley's observations on the relation between chewing side and parotid secretion and, by implication, between chewing force and secretion. In one subject, he demonstrated that anaesthesia of the inferior alveolar and lingual nerves on one side reduced by about 50% the parotid output in response to chewing. Fischer and Kapur [26] reported that there was a 60% reduction in parotid flow following anaesthesia of the same nerves but provided no supporting data. Kerr [10] came to the conclusion that the receptors within the periodontal ligament were responsible for the afferent information on which this secretion depended. In all of these early studies, the mechanical stimulus provided by chewing various materials was clearly not confined to the teeth and their supporting structures and must have spread to involve other intra-oral mechanoreceptors. In all studies since Lashley [25] there has been no contradiction to his observation that unilateral chewing between the molar teeth in man results in an increase in flow on both sides, but the ipsilateral response to chewing is greater than the contralateral. This pattern of secretion continues even during prolonged periods of unilateral chewing (fig. 3). Since the late 1980s, attempts have been made to focus progressively more closely on the possible role of the various intra-oral mechanoreceptors in the masticatory-salivary reflex. These attempts have involved the use of defined and controlled mechanical stimuli which limit the stimulus applied to individual or small groups of receptor types and the use of selective intra-oral topical (mucosal), local infiltration and regional nerve block anaesthesia.

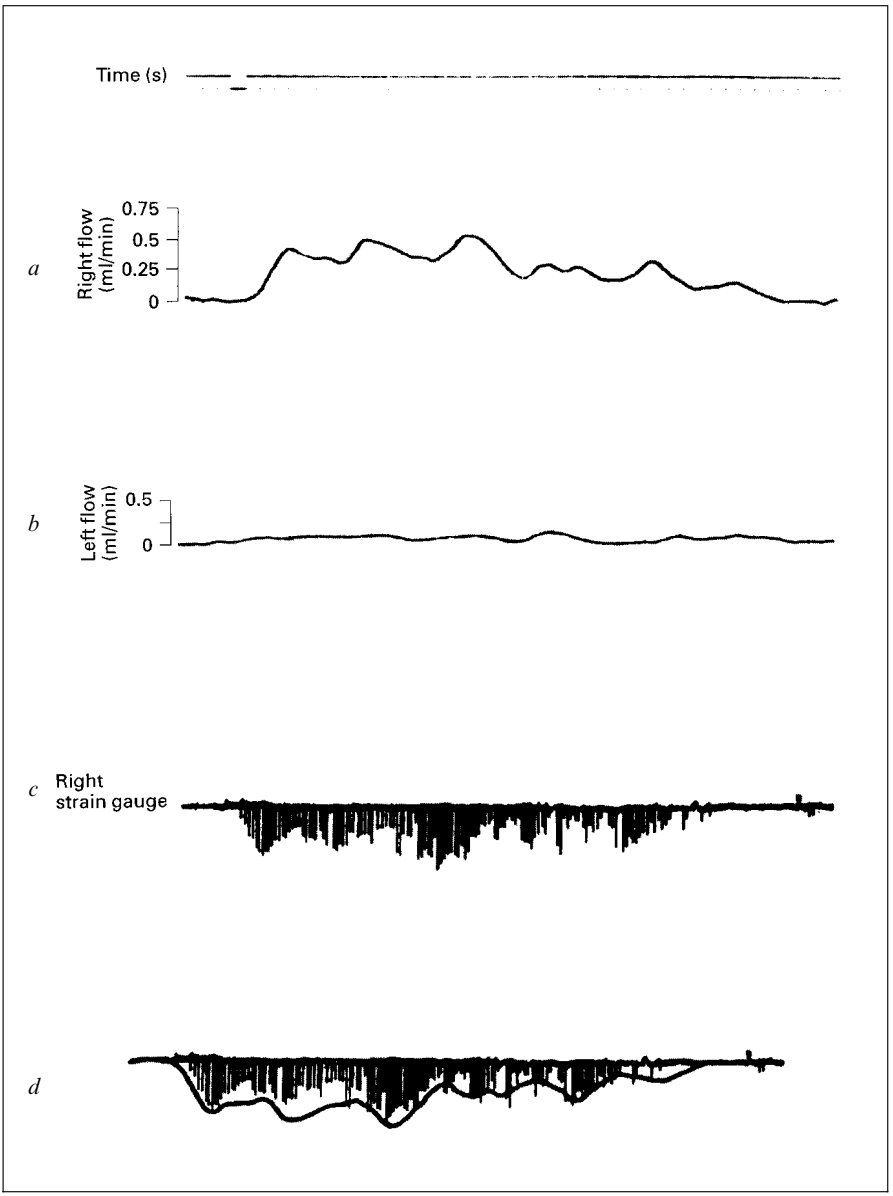

Fig. 2. Masticatory-salivary reflex in the rabbit. Record showing: *a* Right parotid flow. *b* Left parotid flow. *c* Output from a strain gauge attached to the right side of the mandible in a rabbit, during chewing of a single hard pellet on the right side. *d* The remarkably close association between the strain gauge record and ipsilateral flow when the flow record is inverted, shifted (to allow for the latency) and superimposed onto the strain gauge record. Reproduced from Anderson et al. [24].

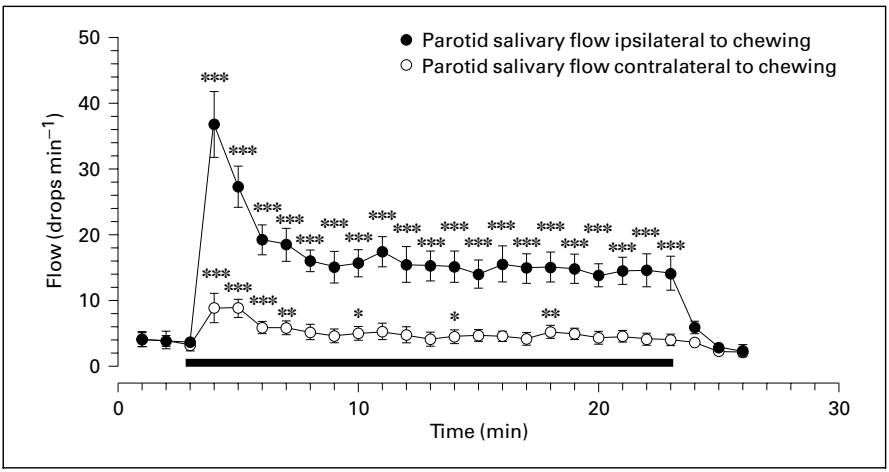

Fig. 3. Masticatory-salivary reflex in humans. Bilateral parotid salivary flow recorded during each minute over a 20-min period of unilateral chewing in humans. The chewing period is shown by the filled bar, parotid salivary flow from each gland is statistically compared with the average of the three rest minutes before and after the chewing period. Each point represents the mean ± SEM drops/min for 10 subjects. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. Unpubl. data from authors' laboratory.

Hector and Linden [27] used a closely fitting bite block placed between the molar teeth and the subject controlled the biting force with the use of visual feedback from the rectified and integrated masseter muscle electromyograph. Parotid salivary flow was recorded bilaterally using modified Lashley cups. The results from this study demonstrated a positive correlation between the masseter electromyographic activity and the ipsilateral parotid flow. Furthermore, resting and mechanically stimulated parotid flows were recorded before and during local anaesthesia of various intra-oral branches of the trigeminal nerve (inferior alveolar, lingual and maxillary nerves). Anaesthesia of two or three inputs always produced significant reductions in ipsilateral flow but anaesthesia of a single input was not always effective. These experiments provided substantial evidence in support of the hypothesis that intra-oral and paritcularly periodontal receptors contribute to the reflex. However, the anaesthesia was not confined to the teeth and periodontal ligament and therefore the involvement of other intra-oral receptors, such as those in the gingival mucosa and tongue, could not be excluded. In a study by Hector [28], a more discrete stimulus was used. In these experiments a small but measurable flow of parotid salivary secretion was recorded when a single uniform piece of breakfast cereal (Grape-nut, Birds General Foods, Ltd.) was crushed between

a pair of opposing molar teeth. Pieces of this cereal when dry are hard, brittle and virtually tasteless. A single piece was placed between a pair of opposing teeth to ensure a minimal input from other oral mechanoreceptors and no gustatory input. By using grape nuts in conjunction with a short-acting local anaesthetic, it was possible to reduce the input from one of these teeth and in all 8 subjects studied there was a significant reduction in the evoked secretion during anaesthesia (for an example see fig. 4). The secretion returned to normal values when the anaesthetic wore off. Removal of part of the afferent information from one tooth always resulted in a reduction in recorded flows without any change in the mechanical factors or afferent information from other intra-oral or extra-oral structures (such as the muscles of mastication and the temporomandibular joint). Thus, this study further supported the role of periodontal receptors in this reflex.

In 1916, as well as concluding that each gland was intimately associated with the receptors of its own side, Lashley [25] proposed that in addition to this excitatory effect on the ipsilateral parotid gland there might also be an inhibitory action on the contralateral gland. His evidence was that when rubber blocks were placed between the molar teeth on both sides the salivary flow recorded from each gland during bilateral chewing was substantially less than that recorded ipsilaterally during unilateral chewing on one block. This could be due to contralateral inhibition as he suggested, but it could also be the result of a smaller input from receptors on both sides due to the distribution of masticatory forces over a larger area. Lashley [25] also observed that something had to be between the teeth during chewing movements in order to evoke a salivary response. This observation was reinforced by the observation made by Hector and Linden [27] that empty clenching generally produces no significant increase in flow above resting levels and frequently below it.

In a recent study, Anderson et al. [29] set out to answer three questions pertinent to the above observations, namely:

(1) Is a lateral component of force required to evoke a flow during empty clenching?

(2) Does contralateral inhibition of salivary secretion explain this observation?

(3) What is the threshold for the masticatory-salivary reflex?

With regards to the first question, the pattern of normal chewing usually includes a lateral component of jaw movement. Periodontal mechanoreceptors are known to be sensitive Ruffini endings, which respond maximally to stretching of the periodontal tissues, most readily produced by lateral movement of the teeth [30]. Lateral movements of the teeth are minimal during empty clenching. It is possible therefore that grinding the teeth together with nothing between them (simulated bruxism) may increase the receptor input and there-

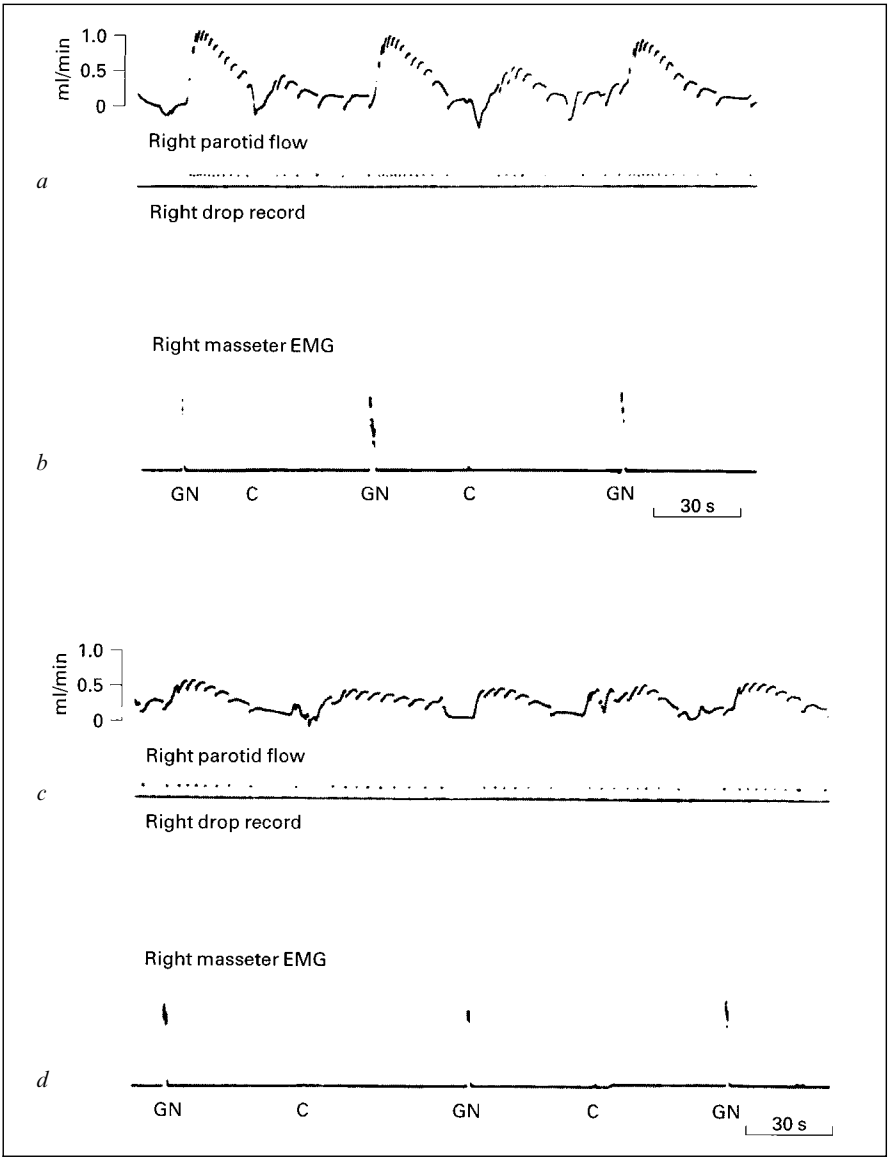

Fig. 4. Evidence for periodontal receptor involvement in the masticatory-salivary reflex in humans. Records from one subject illustrating the right parotid response to crushing 3 grape nuts (as indicated by right masseter electromyographic record) between the right first molar teeth (GN) before (*a*) and during (*b*) anaesthesia of the maxillary molar tooth. Note that during the period of anaesthesia the masseter electromyographic activity was unchanged but the ipsilateral parotid flow was halved compared with the pre-anaesthetic period. The subject cleared the mouth of debris at (*c*). Reproduced from Hector [28].

fore evoke a greater secretion than that seen with empty clenching. However, the first series of experiments carried out by Anderson et al. [29] demonstrated that simulated bruxism did not increase flows above those recorded during empty clenching or at rest. Thus not supporting the first hypothesis. With regard to the second question, if contralateral inhibition is involved in the reduced flows seen during bilateral chewing and empty clenching, this inhibition would be expected to be reduced during anaesthesia of the ipsilateral teeth and therefore one would expect an increase in salivary flow from the opposite gland. However, the results from Anderson et al. [29] produced no evidence for a significant change in contralateral flow during anaesthesia of the opposite side.

Having shown that the output of saliva is directly proportional to masticatory forces an alternative explanation for the low flow seen in empty clenching is that with tooth contact throughout the whole arch, the force per unit area and therefore presumably the periodontal mechanoreceptor discharge does not reach the threshold for reflex parotid secretion. The forces required to reach the threshold for periodontal mechanoreceptors (i.e. the afferent component of this reflex) have been determined and are considerably lower than normal masticatory forces [31]. In the third series of experiments Anderson et al. [29] demonstrated that at less than 5% of comfortable chewing electromyographic activity, salivary flow was evoked in all subjects and at the 20% level of electromyographic activity it was possible to record values as high as 40% of those flows seen at comfortable chewing levels (fig. 5). The observation that salivary flow can be evoked by forces as low as 5% of comfortable chewing means that it is very unlikely that the threshold is not reached during empty clenching over the whole arch. This is despite the fact that the forces are distributed over the whole arch with a consequent reduction in the force applied per unit area of the tooth and therefore to the periodontal mechanoreceptors. All of the subjects found it extremely difficult to bite on the silicone-based block with forces less than 5% of the confortable chewing force. This level was barely more than was necessary to keep the teeth (upper and lower) in contact with the silicone-based block. Even at this very low level of muscle activity, all subjects produced parotid responses, which were higher than resting flows. This suggests that the threshold for the masticatory-parotid salivary reflex is very low. The explanation for the lack of flow during empty clenching remains elusive.

The role of mucosal mechanoreceptors in the masticatory-parotid salivary reflex has recently been described [32, 33]. The first of these papers [32] confirmed the presence of a masticatory-parotid reflex in edentulous patients as seen in earlier studies [34–36]. Anaesthesia of the mucosa of the maxillary and mandibular denture bearing area using topical anaesthetic ointment produced a reduction in salivary flow in response to chewing. This suggests

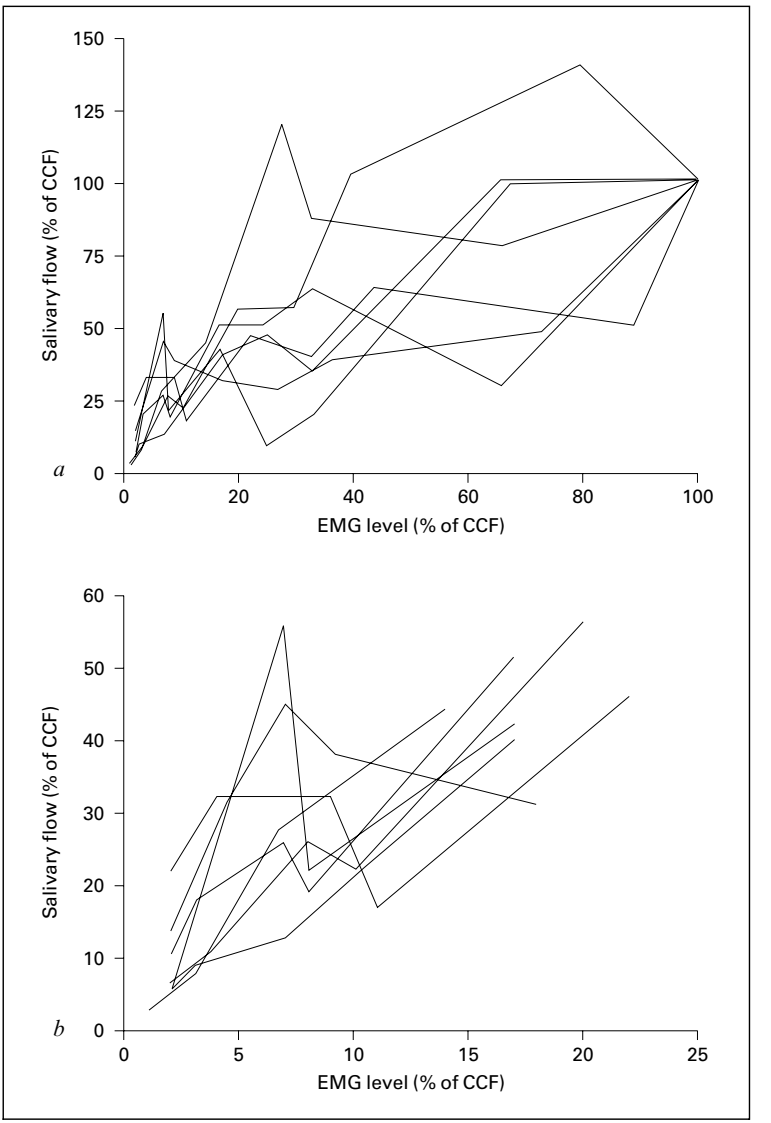

Fig. 5. Threshold of the masticatory-salivary reflex. *a* Relationship between the peak of the rectified and integrated masseter electromyographic activity (expressed as a percentage of the peak activity achieved at a comfortable chewing force: CCF) and salivary flow (100% is the flow recorded at the comfortable level of chewing force). Each line represents the data from one subject, and each point the mean of two observations at each force level. *b* Same as for *a* but limited to the electromyographic levels below 25% of CCF. Reproduced from Anderson et al. [29].

that mechanoreceptors in the oral mucosa are involved in the reflex seen in edentulous subjects. Whether mucosal receptors are involved in the dentate subject was investigated in the paper by Scott et al. [33] in which topical anaesthesia of the lingual gingival tissues alone and both lingual and buccal gingival tissues together resulted in a significant reduction in flow during chewing. However, anaesthesia of buccal gingival tissues alone did not produce a similar reduction. Taken together these results suggest that not only are periodontal ligament mechanoreceptors involved in the masticatory-salivary reflex but so are gingival mucosal tissue mechanoreceptors.

Up to the present day all studies of the masticatory-salivary reflex have involved adult subjects. A recent preliminary study has looked at the reflex in young children with an intact deciduous molar dentition (age 5–8 years). The results demonstrate that receptors associated with the deciduous tooth and supporting structures can contribute to this reflex [37].

The Olfactory-Salivary Reflex

The receptors that are involved in olfaction are termed olfactory receptors and are to be found principally in the olfactory neuroepithelium. In man, the olfactory system responds to airborne, volatile molecules that can stimulate olfactory receptors via nasal flow of air during inspiration and via retro-nasal airflow from the oropharynx or the oral cavity. These odourants stimulate the ciliated dendrites of the olfactory receptor neurones having diffused through a thin layer of mucus. The transduction mechanism involves olfactory proteins on the cilia where odour ligand-receptor interactions take place and lead to olfactory nerve stimulation via a secondary messenger cascade system (for a detailed account of the mechanisms see Wilson and Sullivan [38]). The term olfaction should be used when describing stimulation of the olfactory receptors alone. Common chemical sense has been defined as the sensation caused by the stimulation of trigeminal epithelial or mucosal free nerve endings by chemicals. Smell is defined as nasal chemoreception, which includes the combination or interaction of both olfaction and common chemical sense.

Since the classical work of Pavlov [39, 40], in the late 1920s, on the conditioned reflex, it has been assumed that the smell of food causes salivation in man. Many widely read textbooks state that an olfactory-salivary reflex exists in humans [41–44] and significant increases in whole salivary flow have been recorded in response to olfactory stimulation [10, 45]. However, until recently the evidence available for the existence of an olfactory-parotid reflex was confused and inconclusive. Kerr [10], Shannon [46] and Pangborn et al. [45] all reported that olfactory stimulation caused an increase in parotid sali-

vary flow. However, Pangborn and Berggren [47] demonstrated that non-irritating odours had no effect on parotid salivary flow. Furthermore, Lashley [25], Winsor [48] and Elsberg et al. [49] could not find any consistent increase in parotid salivary flow in response to non-irritating odours. Evidence for the existence of an olfactory-submandibular salivary reflex was sparse [10, 45], but many unsubstantiated reports have been made concerning an apparent awareness of saliva collecting in the floor of the mouth during olfactory stimulation.

In an attempt to bring some clarity to this subject that is surrounded by much confusion, Lee and Linden [50–52] performed a series of experiments that used very sensitive instantaneous flow meters [24, 27, 28] to record both parotid and submandibular salivary flow during olfactory stimulation. In the first of these studies Lee and Linden [50] were unable to elicit an olfactory-parotid salivary reflex in response to stimulation with a series of pleasant odours (fig. 6). However, an increase in salivary flow was seen when lemon juice or odourless citric acid was sniffed or delivered to the subject at high concentrations, causing irritation to the nasal cavity and/or oropharynx. They concluded that there was no true olfactory-parotid salivary reflex in humans, but acidic stimuli can cause irritation with a concomitant increase in parotid salivary flow. In the second paper in the series, Lee and Linden [51] examined the responses of the submandibular gland to the same series of pleasant odours. They demonstrated a significant increase in salivary flow in response to each of the odours (fig. 7). They concluded that the olfactory-submandibular salivary reflex does exist in humans.

In the third paper in the series, Lee and Linden [52] investigated the possibility that synergism may occur between an olfactory stimulus and a strong salivary stimulus such as mastication or gustation in producing a parotid reflex response. They investigated the effect of two odours (peppermint and orange) on unilateral parotid salivary flow stimulated either by mastication or by mastication with gustation. They found that neither odour stimulated flow above that evoked by mastication or mastication with gustation. They concluded that olfaction had no effect on stimulated parotid flow in humans. This was in contrast with the work of Chauncey et al. [53] which showed that parotid salivary flow, stimulated by fruit-flavoured lozenges, was consistently reduced when a nose clamp was placed over the nares to prevent nose breathing thus removing the smell stimulus. They concluded that the reduction in salivary flow was '… a result of the reduction in availability of the olfactant', and, therefore, that olfactory stimulation contributes to parotid salivary flow in humans. Lee and Linden [52] repeated this experiment but instead of using flavoured lozenges, used odourless chewing gum base. Their results showed that sealing the nares with a nose clip also caused a significant reduction in

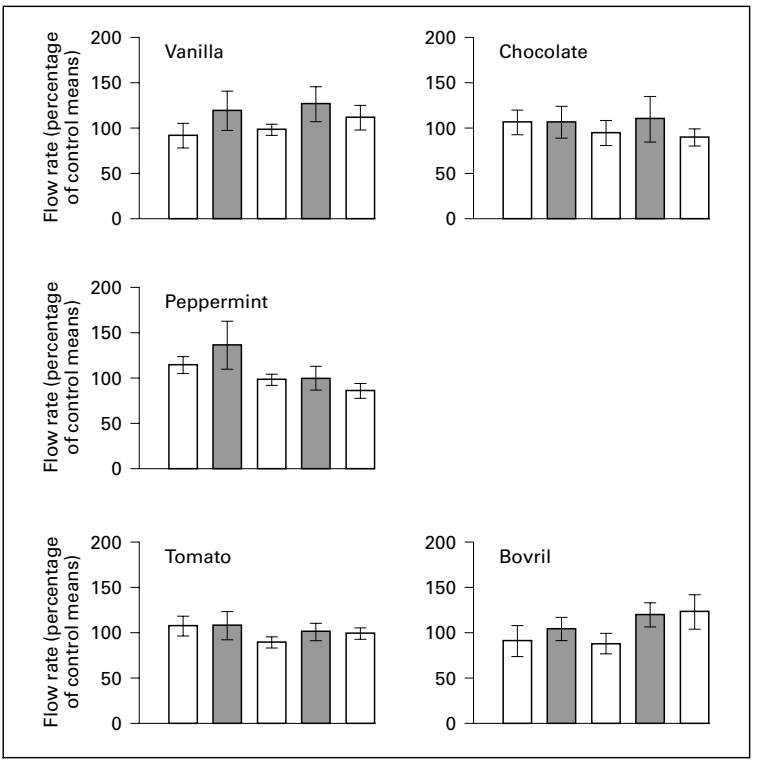

Fig. 6. The olfactory-parotid reflex in humans. The effect of five appetising odours on resting parotid salivary flow in 10 subjects. The flow for each 1-min period is shown as a percentage of the mean of the 3 control periods. The open bars represent the average of two 1-min control periods and the hatched bars represent a single 1-min test period. Bars represent the mean ± SEM. Reproduced from Lee and Linden [50].

parotid saliva elicited by chewing. Furthermore, placing the nose clip over the bridge of the nose without sealing the nares significantly reduced salivary flow. This suggested that the application of the nose clip to the skin overlying the nose caused the reduction in stimulated salivary flow by stimulating trigeminal mechanoreceptors in the skin. Nothing is new under the sun! Lashley [25] reported, albeit in one subject, that tickling the subject's nose or lips caused a reduction in gustatory stimulated parotid flow. He also reported that electrical current applied to the fingertips caused a similar reduction in flow. However, in contrast with the nose clip and the reported tickling, this stimulus was obviously painful since the subjects experienced 'profuse perspiration' and were 'left completely shaken'!

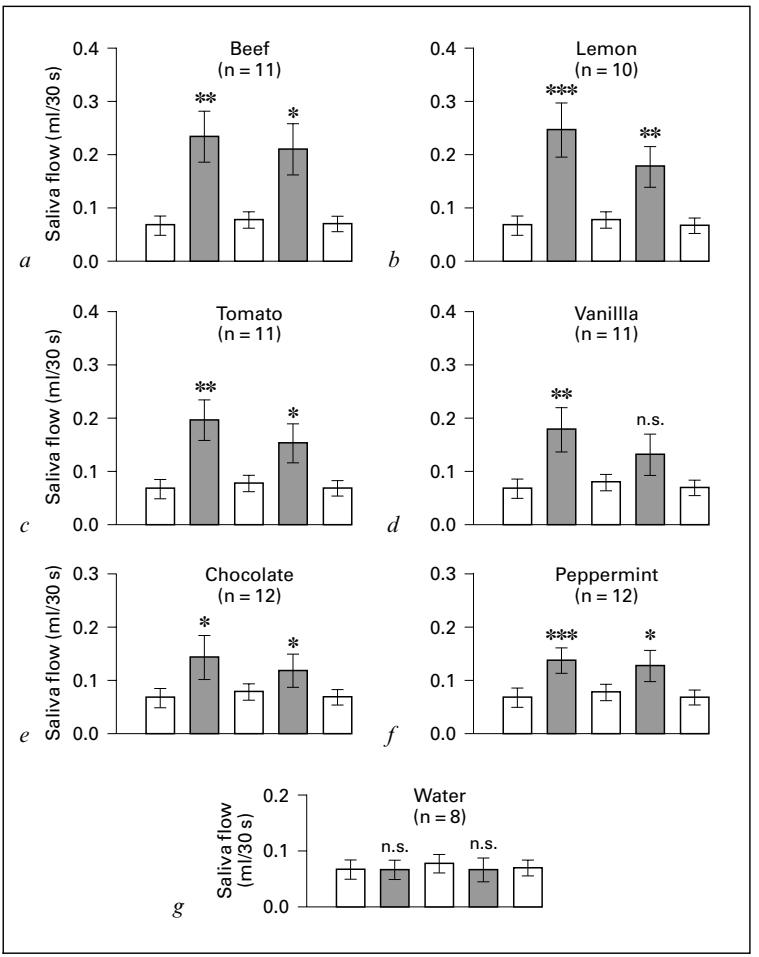

Fig. 7. The olfactory-submandibular reflex in humans. The effect on bilateral submandibular salivary flow of six appetising odours (*a–f*); in *g* distilled water was used as both test and control stimulant. Thge open bars represent the average of two 30-second control periods and the hatched bars represent a single 30-second test period. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ Bars represent the mean $\pm$ SEM. Reproduced from Lee and Linden [51]

The oro-facial region derives most of its sensory innervation from the trigeminal nerve [54]. The mechanism by which the sensory input from the nose clip causes inhibition of salivary flow is unclear, as most animal studies suggest that stimulation of trigeminal sensory nerves results in increased salivary flow.

Additional Afferent Inputs that Effect or Affect Reflex Salivary Secretions

Non-Noxious vs. Noxious Stimuli

As reported earlier, most animal studies suggest that stimulation of trigeminal nerves results in increased salivary flow. Electrical stimulation of trigeminal afferent branches has been shown to elicit increased activity in the secretomotor nerves supplying the submandibular glands [55] and parotid glands [56] in cat, and in monkey [57] electrical stimulation of the trigeminal nerve elicits an increase in submandibular salivary secretion. Furthermore, it has been shown in decerebrate rats [58] and rabbits [59] that noxious mechanical stimulation results in an increase in submandibular flow. These animal studies appear to present very different conclusions from the human studies and may simply be due to species differences. Furthermore, the animal studies involve anaesthetised or decerebrate preparations and the flows observed are those superimposed on basal flows, and use stimuli that could well be of sufficient intensity to stimulate nociceptor fibres. Indeed, the stimuli applied by Kawamura and Yamamoto [59] were noxious. In the human studies described by Lee and Linden [52] the stimuli applied to the nose were non-noxious. In a number of human studies [60–62] in which noxious stimuli have been applied to the oral tissues using solutions of capsaicin (a member of a group of substances known as capsaicinoids, which are found in chilli peppers (*capsicum*)), an increase above resting levels of parotid salivary flow has been reported.

Oesophageal-Salivary Reflex

Patients with gastro-oesophageal reflux typically present with heartburn. They also often experience the 'waterbrash phenomenon' a sudden filling of the mouth with fluid when heartburn is present [63]. There is limited evidence for the effect of oesophageal acid on salivary secretion. In two studies prolonged exposure of the oesophagus to infused hydrochloric acid resulted in increased salivary secretion [63, 64]. In both these studies up to 100 ml of 0.1 *M* hydrochloric acid per hour were constantly infused into the distal oesophagus for up to 2 h. This pattern of exposure to hydrochloric acid does not closely mimic naturally occurring gastro-oesophageal reflux. In an attempt to determine the presence of this reflex under more physiological conditions, von Schönfeld et al. [65] infused 20 ml of 0.1 *M* hydrochloric acid as a single bolus into the distal oesophagus. They observed no increase in either resting or chewing gum stimulated whole mouth salivary secretion when compared with a water control. In contrast to the two earlier studies none of their subjects experienced any discomfort following the infusion of acid.

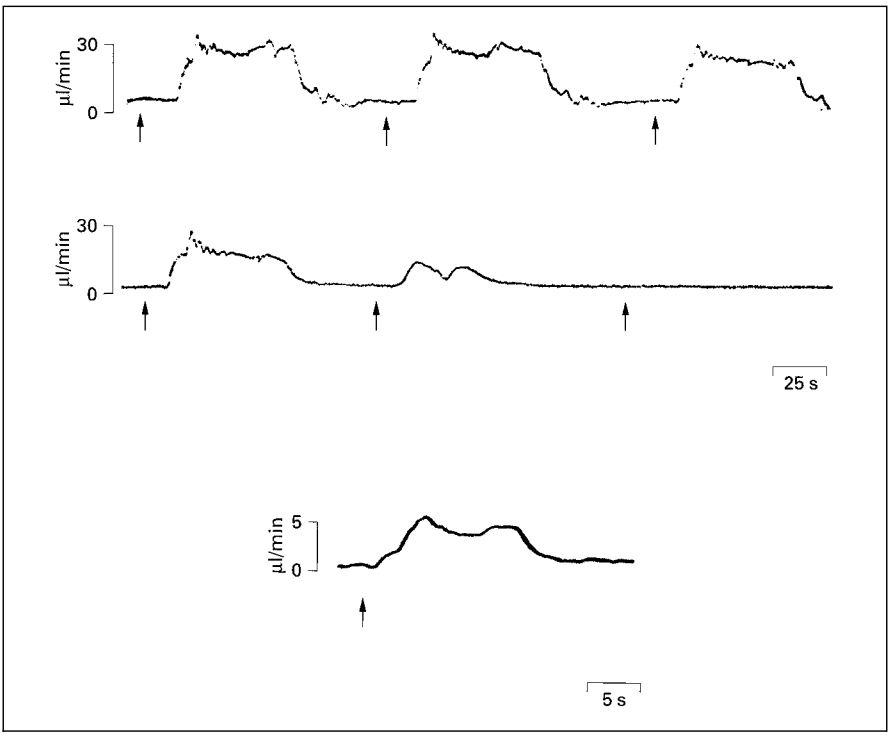

Fig. 8. Conditioned salivary reflex in man. The top two traces show the parotid salivary response to six successive tests (indicated by arrows) with a light and buzzer alone after successful conditioning in man. By the sixth test the conditioned reflex has been extinguished. The bottom trace demonstrates the very small response of a conditioned subject watching the experimenter unwrap and suck a lemon drop. Reproduced from Holland and Matthews [70]

Visual and Psychic Stimuli

Kerr [10] stated that it was widely believed that the thought and sight of food act as strong stimulants to the production of salivation, despite the lack of evidence in the literature. This misconception remains today [44]. There is still no convincing evidence that a non-conditioned salivary reflex in response to the sight or thought of food exists. As suggested by Kerr [10], it is possible that under non-laboratory conditions small increases in salivary flow are a result of unrestricted swallowing and anticipatory mouth movements that cause expulsion of preformed saliva from the dead space of the glands. It is also possible that when individuals think about or see food that not only do they perform anticipatory mouth movements but also

become aware of the presence of saliva in the mouth. The evidence presented by Kerr [10] would suggest that the story of the trumpet player who can be put off his performance by a mischievous schoolboy sitting in front of him and sucking a lemon and thereby evoking an excessive salivary response could at best be described as apocryphal. Any failure in his performance cannot be attributed to an increase in salivary flow caused by an innate reflex response. Even if conditioned the response evoked by such a stimulus is minute (fig. 8).

Nevertheless, there have been a number of studies that have suggested that an increase in salivary flow will occur only under a certain set of conditions that normally accompany a stimulus [66–70]. These studies demonstrated that 'natural' conditioned salivary reflexes are present in man but are extremely small and extinguished rapidly (fig. 8). Holland and Matthews [70] showed that the problem lay not in measuring and recording the response but in the process of establishing the reflex. This is in total agreement with Pavlov [39, 40] who stressed that a reflex could only be established if a consistent response was obtained with each conditioning stimulus. Because of this it is highly unlikely that normal individuals, going about their daily lives, experience an increase in salivary flow when subjected to the sight or thoughts of food.

Concluding Remarks

This chapter has concentrated on the physiology of the reflexes of salivation and in particular the stimuli that cause salivary secretion. We have reviewed the evidence for the contributions each of these stimuli make to the reflexes of salivation in man. Most of these stimuli are related to eating and there is little experimental evidence concerning the integration of these stimuli when we are having a meal. Furthermore, there is little experimental evidence for the additive or synergistic effect on the final secretions of combining the different stimuli not only in terms of volume but also in terms of its composition. It must always be borne in mind that saliva has numerous specialised functions such as lubrication, cleansing, digestion, re-mineralisation of the dentition, maintenance of mucosal integrity and antimicrobial properties and the composition of the saliva is important in all of these. Future research into the stimuli that cause salivary secretion must involve analysis of the resultant saliva and not just the volumes secreted.

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Chapter 12

Glandular and Neural Mechanisms of Salivary Secretion

Past, Present and Future

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> 'Science is built up with facts as a house is with stones, but a collection of facts is no more a science than a heap of stones is a house.'

This delightful aphorism, written by the 19th century mathematician Poincaré, was quoted by the late 'biology watcher' and author, Lewis Thomas, in his last book, 'The Fragile Species' [1]. Lewis cited this passage to advance the argument that while a reductionist approach to research is crucial to our understanding of the fundamental elements of knowledge, science itself must be viewed as a 'holistic' pursuit. Regrettably for contemporary medical science, the term 'holistic' has become debased and lost its respectability. Nonetheless, it remains true that biological systems are greater than the sums of their individual parts.

Each of the authors who contributed to these two volumes[1] was asked to give an integrated assessment of present knowledge, and to do so within a historical context. It is all too prevalent in modern science to consider as 'dusty history' anything that has not been published within the last 10 years. The historical introduction to the first volume [2] clearly illustrates, however, the great debt we owe to Ludwig, Bernard, Langley, Emmelin and others, and the remarkable understanding of salivary gland physiology that was achieved without benefit of

[1] The first volume, 'Glandular Mechanisms of Salivary Secretion' was published as volume 10 of the *Frontiers of Oral Biology* Series, Karger, 1998.

the modern techniques of molecular and cellular biology. Just as lamentable as this loss of history is the fact that we are training a generation of researchers who have little or no understanding of structure as it relates to organ and whole body physiology. We may also be in danger of losing the skills necessary to carry out whole animal work. Thus, there was a need for an historical and structural framework within which current and future research into salivary gland structure and function could be placed.

Glandular Mechanisms of Salivary Secretion

Glandular Variablitlity

The first great truth about salivary glands is that they are wonderfully variable, from one gland to another as well as between one species and another. This fact is sometimes not fully appreciated, but most of the contributors to this series referred to, if not actually dealt with, interglandular and interspecies variability. We may never understand the full importance of many of these differences, but the rules of evolution dictate that for the most part variability arises stochastically. Random mutations that result in adaptations which are beneficial or at least neutral in their effects are perpetuated in the following generations. Tandler and Philips [3] reviewed the microstructure of mammalian salivary glands, and then raised the interesting question of why does such wide phenotypic variation exist. They concluded that the variation is not explainable simply on the basis of a single evolutionary pressure, for example diet, but by studying several related species of bats having specialized diets, Tandler and his colleagues were able to demonstrate effects of natural selection on salivary gland structure.

Just as we don't fully comprehend the basis for interspecific phenotypic variation, there still remain significant questions as to the physiological functions of the amazing array of salivary proteins. This is particularly so for several of the biologically active proteins produced in the granular duct cells of rodent submandibular glands, including tissue kallikrein, epidermal growth factor and nerve growth factor. The 'functions' of salivary glands are often described in terms of the molecular and cellular events involved in exocytosis and fluid transport. However, it is the roles that saliva plays in digestion, the defense of the oral cavity, wound healing and internal homeostasis that really constitute true salivary gland function.

Electrolyte and Fluid Transport

The molecular and cellular mechanisms involved in signal transduction, which lead to the secretion of protein and water, have been the subjects of

intensive study over the past 40 years. Electrophysiological techniques have given us a much clearer understanding of the secretory process, and intense research activity in this arena is now directed towards illuminating the mechanisms of control (Smith and Gallacher [4]). Using patch-clamp techniques, it is possible to investigate individual ion channel activities and, as a result, we are beginning to understand how acinar cells control their intracellular ionic environments in such a way as to optimize fluid and electrolyte secretion. Following this discussion of ion channels, Poulsen [5] summarized the elegant studies that led to and have since refined the classic 'two-step hypothesis' of salivary secretion. In the first step, membrane transport systems and ion channels in the acinar cells are responsible for the formation ionically of plasma-like primary saliva, which in the second step is modified in the duct system. Challenges remain, however. The mechanism of water secretion, as well as that of ion transport in the ducts, is still not clearly understood.

Salivary Gland Blood Flow

Salivary gland fluid production involves the movement of water out of the capillaries into the interstitial tissue, and thence across the glandular epithelium into the lumen, and the rate of this transfer can be impressive. The importance of autonomic nerves in the regulation of salivary gland blood flow was established by the work of Bernard [6] and Heidenhain [7]. This and subsequent observations revealed that resting blood flow is determined largely by tonic sympathetic activity, whereas the flow of saliva and vasodilatation is mediated mainly by parasympathetic impulses. Evidence also supports the belief that there are separate populations of vasomotor and secretomotor nerves to the salivary glands. With respect to the role of nerves in vasoregulation, the activities of nonadrenergic, noncholinergic transmitters and of nitric oxide (NO) in salivary vascular phenomena have come to the forefront [8]. Peptides such as vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP) and substance P are potent vasodilators, and their distribution in vascular parasympathetic nerves varies among glands and species. Nitric oxide is also a potent vasoactive agent whose release is evoked by both parasympathetic and sympathetic stimulation. However, one of the most important questions may be what are the contributions of NO and other vasoactive peptides under reflex conditions?

Saliva must derive from the plasma, but what drives this fluid across the capillary wall and why is there such a large increase in blood flow upon nerve stimulation? Without a concomitant and substantial increase in glandular blood flow the process of salivary secretion quickly comes to a halt. Thus, the huge increase in blood flow may be required to ensure that sufficient fluid is supplied to prevent the creation of gradients countering net fluid transport

across the epithelium and into the lumen. Building on the theoretical considerations underlying the net exchange of water, and in particular the mathematical model of fluid flux first enumerated by Starling [9] in 1986, Smaje [10] summarized the studies which demonstrated that the dramatic increase in transcapillary fluid flux during stimulation of secretion is due to increases in both capillary pressure and tissue oncotic pressure.

Most of the capillaries in salivary glands are fenestrated, which accounts in part for their considerable permeability to water and small solutes, and a movement of substances, such as steroid hormones, drugs and toxins from the blood into saliva does occur and may have potential functional and diagnostic significance. Such glandular permeability was shown by Bernard in 1856 [11], and, based on a number of observations, Bernard concluded that the salivary glands are 'selective' in what they allow to pass out of the blood and into the saliva. Several factors influence glandular permeability, including the lipid solubility, molecular size and ionization of the solute. Many substances reach the saliva by simple diffusion across the plasma membranes of the acinar cells, but others take a paracellular route and traverse the tight junctions or, as in the case of secretory IgA, there is a special transcellular vesicular mechanism [12, 13]. The permeability barrier for solutes seems also to have 'plasticity' that, under certain conditions (especially sympathetic stimulation), enables the passage of larger molecules into saliva. The mechanisms of these changes are not understood, nor do we know whether the movement of specific substances into saliva is purposeful, or whether it is incidental to the normal movement of fluid.

Protien Synthesis and Secretion

Just as electrophysiology made possible an understanding of water and electrolyte secretion, so electron microscopy and radioautographic techniques led to fundamental discoveries about the synthesis and secretion of proteins [14, 15]. Recent evidence demonstrates that coordinated sympathetic and parasympathetic nerve activity regulates not only secretion, but also synthesis at both the transcriptional and translational levels [12]. It is quite intriguing that transcriptional regulation exerted by neural stimuli varies depending on the protein being investigated, thereby highlighting the necessity for further study of the individual promoter and other regulatory sequences involved in the control of gene expression in salivary glands.

The classical exocytotic passage of prepackaged secretory granules is clearly predominant in terms of salivary protein secretion, and a concentrated effort has been made to define the molecular events involved in this process. Nonetheless, there is much more to learn about the cAMP- and Ca^{2+}-mediated events involved in signal transduction. One of the most interesting areas under

current investigation is that of the role played by small, monomeric GTP-binding proteins (ARFs, etc.), attachment proteins (SNAPs), fusion proteins (SNAREs) and others in the targeting and fusion of secretory granules to the apical membrane [16, 17]. Segawa and Yamashina [18] used video-enhanced microscopy and confocal laser microscopy to study the dynamics of secretory granule release from living cells. The results of their elegant work suggest that the release of these secretory proteins is a relatively slow process, and that cytoplasmic microfilaments may regulate both pre- and postfusion processes in granule exocytosis.

Despite the predominance of granule exocytosis in salivary protein secretion, other routes for secretory protein release exist in salivary acinar cells, including (1) a 'constitutive-like' pathway; (2) a constitutive pathway to the apical membrane; (3) a constitutive pathway to the basolateral membrane, and (4) transcytosis from the basolateral to the apical membrane [12]. The functions of these vesicular pathways, with the exception of transcytosis (for secretory IgA), are largely speculative. Their addition to the protein composition in saliva per se must be minor, but do they contribute to homeostatic or 'endocrine-like' functions either within the glands themselves or more widely?

Hormones and Salivary Glands

Rodent submandibular glands, in particular, are a rich source of biologically active proteins, including kallikreins and EGF in rats and mice, and NGF and renin in mice. Although many of these proteins are released into the blood in small amounts, as well as secreted into saliva [11, 12], their physiological functions have yet to be fully explored. There is little direct evidence to support a classical 'endocrine' function for salivary glands, but a constitutive release of biologically active proteins and peptides may contribute to the regulation and maintenance of homeostasis [19]. In addition to homeostatic mechanisms, allostatic processes that may be entirely inappropriate for normal function might become operative under pathological conditions [20]. Under this allostatic model, changes in the exocrine or 'endocrine' functions of salivary glands could actually contribute to the development of pathology.

While salivary secretion is not initiated by circulating hormones, there are significant endocrine influences on the development, structure and function of salivary glands. Experimental animal models of diabetes mellitus have been used to study all aspects of diabetic pathophysiology, and there is now a considerable body of evidence demonstrating the effects of diabetes on rodent salivary glands [21]. The effects of diabetes on salivary glands appear to be related as much to the indirect consequences of insulin insufficiency on the circulating levels of other hormones, and autonomic nerve function, as to the direct actions of insulin. Of more general significance, however, are studies

which suggest that salivary glands may be useful models to study the development of two major complications of diabetes mellitus, microangiopathy and autonomic neuropathy. This hearkens back to the use of salivary glands during the 19th century to study general physiological principles.

Immunological Aspects of Salivary Gland Function

In contrast to the rather uncertain evidence that salivary glands are 'endocrine organs' our view of salivary glands as 'immunological organs' is firmly established [13]. The importance of mucosal immune system to the defense of the oral cavity, and in all likelihood the maintenance of general health, is unquestionable. Nevertheless, the secretory immune system is under complex regulation that is only partially understood. For example, we have yet to determine which lymphoepithelial tissues (e.g. gut-associated lymphoid tissues, GALT) are most important for the induction of secretory immunity, and recent evidence [22] suggests that nerves may influence the process of transcytosis of the IgA molecule.

Myoepithelial Cells

One final and rather intriguing aspect of glandular mechanisms in salivary secretion is the presence of myoepithelial cells [23]. Their presence in salivary glands was first described in 1865 by Krause [24], but they are seldom considered in functional studies on salivary secretion. Electron-microscopic studies on the changes in intraglandular nerves after postganglionic denervations indicated that myoepithelial cells commonly have a dual innervation, which has been corroborated by functional studies. The latter showed that parasympathetic impulses, and commonly sympathetic impulses, cause myoepithelial cells to contract and this precedes the overt secretion of fluid from the parenchymal cells. Consequently, their action must normally affect the dynamics of secretion, a fact that is usually ignored. As with all other facets of salivary gland biology, species and glandular differences exist in myoepithelial cell distribution, their extent, innervation patterns, and neuroeffector-parenchymal cell relationships (epilemmal vs. hypolemmal).

Neural Mechanisms of Salivary Secretion

A system producing only a limited amount of product and controlled by a single agonist would be very vulnerable, but salivary glands (like so much else in biology) are not parsimonious in either production of saliva or redundancy in regulation. Numerous neurotransmitters from two anatomical routes, parasympathetic and sympathetic, act collaboratively to maintain synthesis

and secretion. As a result, the salivary system will continue to perform adequately under a wide range of conditions, excepting severe and destructive pathological influences. Given the importance of autonomic nerves in regulating most aspects of salivary gland function, it seemed not only fitting but also necessary to devote the present volume to those areas of neurobiology (anatomy, biochemistry and physiology) that are relevant to salivary glands.

Two main concepts have emerged from this work: (1) despite considerable variability in structure and innervation there is remarkable similarity of gland secretory function and (2) under reflex conditions there is a synergism and cross-talk between sympathetic and parasympathetic nerves, transmitters, receptors and intracellular transduction mechanisms. Thus, we must be willing to embrace these complexities, rather than continue to view salivary gland function only from the rather limited perspectives offered by the use of specific glands, single agonists, or isolated nerve stimulations.

The Neuroanatomical Basis of Salivary Secretion

The gross anatomy of the nerves supplying the submandibular glands was known at least as early as 1850 [25] and over the succeeding 50 years the gross innervation of all of the main salivary glands was worked out (see chapter 1). It was also recognized that each gland receives a sympathetic and a parasympathetic input, but salivary gland innervation cannot be viewed quite so simplistically for two reasons. First, the pathways taken by the postganglionic fibers are not confined to the conventional routes. Second, and possibly more important, vascular nerves in salivary glands should be considered separately from secretomotor nerves. The central sympathetic connections for vascular nerves are different, and for parasympathetic nerves electrophysiological studies suggest that the firing patterns underlying secretomotor and vascular nerves are distinctive [26].

Microscopic knowledge emerged slowly, because of the need to develop consistent light- and electron-microscopic techniques, which did not occur until the second half of the 20th century. Electron microscopy, in particular, was required to provide information about the neuroeffector arrangements (a intimate hypolemmal type vs. the somewhat more distant epilemmal relationship, for example). Here again, glandular and species variability abounds. Immunohistochemical techniques have replaced the earlier histochemical methods and this has led to a revolution in our understanding about the coexistence and cofunction of neuropeptides and the classical neurotransmitters (acetylcholine and noradrenaline). However, the presence and intraglandular distribution of different peptides varies not only between species, but also between and within glands. In addition, we cannot say whether all axons of any given type (i.e. sympathetic or parasympathetic, vascular or secretomotor)

contain similar amounts of transmitters in similar proportions. Complicating all of this is the fact that impulse rates influence the amounts of transmitter released. For example, at lower frequencies the release of conventional transmitters such as noradrenaline and acetylcholine predominate, whereas higher frequencies and burst stimulation are associated with the detectable but exhaustible release of neuropeptides. Thus, while no general pattern is discernible at this time, neuropeptide and conventional transmitter release must be adaptable to the functional requirements of the species, the gland and the cell type that is being innervated. As new candidate peptides are discovered, the need to come to some general consensus about neuropeptides and salivary gland function will continue to grow. However, it is manifestly clear that attempts to attribute sole responsibility for any activity to a single transmitter are no longer tenable.

The central connections of salivary glands have also been known for more than 100 years, yet the central control of sympathetic and parasympathetic impulse generation remains to be fully explored (see chapter 2). Efferent impulses originate from preganglionic neurones in the medulla (the parasympathetic primary centers) and in the upper thoracic spinal cord (the sympathetic relay). Each of these neuronal systems receives both excitatory and inhibitory inputs from other neural structures in the brainstem (oral sensory information) and forebrain (regulation of feeding, drinking and body temperature). Functionally, inputs from these areas converge on the primary salivary neurones simultaneously, and considering the multiple convergences of synaptic inputs, the primary salivary centers should be able to produce various patterns of efferent impulses. To date, electrophysiological experiments have focused primarily on only a few reflex pathways involving oral sensory inputs, and neither complex firing patterns nor inhibition have been detected, but they may occur in vivo. Nonetheless, we expect that these very sophisticated electrophysiological studies will continue to contribute greatly to our understanding of reflex salivary secretion (see below).

Neurotransmitter and Neuropeptide Function

Attempts to work out the roles of various transmitters in salivary secretion have involved multidisciplinary approaches, including selective denervations, as well as pharmacological and direct nerve stimulations.

Initially, attempts were made by Heidenhain and others to correlate the histological changes that accompanied electrical stimulation of the salivary glands with the secretory events themselves (see chapter 4), and these earlier observations have been extended through the use of electron microscopy. Studies of salivary flow rate and biochemistry have continued, and together with data from pharmacological experiments, both in vivo and vitro, an apparently orderly scheme of transmitter and function was elaborated. Until quite

recently, however, the resulting sympathetic (β-adrenergic)-parasympathetic (cholinergic) dichotomy has in fact hindered a true understanding of the neurobiology of salivary secretion, largely because the limitations of the experimental protocols were either unappreciated or dismissed as insignificant.

A different approach, selective denervation, is based on the premise that changes occurring after sectioning of one or the other autonomic nerve division, thereby removing normal reflex impulse traffic, shed light on the role of these nerves in salivary gland function. The structural and physiological effects of denervations on dog submandibular glands were reported by Bernard [27, 28]. Since then, long-term denervation studies showed that normal reflex activity is usually required to maintain structural integrity, with parasympathetic denervations having the greatest effect (see chapter 7). Loss of the sympathetic innervation produces more subtle effects. Recently, a substantial body of evidence indicates that neuropeptides, rather than acetylcholine often have a more important role with respect to the trophic effects of autonomic nerves (see chapter 6). Nonadrenergic, noncholinergic (NANC) mechanisms may also play an important role during gland development.

The effects of denervation on protein synthesis and secretion are discussed by Proctor (chapter 8), who notes that even in the absence of both the sympathetic and parasympathetic nerve supplies to the salivary glands, some protein synthesis and secretion continue. Pathways other than the regulated exocytosis of secretory granules must remain operative [12]. More importantly, denervation experiments once again demonstrated that the commonly presented dichotomy, sympathetic = protein secretion and parasympathetic = fluid secretion, is an overly simplistic concept. Following sympathetic and/or parasympathetic denervation a postjunctional supersensitivity of salivary secretory cells develops that cannot be explained simply by changes in receptor number. Rather, it appears that cross-talk mechanisms involving intracellular calcium are at work. In addition, studies on reflex-stimulated protein secretion (see chapters 6, 8) have revealed that NANC, not β-adrenoceptor, mechanisms may predominate with regard to the release of protein. This is indeed an iconoclastic view for the moment, but it is one that is gradually gaining experimental support.

Supersensitivity and 'degeneration secretion' were further discussed by Ekström (chapter 9), and again it was pointed out that salivary glands may serve as model organs for the study of various neurobiological phenomena. For example, salivary glands have been used to demonstrate Cannon's 'law of denervation', which states that surgical postganglionic denervation of an autonomic effector produces a more profound sensitivity to chemical agonists than does preganglionic denervation (decentralization). A second interesting aspect of this discussion was its focus on the apparent role of normal reflex activity on neurotransmitter metabolism in general, and the activity of choline

acetyltransferase in particular. The activity of this enzyme in postganglionic parasympathetic nerves is dependent on (1) the stimulation of ganglion nicotinic receptors, and (2) the intensity and duration of nerve impulse traffic. However, seemingly unrelated manipulations, such as surgical sympathectomy, can also influence the activity of choline acetyltransferase, but the mechanisms that underlie these 'local adaptions' are unknown.

Neuroreceptors

Finally, with regard to neuroreceptors in salivary glands there have been three areas of significant recent progress: (1) the finer characterization of α- and β-adrenergic and cholinergic receptors, including the recognition of new postreceptor amplification and activation steps; (2) nonadrenergic, noncholinergic receptors, and (3) receptors for other factors, such as cytokines and steroids (see chapter 3). The binding of transmitters to their membrane receptors activates one or the other of two intracellular pathways, so far described: the first involves the production of cAMP, and the second leads to a rise in intracellular calcium. One of the most significant advancements, however, has been the recognition that these two pathways are highly interactive and interdependent, and that there is a great deal of 'cross-talk' as a consequence of the simultaneous activation by multiple transmitters that must occur during reflex stimulation. Furthermore, for calcium signaling there are distinct spatial and temporal signatures depending on which receptor is activated, and there is evidence for the interaction of multiple second messengers (inositol trisphosphate, cADP ribose, and nicotinic acid adenine dinucleotide phosphate) and the cAMP pathway in the mobilization of intracellular calcium (see chapter 5). Future studies will almost certainly be directed towards understanding this complex scheme for generating unidirectional ion fluxes through a polarized epithelium.

Reflex Stimulation of Salivary Secretion

It has been known since the mid-19th century that salivary secretion is dependent on reflex activity [25, 27]. So it is fitting that this book on neural regulation of salivary glands is concluded by reviewing reflex salivary secretion, since pharmacological and direct nerve stimulation studies offer only approximations of 'real life', and actual physiology must be extrapolated from disparate and sometimes conflicting experimental results. Thus, to study salivary gland responses under reflex conditions, and to corroborate the information derived using pharmacological, denervation and nerve-stimulation protocols is both an immense challenge and imperative. Matsuo (chapter 10) reviewed the neuroanatomical and physiological experiments that underlie our current understanding of the gustatory-salivary reflex. Taste information arising from cranial nerves

VII, IX and X is transmitted to first order neurones in the solitary nucleus (brainstem) and from there impulses are projected to numerous sites within the central nervous system, which in turn send efferent fibers to the salivatory nuclei. Significantly, this reflex arc can be modulated by higher centers.

The final contribution by Hector and Linden (chapter 11) emphasized roles of a number of different sensory receptors in reflex salivary secretion. The gustatory-salivary reflex is probably the most thoroughly documented and, as nearly all salivary researchers know, acid stimulation (sour candy) provides a very effective stimulus for the flow of saliva. However, it's not as well appreciated that the flow rate and composition of saliva is dependent on the quality of the taste stimulation. The masticatory-salivary and olfactory-salivary reflexes have only recently been the subjects of controlled experimentation. One of the difficulties in studying these phenomena has been that such investigations must be carried out in conscious laboratory animals and humans. Since the first study by Colin [29] in the horse and mule, most studies have demonstrated that the effects of chewing on salivary flow are greatest on the ipsilateral side. During the past 20 years, research has focussed on attempts to identify the intraoral mechanoreceptors responsible for the masticatory salivary reflex, and several studies by Anderson, Hector and Linden (see chapter 11) support the hypothesis that peridontal receptors play a dominant role. Nevertheless, mucosal mechanoreceptors also have a role, as shown by Linden and his coworkers. In contrast to other salivary reflexes, the existence of an olfactory-salivary reflex in man has been much debated, and until recently the available data were inconclusive. Despite this, it has been widely and popularly believed that the smell of food causes salivation in humans. Using sensitive instrumentation Lee and Linden [30, 31] demonstrated that there is no true olfactory-parotid salivary reflex in man, but that there is one in the submandibular gland. The question of how the additive or synergisic effects of these various stimuli impact salivary flow rate and composition remains to be answered.

Finally, while most reflex salivary secretion is related to eating, Hector and Linden concluded by noting that saliva has many other specialized functions, including lubrication and protection of mucosal and enamel surfaces, thermoregulation and grooming. Future studies, therefore, must examine the reflex pathways that are involved under special circumstances, and such studies must involve biochemical analysis of the saliva and not just flow rate.

Concluding Remarks

'The salivary glands are a challenging group of organs. They are like the pancreas in producing digestive enzymes and like the kidney in withdrawing constituents from the plasma.

... This complexity of function is coupled with the fact that saliva is easier to collect than any other secretory product, a fact which made it convenient to use salivary secretions in the study of conditioned reflexes.

It is no wonder that the study of these organs has a long history and that salivary glands have often been used in research on general physiological questions concerning the structure and function of secreting organs and the composition, mechanism, and control of secretions.'

This quotation from the preface to *Salivary Glands and Their Secretions* [32], which was published in 1964 as part of the International Series of Monographs on Oral Biology, remains as pertinent today as it was 35 years ago. The challenge to present-day and future investigators also remains the same; to continue to define salivary gland function at the cellular and molecular levels, using all of the sophisticated techniques of modern biology, while at the same time maintaining an understanding of salivary gland physiology at the organ level. It is the second part of this challenge that we hope, in some significant way, to have addressed.

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