On The Innervation of Salivary Glands and Treatment of Dry Mouth
- An Experimental and Clinical Study

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Abstract

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Detailed knowledge of the innervation of the parotid gland is essential in basic studies on various neuroglandular phenomena as well as in various types of orofacial surgery. The innervation is more complex than usually depicted in Textbooks. Using the rat as experimental model, it was shown that not only the classical auriculo-temporal nerve but also the facial nerve contributed to the cholinergic innervation of the gland, and that facial nerve-mediated impulses, reflexly elicited, evoked secretion of saliva. In humans, aberrant regenerating parasympathetic nerve fibres of the facial nerve may, therefore, be a potential contributor to Frey´s syndrome, characterized by sweating and redness over the parotid region. Little is known about the sensory innervation of salivary glands. A co-localization of the neuropeptides substance P and calcitonin gene-related peptide signals sensory nerve fibres in the salivary glands. Though the auriculo-temporal nerve trunk carries sensory fibres from the trigeminal ganglion, denervation experiments showed that those sensory substance P- and calcitonin gene-related fibres that innervate the gland use other routes. The comparison of a number of various types of glands in the ferret revealed large differences in the acetylcholine synthesis, the mucin-producing sublingual, zygomatic and molar glands showing a synthesizing capacity, expressed per gland weight, 3-4 times higher than that of the serous parotid gland and the sero-mucous submandibular gland, implying a high cholinergic tone in the mucin-producing glands. The acetylcholine formation was due to the specific action of choline acetyltransferase, and denervation experiments showed this enzyme to be confined to the nerves. Thus, no support for an extra-neuronal synthesis of acetylcholine by the activity of choline acetyltransferase was found. Dry mouth jeopardizes the oral health. A new approach to the treatment of dry mouth was tested in healthy subjects and in patients suffering from salivary gland hypofunction. The cholinesterase inhibitor physostigmine prevents the breakdown of acetylcholine released from cholinergic nerve endings: acetylcholine accumulates and either evokes an effector response or enhances it. Physostigmine was applied locally on the oral mucosa aiming at activating hundreds of underlying, submucosal minor glands (producing lubricating mucin), while at the same time minimising systemic cholinergic effects. A dose-finding showed that it was possible to obtain a long-lasting secretion of saliva in the two study groups concomitant with a long-lasting relief from oral dryness (as revealed by Visual Analogue Scale-scoring) in the group of dry mouth patients at a dose level, where side-effects were absent or in the form of mild gastro-intestinal discomfort. The local drug application, directed towards the minor salivary glands, seems promising and may develop into a therapeutic option in the treatment of dry mouth.

Keywords: Parotid gland, denervation, acetylcholine synthesis, otic ganglion, auriculo-temporal nerve, facial nerve, neuropeptides, salivary gland hypofunction, physostigmine.

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Original publications

This thesis is based on the following research papers, which will be referred to in the text by their Arabic numerals:

1. Khosravani N, Sandberg M, Ekström J. The otic ganglion in rats and its parotid connection: cholinergic pathways, reflex secretion and a secretory role for the facial nerve. *Exp Physiol* 2006; **91**: 239-247. Erratum in: *Exp Physiol* 2006;**91**:481.

2. Khosravani N, Ekström J. Facial nerve section induces transient changes in sensitivity to methacholine and in acetylcholine synthesis in the rat parotid gland. *Arch Oral Biol* 2006;**51**:736-739.

3. Khosravani N, Ekman R, Ekström J. The peptidergic innervation of the rat parotid gland: effects of section of the auriculo-temporal nerve and/or of otic ganglionectomy. *Arch Oral Biol* 2008;**53**:238-242.

4. Khosravani N, Ekman R, Ekström J. Acetylcholine synthesis, muscarinic receptor subtypes, neuropeptides and secretion of ferret salivary glands with special reference to the zygomatic gland. *Arch Oral Biol* 2007;**52**:417-426.

5. Khosravani N, Ekström J, Birkhed D. Intraoral stimulation of salivary secretion with the cholinersterase inhibitor physostigmine as a mouth spray: a pilot study in healthy volunteers. *Arch Oral Biol* 2007;**52**:1097-1101.

6. Khosravani N, Birkhed D, Ekström J. The cholinesterase inhibitor physostigmine for the local treatment of dry mouth: a randomized study. *Eur J Oral Sci* 2009;**117**:209-217.

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Introduction

Saliva is of outmost importance for the oral health. It lubricates oral structures, maintains neutral pH by its buffering capacity, remineralizes the enamel of the teeth, cleanses the oral cavity, exerts antimicrobial effects, stimulates wound healing, solutes tastants, aids in maintenance of taste buds, takes part in the digestion of the food and protects the oesophageal mucosa from regurgitating gastric secretion. Saliva is a mixture of secretion from parotid, submandibular and sublingual glands and hundreds of minor salivary glands located just under the mucosal epithelium and distributed throughout the mouth. Each type of gland contributes to whole saliva with specific constituencies. The daily salivary output is approximately one liter and the flow rate varies considerably over time (Dawes, 1972; Kaplan & Baum, 1993; Tenovou, 1998; van Nieuw Amerongen *et al.,* 2004; Flink *et al.,* 2005).

The low mucin-rich salivary flow rate during the night-time is maintained by the spontaneous activity of the minor glands. Depending on type and intensity of the reflex stimulus different types of glands are thrown into activity to various extent. At rest a weak reflex driven secretion, in response to mucosal dryness and movements of the lips and the tongue, is superimposed on the spontaneous secretion. In response to a meal a number of salivatory reflexes are set up by stimulation of mechanoreceptors, gustatory receptors, olfactory receptors and nociceptors, and, as a result, large volumes of saliva are secreted from the parotid and submandibular glands (Hector & Linden, 1999).

All types of salivary glands and their secretory elements (acini, ducts and myoepithelial cells) seem to be supplied with parasympathetic nerve fibres. The extent of the sympathetic innervation of the secretory elements varies between species and between the glands in the same species. For instance in humans, the sympathetic nerve supply of the secretory cells is scarce in the labial glands but rich in the submandibular glands. While parasympathetic activity evokes a

rich flow of saliva, the response to sympathetic activity is usually small, if any. Though both the stimulation of the parasympathetic nerve and the stimulation of the sympathetic nerve give rise to protein secretion, the protein concentration will be less in parasympathetic saliva as a consequence of the large fluid production in response to the parasympathetic innervation. Under physiological conditions sympathetic secretory activity is thought to occur during a background of on-going parasympathetic secretion, and positive interactions may occur with respect to fluid and protein output. In contrast, the two divisions of the autonomic nervous system have opposite effects on the blood vessels. Sympathetic stimulation decreases the blood flow through the gland, while parasympathetic stimulation increases the blood flow. However, the sympathetic vasoconstrictor fibres are of different origin than the sympathetic secretory fibres. They are not part of the alimentary reflexes but are mobilized during a profound fall in arterial blood pressure such as that upon massive bleeding (Emmelin, 1967, 1987; Garrett, 1988).

Traditionally, acetylcholine is the parasympathetic transmitter and noradrenaline the sympathetic transmitter. However, in the late 1970s it became apparent that a number of transmission mechanisms besides the classical cholinergic and adrenergic ones are at work in the neuro-effector region of various autonomically innervated organs. Atropine-resistant parasympathetic vasodilatation is a well-known phenomenon in salivary glands, first demonstrated by Heidenhain in 1872. Retrospectively, the observation of Heidenhain is the original demonstration of a so called parasympathetic non-adrenergic, non-cholinergic effector response (Burnstock, 1986). The phenomenon was once explained by the so-called “proximity-theory”, i.e. the contact between nerve-ending and postjunctional receptors was too tight to allow the access of atropine (see Bloom & Edwards, 1980). The parasympathetic atropine-resistant vasodilatation is presently attributed to a number of neuropeptides, notable vasoactive intestinal peptide, and to nitric oxide (Edwards, 1998).

Over more than hundred years, the parasympathetic nerve-evoked flow of saliva has been thought to be completely abolished by atropine, a finding that has been interpreted to imply that acetylcholine is the sole transmitter in parasympathetic nerve-evoked secretion. When shifting focus from classical laboratory animals, such as the cat and the dog, to the rat, an atropine-resistant parasympathetic salivary secretion from the submandibular gland was reported, in passing, by Thulin (1976a) when studying the atropine-resistant blood flow of this gland. Though not immediately recognized at that time, the observation made by Thulin became the beginning of a paradigm shift (Ekström *et al.,* 1983; Ekström, 1999a). A number of parasympathetic peptidergic transmission mechanisms were found to release proteins or, in addition, to evoke fluid secretion and further, to interact positively with each other and with acetylcholine. Moreover, parasympathetic non-adrenergic, non-cholinergic transmitters are involved in gland metabolism and gland growth. In the exploration of the field of the regulation of salivary glandular activities by parasympathetic non-adrenergic, non-cholinergic transmission mechanisms not only the rat but also the ferret became useful experimental animals (Ekström *et al.,* 1988b). Like the glands of the rat, those of the ferret responded with secretion of saliva to neuropeptides, administered to the blood stream, and with an atropine-resistant flow of saliva to parasympathetic stimulation. Non-adrenergic, non-cholinergic mechanisms were also found to act in those glands of the cat and the dog, favoured by the early experimenters in physiology, where no overt secretion of fluid is observed after atropinization in response to parasympathetic nerve stimulation; here, the non-adrenergic, non-cholinergic transmission mechanisms evoked exocytosis of secretory granules and protein secretion (Ekström, 1999a).

The acinar cells of salivary glands are supplied with muscarinic receptors, usually of both muscarinic M1 and M3 subtypes (Tobin *et al.,* 2009). The adrenergic receptors are also usually of two types, α_1 and β_1 (Baum & Wellner, 1999). In addition, the acinar cells may be supplied with various receptors for the peptidergic transmitters, involving vasoactive intestinal peptide, pituitary

adenylate cyclase activating peptide, substance P, neurokinin A, calcitonin gene-related peptide and neuropeptide Y (Ekström, 1999a).

Intracellularly, the various transmitters acting on the receptors of the acinar cells mobilize either Ca^{2+}/Inositoltriphosphate or cAMP. For example, stimulation of muscarinic, α_1-adrenergic and substance P-ergic receptors activate the Ca^{2+}/Inositoltriphosphate- pathway, while β_1-adrenergic and vasoactive intestinal peptide-ergic receptors activate the cAMP-pathway. In addition, agonists mobilizing the cAMP-pathway do also generate nitric oxide by the activity of neuronal type of nitric oxide synthase (but of non-nervous origin). cAMP/nitric oxide causes the secretion of protein with little accompanying fluid, while Ca^{2+}/Inositoltriphosphate does also cause secretion of protein but which, in this case, is accompanied with a large amount of fluid (Baum & Wellner, 1999; Ekström *et al.,* 2007).

The fluid secretion is an active, energy-dependent, process that requires an adequate blood flow. Upon increase in intracellular Ca^{2+}, basolateral K^+ - and apical Cl^- -channels open and the two electrolytes move down their concentration gradients to the extracellular compartment. Cl^- in the acinar lumen will drag Na^+ from the interstitium to the lumen, and the luminal increase of NaCl creates an osmotic gradient that causes large volumes of water to move into the lumen. During its passage through the ducts, the electrolyte composition of the primary saliva is modified and further, proteins are added but the volume of saliva is not affected, resulting in a hypotonic secondary saliva (Poulsen, 1998). Proteins of acinar and ductule cells are secreted by two main routes, the regulated exocytotic route, involving storage granules, and the constitutive vesicular route, involving a direct secretion from the Golgi (Proctor, 1998). Acini and ducts are embraced by myoepithelial cells, increasing - by their contraction - the intraluminal pressure that may be of particular importance for the flow of the viscous mucin-rich saliva (Garrett & Emmelin, 1979). The myoepithelial cells are

activated both by muscarinic and α_1-adrenergic stimuli and, in addition by tachykinins (as shown by physalaemin, Thulin, 1976b).

Little is known about the sensory innervation of salivary glands. In general, nerves showing co-localization of substance P and calcitonin gene-related peptide are usually thought to be of sensory origin (Saria *et al.,* 1985). Though calcitonin gene-related peptide-containing nerve fibres may occur close to acinar cells in the rat parotid gland, most of these fibres are found around secretory ducts and blood vessels and these fibres contain substance P in addition. The bulk of the calcitonin gene-related peptide /substance P-containing nerve fibres is of sensoric origin, since they are destroyed by the sensory neurotoxin capsaicin. Most substance P-containing nerve fibres are devoid of calcitonin gene-related peptide and found close to acini (Ekström *et al.,* 1988a, 1989). Calcitonin gene-related peptide and substance P are found in both the trigeminal ganglion and the otic ganglion (Ma *et al.,* 2001; Hardebo *et al*., 1992). Another neuropeptide of the parotid gland, vasoactive intestinal peptide, which cause a small, protein rich, flow of saliva, is only localized in the otic ganglion (and not in the trigeminal ganglion, Hardebo *et al.,* 1992). The auriculo-temporal nerve is not only conveying secreto-motor fibres for the parotid gland but also sensory nerve fibres from the trigeminal ganglion to the temporal region, auricle, external acoustic meatus, tympanic membrane and temporo-mandibular joint (Greene, 1955; Gray, 1988). Thus, there is the possibility that the auriculo-temporal nerve trunk innervates the parotid gland not only with nerve fibres from the otic ganglion but also with nerve fibres of the trigeminal ganglion.

Nerves do not only exert short-term regulation of the salivary glands but they also exert a long-term regulation of gland metabolism and gland size (Ohlin, 1966). Moreover, by their transmitter bombardement of the glandular receptors over time the nerves are of importance for the sensitivity of the glandular receptors (Emmelin, 1965, see below).

Whereas a long-term endocrine influence on salivary gland size and function is a well-recognized phenomenon, exemplified with the development of dry mouth in post-menopausal women (Johnson, 1988; Eliasson *et al.,* 2003), hormones are not usually thought to take part in the immediate regulation of the secretory activity of the glands (Emmelin, 1967; Johnson & Gerwin, 2001; Ferguson, 1999). Recent animal experiments do, however, show the gastro-intestinal peptide hormones cholecystokinin, gastrin and melatonin to secrete proteins from the parotid glands of rats *in vivo* without any accompanying overt fluid secretion (Çevik Aras & Ekström, 2006, 2008), in analogy to the action of some parasympathetic neuropeptides (Ekström, 1999a).

Acetylcholine and its synthesizing enzyme, choline acetyltransferase - transferring the acetylgroup from acetylCoenzyme A to choline, is traditionally associated with nervous structures. The placenta of higher primates is the unambiguous example of a non-nervous synthesis of acetylcholine, since this tissue lacks an innervation (Hebb & Ratković, 1962). In recent years, a non-nervous acetylcholine synthesis has come in focus (Wessler & Kirkpatrick, 2008; Kawashima & Fujii, 2008). A number of epithelial, endothelial, mesenchymal and immune cells are reported to show immunoreactivity for choline acetyltransferase and to contain acetylcholine. Among the many functions attributed to non-neuronal acetylcholine are skin regeneration, wound healing, airway ciliary activity, blood flow control, antibody generation and inhibition of release of pro-inflammatory mediators. In the context of the present *Thesis*, it may also be noted that non-neuronal acetylcholine is implied in modifying fluid and electrolyte movements in mucosal and glandular epithelial cells of the airways and in increasing paracellular permeability in the pancreas by an action on tight junctions. The demonstration of acetylcholine synthesis in a number of tissues raises the question whether in some of these tissues “contaminating” cholinergic nerves are present. In homogenates of denervated skeletal muscles, a capacity to synthesize acetylcholine of 5-8% remains. This persisting synthesis is due to an unspecific synthesis of acetylcholine by extraneuronal carnitine

acetyltransferase (Tuček, 1982). Of anatomical reasons studies on parasympathetically denervated structures are few. The pathways of the postganglionic nerves may in part be unknown or the location of the relay between pre- and postganglionic nerves not easily accessible. In fact, in most cases the ganglia are located within the organ. The urinary bladder of the male rat and the parotid gland belong to those structures where the respective ganglion is situated outside the effector organ.

Though the parotid gland has been used as neurobiological model organs since the days of Claude Bernard, for example to study various denervation phenomena such as supersensitivity, no studies have been made on the otic ganglionic connection with the gland. By studies of Holmberg (1971, 1972), the anatomical routes for the parasympathetic innervation of the dog´s parotid gland were shown to include not only the auriculo-temporal nerve but also nerve fibres reaching the gland via the internal maxillary artery. Twigs of the facial nerve transverse the parotid gland and in the dog´s parotid gland this nerve seems to contribute to the secretory response of the gland (Ekström & Holmberg, 1972). Knowledge of the parotid innervation is not only of interest to the experimenter but must also be of major interest for the oro-facial surgeon.

Frey´s syndrome, also called the auriculo-temporal syndrome or gustatory sweating, named after the Polish neurologist Lucja Frey, who was the first to identify the role of the auriculo-temporal nerve (1923) in a syndrome characterized by sweating, redness, flushing and warming over the parotid region in connection with eating. However, the first case report may be that of Kastremsky in 1740, describing a patient with perspiration when eating salty food, though the report by Duphenix in 1757 is usually considered as the first case of gustatory sweating (Dunbar *et al.,* 2002). Both the patient of Duphenix and of Frey had been injured by a bullet penetrating the parotid gland, followed by chronic inflammation. Frey´s syndrome is most frequently observed after parotid gland surgery, the incidence varying between 3% and 98%, neck

dissection, blunt trauma to the cheek and chronic infection of the parotid area. It appears over a period of several months. Frey´s syndrome is thought to reflect an aberrant regeneration of postganglionic (cholinergic) parasympathetic fibres of the auriculo-temporal nerve innervating sweat glands and skin vessels following loss of the sympathetic postganglionic (cholinergic) innervation. It may also be noted that Frey´s syndrome has been reported in infants as sequale to forceps delivery (Johnson & Birchall, 1995). Medical treatment includes topical facial application of anticholinergics and botulinum toxin. Surgical treatments include division of the branches of the tympanic plexus (Sood *et al.,* 1998; de Bree *et al.,* 2007).

When the amount of a drug required to evoke a certain (submaximal) biological response diminishes the tissue is referred to as being supersensitive. Postjunctional supersensitivity is non-specific and develops over a period of some weeks. Salivary glands have been useful model organs in exploring the phenomenon of supersensitivity, particularly that following interference with the parasympathetic innervation (Emmelin, 1961, 1965). It is more marked after postganglionic denervation than after preganglionic denervation, since after postganglionic denervation the target cells has lost the transmitter bombardement not only of that fraction continuously released from the postganglionic nerve endings but also of that fraction released upon the arrival of the reflexly elicited nerve impulses. Sensitization may be used as a diagnostic test for nerve damages (Lapides *et al.,* 1962). For experimental purposes a presumptive secretory nerve may be caused to degenerate to allow the development of supersensitivity to mark a functional influence of that nerve on the secretory cells.

In the clinic, salivary flow rate is categorized as resting/unstimulated (i.e. spontaneous secretion combined with a low-graded reflex secretion) and stimulated (reflexly elicited by chewing or citric acid). An unstimulated flow rate of whole saliva < 0.1 ml/min and a stimulated flow rate of whole saliva < 0.7 ml/min

are considered to reflect salivary gland hypofunction (Ericsson & Hardwick, 1978). Xerostomia is the subjective feeling of dryness of the oral mucosa. Xerostomia and salivary gland hypofunction may or may not be related (Fox *et al.,* 1987). The term “dry mouth” refers to the subjective feeling of dryness with or without demonstration of hyposalivation. The prevalence of dry mouth is 15-40%, and is more common among women than men and increases with age (Österberg *et al.,* 1984; Nederfors *et al.,* 1997). With a dry mouth, mastication, swallowing and speaking become difficult. Taste acuity weakens and oral mucosal infections, dental caries and halitosis are common. The quality of life is dramatically impaired (Ship *et al.,* 2002; Wärnberg *et al.,* 2005). Among known causes of dry mouth are chronic gland inflammation (e.g. Sjögren´s syndrome), diabetes, depression, head and neck radiotherapy, radioiodide therapy, HIV/AIDS, orofacial trauma, surgery and use of medications (Grišius & Fox, 1988). In about 20% of those complaining of dry mouth, the cause is unknown (Longman *et al.,* 1995; Field *et al.,* 1997).

The options to treat dry mouth are limited and often focused on flavored gums and lozenges, artificial saliva, oral rinses and oral gels. These treatments are of short duration. A number of drugs for systemic treatments have been suggested such as parasympathomimetics, cholinesterase inhibitors, anethole trithione - a bile-stimulating agent, bromhexine and guafensin – both mycolytic agents and further, the immune-enhancing substance alpha interferon, the cytoprotective amifostine, the antimalarial hydroxychloroquine. In many cases, definite clinical effects have not been established and further, the use of some of these drugs is associated with serious adverse effects. The parasympathomimetic drugs pilocarpine (Salagen®) and cevimeline (Evoxac®) are commercially available but a number of side effects are observed. In addition, positive results have been reported with the use of acupuncture but further clinical trials seem necessary (Fox, 2004). A device mounted on an intra-oral removable appliance to stimulate the lingual nerve to evoke secretion is presently under clinical trial (Strietzel *et al.,* 2007).

The amount of acetylcholine continuously released from the cholinergic nerve endings in the salivary glands, in the absence of nerve impulse traffic, is subliminal for evoking secretion of saliva. It may, however, be revealed by the intraductal injection of an acetycholinesterase inhibitor, which prevents the degradation of released acetylcholine, and thus, accumulated acetylcholine in the neuro-effector region reaches suprathreshold levels for evoking secretion as demonstrated in parotid and submandibular glands (Emmelin *et al.,* 1954; Ekström & Emmelin, 1974a,b). Likewise, during on-going nerve stimulation, the cholinergic secretory response will be enhanced by a cholinesterase inhibitor (Månsson & Ekström, 1991).

Cholinesterase inhibitors may be divided into two groups, reversible and irreversible inhibitors. War gases and pesticides are found in the group of irreversible inhibitors. The classical reversible inhibitor is physostigmine, also called eserine. It is an alkaloid, originally extracted from the Calabar bean of a plant growing in West Africa. Physostigmine is a tertiary amine with lipohilic properties that readily passes biological barriers (Taylor, 1996). Therefore, it has been considered as a therapeutic option in the treatment of Alzheimer's disease (Nordberg & Svensson, 1998). Synthetic congeners of physostigmine are the quaternary ammonium derivates neostigmine and pyridostigmine, which exert more long-lasting actions but are poorly absorbed. Neostigmine and pyridostigmine are made use of clinically, neostigmine to reverse the paralytic action of non-depolarising neuromuscular-blocking and to lower the intra-ocular pressure and pyridostigmine to enhance the neuro-muscular cholinergic transmission in myasthenia gravis.

As mentioned above, the pharmacological treatment of dry mouth involves the systemic administration of drugs aiming at activating the parotid and submandibular glands. Since it is the mucin-rich saliva rather than the watery saliva that protects the oral mucosa from dryness (Collins & Dawes, 1987;

Sreebny & Broich, 1988) a therapeutic approach to the treatment of dry mouth would be to selectively activate the mucin-producing minor glands located just below the oral epithelium. By local application of the drug onto the mucosa, followed by the diffusion of the drug through the mucosal barrier, systemic effects would be kept at a minimum. In the development of such a treatment, the ferret submucosal glands have served as model organs. Both in humans and in the ferret, local application of physostigmine on the mucosa causes the underlying submucosal glands to secrete (Hedner *et al.,* 2001; Ekström & Helander, 2002).

The first division of this *Thesis* focuses on the cholinergic and peptidergic innervation of the rat parotid gland with special emphasis on the effect of otic ganglionectomy, reflex secretion, the secretory role of the facial nerve and the sensory innervation. In the second division, comparisons are made between mucin-producing salivary glands and the serous/seromucous producing glands of the ferret with respect to the acetylcholine-synthesizing capacity, secretory capacity, cholinergic receptor populations and gland contents of some neuropeptides with particular focus on the zygomatic gland. The third division is devoted to neuronal and non-neuronal acetylcholine synthesis in salivary glands studied in denervation experiments and by the inhibition of choline acetyltransferase activity. Finally, the fourth and fifth divisions deal with the secretory effect of physostigmine topically applied in healthy subjects and in patients suffering from dry mouth including objective measurements of salivary secretion and subjective measurements of the feeling of mouth dryness by the use of a Visual Analogue Scale.

To summarize, the specific aims of this *Thesis* are to define:

- the otic ganglionic connections with the parotid gland
- the secretory role of the facial nerve for the parotid gland
- the contribution of the auriculo-temporal nerve to the sensory innervation of the parotid gland
- characteristic features of mucin-producing and water-producing salivary glands
- neuronal and non-neuronal acetylcholine synthesis in salivary glands
- the role of physostigmine as a potential drug for the treatment of dry mouth.

Material and Methods

Observations on animals

Adult ferrets and Sprague-Dawley rats (B & K Universal, Sollentuna, Sweden) were used. The animal experiments were approved by the Local Animal Welfare Committee. To perform preliminary surgery the animals were anaesthetized with sodium pentobarbitone (25-30 mg/kg I.P.) combined with ketamine (50 mg/kg I.M.). Postoperatively, they were given bupvenorphine (0.015 mg/kg S.C.) as an analgesic. In acute experiments, the animals were anaesthetized with sodium pentobarbitone (50-55 mg/kg I.P. - further anaesthetic was injected I.V. as required). During anaesthesia the body-temperature, measured by a rectal probe, was maintained at 37.5- 38°C using a thermostatically controlled blanket. Drugs were injected intravenously. Under deep anaesthesia, the aorta was cut and the animals were killed by exsanguination. The glands were rapidly removed, cleaned, and briefly dried on filter paper, weighed and stored at -70 °C until analyzed.

Preliminary surgery

The denervation procedures were performed 7-9 days (1,2,3) or 2-3 weeks (2,4) before the acute experiment. In rats, the auriculo-temporal nerve was either cut where it emerges from the base of the skull or avulsed by swiftly pulling out a hook placed under the nerve trunk (taking care to avoid damage to the chorda tympani nerve) aiming at parasympathetic (postganglionic) denervation. The parasympathetic otic ganglion, located in the oval foramen (Al-Hadithi & Mitchell, 1987), was extirpated. The facial nerve was cut at the level of the stylomastoid foramen and the great auricular nerve was cut where it emerges along the posterior border of the sternocleidomastoid muscle. The various types of surgery were combined in some series of experiments (1,3). In ferrets, the auriculo-temporal nerve was avulsed, where it emerges from the base of the skull. The buccal branch of the mandibular nerve was approached from the mouth and cut as it appears between the pterygoid muscles. Following surgery the wounds were sutured.

Duct preparation

The parotid duct was exposed by a skin incision in the cheek close to the mouth in rats (1,2) and ferrets (4). In ferrets, the submandibular duct was exposed in the neck (Ekström *et al.,* 1988b). The lateral duct of the zygomatic gland, draining 80% of the gland (Ekström & Helander, 2002), was cannulated from the mouth. The ducts were cannulated with polyethylene tubes.

Stimulation of nerves

In rats (1), the facial nerve was exposed at the level of the stylomastoid foramen, ligated and cut. The great auricular nerve was exposed as it emerged at the posterior border of the sternocleidomastoid muscle, ligated as far from the gland as possible and cut. The peripheral end of each nerve was passed trough a ring electrode and stimulated at high frequency (40 Hz) using varying voltage (2-8 Hz) and time periods of stimulation (2-10 min). In ferrets (4), the buccal nerve was dissected as it appears between the ptergoid muscles. The auriculotemporal

nerve was dissected medial of the mandible. The chorda-lingual nerve was dissected as far as possible from the submandibular duct. Each nerve was ligated and cut. The peripheral nerve end was stimulated with a ring electrode at 20 Hz (6-8V) either intermittently in periods or continuously up to 80 min.

Reflex secretion

In rats, the parotid ducts on both sides were cannulated with a fine polyethylene catheter (1). About 2-3 hours after surgery, when the animal was awakening and licking could be evoked, ascorbic acid was applied on the apex of the tongue every 30 s for 10 min, followed by a pause of 10 min and then, a new stimulation period of 10 min. Before the start of the application of ascorbic acid, the α-adrenoceptor blocker phentolamine and the β-adrenoceptor blocker propranolol were administered via the cannulated tail vein. When appropriate, the second stimulation period was, in addition, performed in the presence of the muscarinic receptor blocker metylscopolamine.

Collection of saliva

Saliva secreted was collected on preweighed filter paper or in ice-chilled preweighed tubes, which were then reweighed. The amount of saliva secreted was expressed in µl; the specific density was taken to be 1.0 g/ml. In some experiments the amount secreted was related to unit time per gland weight.

Acetylcholine synthesis

Usually tissues were homogenized in 1 ml of ice-chilled Na-phosphate buffer 0,05 M, PH 6,5, containing NaCl 200 mM, dithiotreitol 1,2 mM and 0,5% Triton, 100-X using an Ultra-Turrax homogenizer for 20 s at high speed. In case of nervous tissue a Potter-Elvehjem all-glass homogenizer was used. The homogenates were frozen and thawed before they were centrifuged (5min, 3000 x g). The supernatant were transferred to Eppendorf®-tubes and stored at -20 °C before being analysed. Briefly, the incubation occurred under optimal conditions, and the medium was that of Banns *et al.* (1979) but dithiotreitol was omitted; the

control incubation contained acetylcholinesterase instead of the cholinesterase inhibitor physostigmine (eserine). The reaction was started by the addition of 10 µl [^{3}H]- acetylcoenzyme A (specific activity 180 mCi mmol $^{-1}$) and after 30 min at 39 °C it was stopped by transferring the incubate to glass tubes containing 2 ml of an ice-cold mM acetylcholine chloride, followed by cooling on ice. Acetylcholine was extracted using tetraphenylboron (Fonnum, 1969). To separate the organic and aqueous phases, the tubes were centrifuged. A sample of the organic layer was transferred into scintillaion vials and measured in scintillation liquid. The salivary gland homogenate does not only form radiolabelled acetylcholine but also radiolabelled acetylcarnitine (Banns *et al.,* 1979; Banns & Ekström, 1981). The true reading for acetylcholine formation was obtained by subtracting the radioactivity left in the control incubation, where acetylcholine was continually destroyed by acetylcholinesterase, from that obtained in test incubations, where acetylcholine was preserved by eserine. The acetylcholine formed (1,2,4) was expressed in nmol per gland per hour or in terms of concentration in nmol per 100 mg wet gland tissue. Unless otherwise stated, the acetylcholine synthesis is expressed per gland in the text.

Effects of the choline acetyltransferase inhibitor bromoacetylcholine

To find out whether the acetylcholine synthesis in normally innervated and in chronically denervated glands as well as in intact nerves was due to choline acetyltransferase activity, the choline acetyltransferase inhibitor bromoacetylcholine (0.02–2000 µM, final concentration) was included in the incubate (Henderson & Sastry, 1978). The inhibitor was added to the homogenate before other components of both test and control medium (1,2,4).

Immunoblotting

Pieces of gland tissue were homogenized on ice. Gel electrophoreses was used to separate proteins. The proteins were then transferred to a membrane (PVDF, Hypobond-P, Amersham Bioscience), where they were probed using antibodies (primary rabbit polyclonal antibodies, anti subtype M1, M2, M3, M4 and M5,

respectively diluted 1:1000 (Santa Cruz Biotechnology). To visualize the proteins alkaline phosphates-conjugated secondary goat anti-rabbit antibody (diluted 1:40 000, Tropix). To exclude unspecific binding, membranes were not exposed to primary antibodies. Semi-quantitative measurements of proteins were made by densitometry (4).

Measurements of neuropeptide gland contents

Antiserum raised against synthetic rat calcitonin gene related peptide conjugated to bovine serum albumine, was used (4). The antiserum does not recognize calcitonin, vasoactive intestinal peptide, somatostatin, gastrin-releasing peptide, enkephalins or tachykinins. Antiserum directed against the C-terminal part of Substance P was used. The antiserum does not cross-react with other known tachykinins. The antiserum recognizes the N-terminal 15-amino acid sequence of vasoactive intestinal peptide and does not cross-react with peptidine histidine isolucine amide or any known regulatory peptide (Ekström *et al.,* 1984)

Observations on humans

The studies took place at the Department of Cariology, Institute of Odontology, Sahlgrenska Academy at Göteborg University. The protocols were reviewed and approved by the Ethics Committee at Göteborg University. The studies were performed with the signed consent of the participants. The subjects were free to discontinue their participation in the studies.

Study 1- Healthy subjects

Seven healthy female volunteers took part; six were aged between 18 and 24, whilst one was 53 years old (5). The mean age was 27 years. The volunteers did not experience mouth dryness. They produced a normal salivary flow in response to paraffin-chewing (>1ml/min for whole saliva).

Study design

The subjects were treated on four separate days with a spray solution of either placebo or physostigmine in various concentrations to the oral mucosa in three puffs, one puff in each cheek and one puff under the lip given by the staff. The mean weight of the solution sprayed was 166 ± 3 mg (n=28). The total amount of physostigmine, as base, administered at three different concentrations was 0.9 mg (0.5%), 1.8 mg (1.0%) and 3.6 mg (2%). After administration of placebo and physostigmine, the subjects were asked to roll their tongues along booth cheek surfaces to distribute the solution more effectively on the mucosa. Physostigmine has a bitter taste. A grapefruit-like taste correction was therefore made for both placebo and physostigmine to minimize the difference in taste between the solutions.

Collection of saliva

Whole saliva secretion was measured every 15 min up to a maximum of 3 hours by placing one pre-weighed dental roll in each lower jaw vestibulum for 5 min (10-15 min, 25-30 min, 40-45 min, etc). The dental rolls were then weighed to calculate the amount of absorbed saliva. The administration of physostigmine/ placebo was preceded by a period of 35 min, during which saliva was collected three times as above; the amount collected during the period “-5 min to 0 min” was set to basal value.

Safety assessment

The subjects were asked to report any discomfort.

Study 2 - Dry mouth patients

The study group comprised of twenty volunteers, eleven females and nine males (6). The age varied between 24 and 70 years, the mean age being 58 years. The subjects had experienced mouth dryness for at least six months prior to screening. All subjects were able to secrete but their resting secretion was less

than 0.1 ml/min, indicating hyposecretion. Intra-oral examination showed a dry mucosa.

Study design

This study comprised of two phases, A and B. In Phase A, the subjective feeling of mouth dryness was assessed by a Visual Analogue Scale. As a result of the Visual Analogue Scale-scoring and safety assessment, a dose of physostigmine was selected to objectively measure the amount of saliva secreted (Phase B). Physostigmine solutions, in a gel formulation, were prepared in a standard volume of 300 ul, which was dosed, by the staff, in two equal portions (150 ul) inside the upper and lower lips, respectively. The subjects were asked to distribute, with the tongue, the solution and to retain it in the mouth. The placebo solution was prepared in an identical volume as physostigmine and administered in the same manner as physostigmine. Also in this study, a grapefruit-like taste correction was made for both placebo and physostigmine.

Phase A

In phase A, physostigmine (0.9 mg, 1.8 mg, 3.6 mg and 7.2 mg) and placebo were compared. Three different doses of physostigmine or placebo and two doses of physostigmine were administered to each subject according to a randomisation schedule. At each treatment visit, subjective assessments were done at 15 min and 0 min before administration of physostigmine/placebo, and again at 15, 30, 60, 90, 120 and 180 min after administration. At each time point, the subjects were first asked to estimate the feeling of dryness in the mouth and then to estimate the feeling of dryness on the inside of the lips by the questions “How do you feel in the mouth right now” (“Hur känner du dig i munnen nu”) and “How do you feel on the inside of the lips right now” (“Hur känner du dig på läpparnas insida nu”), respectively. The subjects answered by using a Visual Analogue Scale. The subject was not allowed to see the immediate preceding scores. The answers were documented, by the subject, with one pencil mark

across a 100 mm horizontal line that was marked at its extreme end with “Extremely dry” (“Extremt torr”) and “Not at all dry” (“Inte alls torr”)

Phase B

Physostigmine 1.8 mg was selected for the quantative measurements of salivation. Pre-weighed dental rolls were placed in the vestibulum of the lower jaw on both sides and left to absorb saliva for 15 min. Before application of the study drug or placebo, rolls were applied at 30 min and at 15 min, respectively. The volume obtained between -15 min and 0 min was set to basal value. After the application of the drug or placebo, rolls were placed again at 15, 45, 75, 105 and 165 minutes.

Safety assessment

Signs of systemic effects were recorded both in Phase A and Phase B and were focused on bradycardia, fall in blood pressure, change in mental alertness (sedation, nervousness), respiratory distress (asthma), gastro-intestinal discomfort (nausea, stomach pain) and excessive sweating. Heart rate and blood pressure were measured automatically.

Evaluation - study 2

The material with respect to the objective measurement of saliva volumes and the subjective estimation of oral dryness was analysed “per protocol”.

Statistics

Statistical significances of differences were calculated either by Student´s *t*-test for paired or unpaired values, one-way analysis of variance (ANOVA) followed by Fisher´s protected least-significant difference, Wilcoxon´s signed-rank test for paired comparisons or Wilcoxon´s rank-sum test for unpaired comparisons using Statview™ SE+. The area under the curve was calculated using KaleidaGraph

version 3.51. Probabilities of less than 5% were considered significant. Values presented are means ±S.E.M.

Substances

Acetylcholinesterase type V-S, atropine sulphate, bromoacetylcholine bromide, hexamehonium, methacholine, methylscopolamine, physostigmine (eserine), propranolol were from Sigma. Physostigmine base (to be applied in humans) was from Lonza. Radiolabelled acetylCoenzyme A was from Amersham. Phentolamine mesylate was from Novartis Pharma.

Results and Discussion

Observations on animals

I. On the innervation of the rat parotid gland

1. Acetylcholine synthesis and Gland weights

(a) The otic ganglion and the auriculo-temporal nerve

Otic ganglionectomy reduced the total acetylcholine synthesizing capacity of the parotid gland by 88% and the gland weight by 33%, when examined 7 days postoperatively (1). In response to *division of the auriculo-temporal nerve* the effect was less conspicuous, the acetylcholine synthesis being reduced by 76% and the gland weight by 20%. *Avulsion of the auriculo-temporal nerve* was more effective than otic ganglionectomy with respect to the acetylcholine synthesis (94%), while the effect on the gland weight was about the same as after ganglionectomy (39%). Acetylcholine synthesis and gland weights of contralateral, unoperated glands were unchanged.

(b) The facial nerve

Seven days after division of the facial nerve, the total acetylcholine synthesizing capacity of the parotid gland was reduced by 15%, whereas the gland weight was unaffected (1). The decrease in the synthesizing capacity upon otic ganglionectomy (88%) was even more reduced in combination with facial nerve division (98%). Also the combined division of the auriculo-temporal nerve and the facial nerve caused a greater fall in the acetylcholine synthesis (89%) than division of just the auriculo-temporal nerve (76%).

(c) The great auricular nerve

Neither the acetylcholine synthesis nor the gland weight was affected by division of the great auricular nerve (1).

(d) The distribution of the acetylcholine synthesizing capacity

In the normally innervated parotid glands the concentration of acetylcholine synthesis was evenly distributed in the three lobes of the gland. Otic ganglionectomy reduced the synthesizing capacity in all three lobes to the same extent. Combined with facial nerve division a further even distributed reduction was observed in all three lobes, suggesting that the facial nerve reaches the whole gland (1).

2. Reflex secretion

Reflex secretion was elicited after elimination of the influence of sympathetic noradrenaline and circulating catecholamines on the secretory cells (1). Citric acid evoked a high flow of saliva from innervated glands, which was not affected by interference with the nervous secretory pathways on the contralateral side, suggesting that the glands were exposed to maximal secretion. After acute *otic ganglionectomy*, the flow rate of the denervated gland was reduced by as much as 99%. Just division of *the auriculo-temporal* reduced the flow rate by 88% and combined with division of *the facial nerve* by 95%. The reduction in flow rate

(99%) in response to *avulsion of the auriculo-temporal nerve* was of the same magnitude as after otic ganglionectomy.

3. Electrical stimulation of the facial nerve

Stimulation of the peripheral facial nerve (40 Hz), divided at the level of the stylomastoid foramen, evoked a flow of saliva (1), in the presence of adrenoceptor blockade, that was about 10% of that in response to stimulation of the auriculo-temporal nerve (Månsson & Ekström, 1991). When preceded by otic ganglionectomy one week in advance, the facial nerve still evoked secretion albeit at a reduced rate, a response that was exaggerated due to supersensitivity. Analytic pharmacology showed the facial nerve-evoked secretion to be unaffected by the ganglion blocker hexamethonium, but (almost) completely abolished by atropine. The facial secretory response was not due to electrical irradiation from the stimulating electrode, since a) firm ligation of the peripheral nerve stump distal to the electrode completely abolished the flow of saliva and further, (b) stimulation of the peripheral end of the facial nerve, divided 7 days in advance, produced no flow of saliva from the gland.

4. Stimulation of the great auricular nerve

No support was gained for a secretory role for the great auricular nerve, since stimulation (40 Hz) of the nerve in innervated glands or sensitized glands, by otic ganglionectomy one week in advance, caused no flow of saliva (1).

5. Further evidence for a facial nerve influence on the secretory cells

Nerves exert long-term influences on the gland cells as revealed by the development of a postjunctional supersensitivity over a period of 2-3 weeks following parasympathetic or sympathetic denervation (Emmelin, 1965; Ekström, 1980). However, no supersensitivity to the intravenous injection of methacholine was demonstrated 2-3 weeks following division of the facial nerve. Furthermore, no difference in the acetylcholine-synthesizing capacity existed between glands on operated and non-operated sides. In contrast, one week postoperatively, the

parotid gland showed a slight sensitization to methacholine (as judged by the increase in volume response (25%) to a suprathreshold dose of this drug, a finding to be combined with the 15%-decrease in acetylcholine-synthesizing capacity of the gland on the operated side (1,2). For comparison, the secretory response to methacholine following otic ganglionectomy increased more than tenfold (I). The rapid restoration of the acetylcholine synthesizing capacity and the decrease in sensitivity may reflect compensatory impulse traffic in the remaining nerves and (or) collateral sprouting from these nerves (Ekström, 1999b).

6. Sensory contribution to the peptidergic gland innervation

In the rat, vasoactive intestinal peptide is found in the otic ganglion but not in the trigeminal ganglion, whereas both calcitonin-gene related peptide and substance P are found in both the trigeminal ganglion and the otic ganglion (Ma *et al.,* 2001; Hardebo *et al.,* 1992). Almost all of the substance P- and vasoactive intestinal peptide-containing nerve fibres of the rat parotid gland reached the gland via the auriculo-temporal nerve trunk, while only a minor proportion of the calcitonin gene related peptide-containing nerve fibres did so (3): seven days after division of the auriculo-temporal nerve, the gland contents of vasoactive intestinal peptide, substance P and calcitonin gene related peptide were reduced by 88%, 93% and 37%, respectively. Virtually all of the substance P- and vasoactive intestinal peptide-containing nerve fibres originated from the otic ganglion, while, once again, only a minor proportion of the calictonin gene related peptide-containing nerve fibres was of otic origin: after otic ganglionectomy, the gland contents of substance P and vasoactive intestinal peptide were reduced by 98% and the gland content of calcitonin gene related peptide by 32%. No support for the idea that the auriculo-temporal nerve trunk supplied the gland with substance P- and/or calcitonin gene-related peptide-containing nerve fibres originating from another source than the otic ganglion was found: the division of the auriculo-temporal nerve combined with otic ganglionectomy did not further lower the gland content of substance P (97%) and calcitonin gene related peptide (23%).

II. On the innervation of ferret salivary glands and their secretion: comparisons between glands, and with special focus on the submucosal zygomatic gland and the acetylcholine synthesis.

1. Acetylcholine synthesis and Gland weights.

(a) Intact glands

Of the five types of glands studied the zygomatic and molar glands were heavier than the sublingual glands but lighter than the parotid and submandibular glands. The acetylcholine synthesis expressed per gland weight was three to four times higher in the mucin-producing sublingual, zygomatic and molar glands than in the serous parotid and seromucous submandibular glands (4).

(b) Effects of division of the auriculo-temporal nerve and the buccal nerve

Seven days postoperatively (4), the total amount of the acetylcholine-synthesizing capacity was reduced by 97% in the parotid gland (auriculo-temporal nerve), 95% in the zygomatic gland (buccal nerve) and 85% in the molar gland (buccal nerve). The parotid gland lost 15% in weight, while the weight loss was more pronounced in the zygomatic (46%) and molar glands (23%).

2. Expression of muscarinic subtypes

All five subtypes of muscarinic receptors were expressed in the five types of glands (4). The semiquantitative comparison within each gland showed the M3-receptor subtype to dominate. The concentration of M5-receptor subtype was less in the mucinproducing glands than in the seous/sero-mucous glands.

3. Secretory responses to nerve stimulation

A resting viscous flow from the zygomatic gland was observed, while no resting secretion occurred from the parotid and submandibular glands (4). Expressed per gland weight, the parotid and submandibular glands secreted larger volumes

to stimulation of the auriculo-temporal nerve and chorda-lingual nerve, respectively, than the zygomatic gland to stimulation of the buccal nerve. A certain fraction of the secretory response of the zygomatic gland was due to mobilization of non-adrenergic, non-cholinergic transmitters, since in the presence of atropine (and α- and β-adrenoceptor blockers) 25% of the secretion persisted, to be compared with previous findings of the submandibular gland of 30% and the parotid gland of 5% (Ekström *et al.,* 1988b).

4. Neuropeptides

The three peptides vasoactive intestinal peptide, substance P and calcitonin gene related peptide were all present in the parotid, submandibular, zygomatic and molar glands, while the sublingual gland lacked detectable amounts of calcitonin gene-related peptide (4). Vasoactive intestinal peptide is associated with salivary protein secretion. However, no uniform pattern with respect to mucin producing and non-mucin producing glands was found; *e.g.* the vasoactive intestinal peptide concentration was low both in the parotid gland and in the zygomatic gland.

III. On the origin of acetylcholine and its specific synthesis

The acetylcholine synthesizing capacity almost completely disappeared in the rat parotid gland in response to otic ganglionectomy (combined with division of the facial nerve) or to avulsion of the auriculo-temporal nerve (1), in the ferret parotid gland to avulsion of the auriculo-temporal nerve and in the ferret zygomatic gland to division of the buccal nerve (4).

In salivary gland tissue extracts, the radioactive labeled acetylgroup is not only transferred to choline by the activity of choline acetyltransferase but also to carnitine by the activity of carnitine acetyltransferase (Banns & Ekström, 1981). Though the radioassay method of Fonnum (1975) is thought to avoid contamination of acetylcarnitine, comparisons between incubates where

acetylcholine is preserved (by physostigmine) and continuously destroyed (by cholinesterase) were always made (Banns *et al.,* 1979). In incubates of innervated gland tissue extracts (of rat parotid gland and ferret parotid, submandibular, sublingual, zygomatic and molar glands) supplied with bromoacetylcholine, a specific inhibitor of choline acetyltransferase (Hendersson & Sastry, 1978), the enzyme activity was virtually abolished (95-99.5%). Likewise, the acetylcholine synthesis of the auriculo-temporal nerve (not containing carnitine acetyltransferase) showed a similar reduction in response to bromoacetylcholine (1, 4). In homogenates of denervated skeletal muscles a capacity to synthesize acetycholine of 5-8% is known to persist. In this case, bromoacetylcholine inhibited only 5% of the acetylcholine synthesis in the denervated muscle (Tuček, 1982). In contrast (1), and at the same concentration of bromoacetylcholine, the residual acetylcholine synthesizing capacity (2%) of the denervated rat parotid gland (otic ganglionectomy combined with section of the facial nerve) was reduced by 85% (20 nM), and at a higher concentration (200 nM) by 97%.

Observations on humans

IV. Healthy subjects: topical administration of physostigmine

1. Secretion of saliva

The volume response to all three doses of physostigmine (0.9 mg, 1.8 mg and 3.6 mg), applied as spray, was increased, peak values being 1 ½ -2½ above basal level, while the response to placebo decreased over time (5). One hour and forty-five minutes after the administration the curves of placebo and of the various physostigmine doses met. Based on AUC-values (0-105 min), the response to 0.9 mg, 1.8 mg and 3.6 mg was elevated by 66%, 91% and 62% over placebo. Interestingly, that subject, which showed the largest basal secretion (three times that of the subject showing the second largest increase) did not show any increase to physostigmine. There is the possibility that an

existing film of saliva, covering the mucosa, may attenuate the uptake of physostigmine. Thus, the result of a physostigmine spray may be more effective in patients suffering from dry mouth.

2. Safety results

At the highest dose (7.2 mg) of physostigmine, two subjects complained of nausea and one of them discontinued the test. A third subject complained of a transient slight chest discomfort at 0.9 mg and 7.2 mg, but she experienced no respiratory distress. Neither blood pressure nor heart rate was affected over time by the three physostigmine doses. There were no complains in response to placebo.

V. Salivary gland hypofunction: topical administration of physostigmine

In the first part of this study, the effect of various doses of physostigmine on the subjective feeling of dryness in the mouth or on the lips was estimated and reported or objectively noted signs of systemic effects were recorded (6). Based on safety results and relief in the feeling of dryness a dose of physostigmine was selected, to objectively estimate the effect on the volume of saliva in the second part of the study.

1. Subjective assessment of dryness

The baseline mean VAS scores for mouth dryness (“How do you feel in your mouth right now”) were not significantly different from each other in response to placebo and physostigmine at the dose levels of 0.9 mg, 1.8 mg, 3.6 mg and 7.2 mg - varying between 75 and 68. The corresponding baseline VAS scores for lip dryness (“How do you feel on the inside of your lips right now”) were in the same range as those for mouth dryness - varying between 72 and 59. In response to physostigmine, the scores for both mouth and lips changed in tandem. Physostigmine at the dose levels of 0.9 mg, 1.8 mg and 3.6 mg caused significant and long-lasting relief in the feeling of dryness; placebo induced a

transient reduction in scores of about 5. In response to physostigmine, the reduction in scores was 10-15 at 0.9 mg (up to 120 min), about 25 at 1.8 mg (up to 120 min) and 15-20 at 3.6 mg (up to 180 min). The decrease in VAS scores was reflected in significant reductions in the area under the curve. When calculated over 180 min, as compared to placebo, the AUC-value at physostigmine 1.8 mg and 3.6 mg, respectively, was 6 and 5 times that of placebo. At the dose level of 7.2 mg of physostigmine, the subjects reported only a little relief from their dryness. Probably, the adverse effects that appeared at the higher doses (see below), distracted the subjects, thereby shifting their focus from oral sensations.

2. Salivary secretion

Following, placebo a small increase above baseline was initially (30 min) observed. In response to physostigmine 1.8 mg, elevated levels of secretion were found between 30 min and 180 min. The AUC values in response to physostigmine over 180 min was about five times that in response to placebo.

3. Safety results

In the first part of the study, no signs of systemic effects were recorded at the dose levels 0.9 mg and 1.8 mg. At 3.6 mg and 7.2 mg of physostigmine gastro-intestinal disorders, mainly of mild character, were frequent. In the second part of the study, mild gastro-intestinal disorders were also reported to physostigmine 1.8 mg. Most likely, the subjects were “primed” by the preceding tests, paying extra attention to the occurrence of anything unusual. Placebo was in both parts of the study without systemic effects. Neither blood pressure nor heart rate were affected by the various doses of physostigmine.

General Discussion

Textbooks usually depict the parasympathetic secretory nerve fibres of the parotid gland, arising from the inferior salivatory nucleus, to travel via the tympanic branch of the glossopharyngeal nerve (Jacobson´s nerve), the tympanic plexus, the small superficial petrosal nerve and, after relaying in the otic ganglion, the auriculo-temporal nerve to reach the gland cells (Bradley, 1995; Ferguson, 1999), a concept originating from early observations in the dog (see Holmberg, 1972).

The nerve routes of the parotid gland seem, however, to be more complex than outlined above. The human parotid gland is supplied not only by fibres of the glossopharyngeal nerve but also by fibres of the chorda tympani branch of the facial nerve, since reflex parotid secretion is affected not only by the division of the glossopharyngeal nerve intracranially but also by the division of the chorda tympani in the tympanic membrane (Reichert & Poth, 1933; Diamant & Wiberg, 1965). In the rat, there is no direct connection with the glossopharyngeal nerve and the otic ganglion; the tympanic branch of the glossopharyngeal nerve does not exist (Contreras *et al.,* 1980; Al-Hadithi & Mitchell, 1987). The only visible connection between the otic ganglion and the cranial nerves seems to be a branch of the facial nerve. A further connection between the facial nerve and the glossopharyngeal nerve has also been described in this species, which tentatively may offer a passage for salivatory preganglionic axons from the IXth nerve to the VIIth nerve (Al-Hadithi & Mitchell, 1987). In the dog (Emmelin *et al.,* 1968) as well as in the cat (Ekström & Emmelin, 1974a,b), reflexly elicited secretion is still obtained, albeit markedly reduced, from the parotid gland after division of the auriculo-temporal nerve - not depending on the sympathetic innervation and blockable by atropine - showing that cholinergic nerve fibres outside the auriculo-temporal nerve innervate the gland. Extensive studies by Holmberg (1971, 1972) showed cholinergic nerve fibres, detached at an early stage from the auriculo-temporal nerve, to reach the gland via the internal

maxillary artery, a finding later also observed in the cat (Ekström & Emmelin, 1974b). The present *Thesis*, using the rat as experimental animal, focused on the otic ganglion and yet another peripheral secretory pathway: the facial nerve.

The history of salivary glands and nerves began with the discovery of Carl Ludwig in 1850, when he described that electrical stimulation of the chorda-lingual nerve caused a copious flow of submandibular saliva in the dog (Garrett, 1999). In submandibular glands, relays between pre- and postganglionic nerve fibres occur intraglandularly. Apart from a recent study concerned with the influence of sympathectomy on the neuropeptide content of the parotid gland of the rat (Ekström & Ekman, 2005), the effect of otic ganglionectomy on the parotid gland innervation has not previously been reported. The otic ganglion in the rat is a discoid structure of about 1 mm in diameter located in the foramen ovale. It lies medial to the pterygopalantine branch of the internal carotid artery (corresponding to the pterygopalatine portion of the internal maxillary branch of the external carotid artery in man) and is separated from the trigeminal ganglion by the sphenoid bone (Al-Hadithi & Mitchell, 1987). Otic ganglionectomy affected the parotid gland more profoundly than division of the auriculo-temporal nerve with respect to loss in gland weight, synthesis of acetylcholine and reflex secretion (1); the latter was, in fact, almost abolished (in the presence of α- and β-adrenoceptor blockade). As judged from the acetylcholine synthesis, the proportion of cholinergic nerve fibres to the parotid gland outside the auriculo-temporal nerve is about the same in the rat (24%) and the dog (29%, Ekström & Holmberg, 1972) but lower in the cat (11%, Ekström & Emmelin, 1974b). In the present investigation, no attempt was made to trace nerve fibres on the artery to the gland. Since otic ganglionectomy combined with division of the facial nerve reduced the acetylcholine synthesis of the parotid gland even more than division of the auriculo-temporal nerve combined with division of the facial nerve, a minute contribution seems to occur by additional routes to the parotid gland in the rat. The great auricular nerve was not such a route as to the cholinergic

innervation (1). Neither is the sympathetic innervation a route for cholinergic nerves to the gland (Ekström, 1972).

The facial nerve (1,2) contributed to the acetylcholine synthesizing capacity of the three lobes of the gland, evoked secretion upon electrical stimulation (about 10% of that to stimulation of the auriculo-temporal nerve), conveyed reflexly elicited impulses for secretion, via the otic ganglion, and exerted trophic effects on the secretory cells (as shown by the development of denervation supersensitivity). The secretion was of cholinergic muscarinic nature and the secretory nerve fibres originated mainly from the otic ganglion. However, the present findings did also suggest that some parasympathetic cholinergic nerve fibres relayed outside the otic ganglion, since after otic ganglionectomy, the facial nerve still contributed to a certain fraction of the acetylcholine synthesis of the gland and the stimulation of the facial nerve still evoked a small secretory response (blockable by atropine). It cannot be excluded that some facial somato-motor fibres contributed to the residual acetylcholine synthesis. However, the even distribution of the facial nerve in the gland, as judged by the contribution of the facial nerve to the acetylcholine synthesis of the three lobes of the gland seems to favour the idea that somato-motor fibres, escaping dissection, contribute but little to the acetylcholine synthesis of the gland. There is not necessarily a direct relationship between the acetylcholine synthesizing capacity of the gland and its cholinergic innervation stimulating to fluid secretion. Cholinergic nerve fibres supply vessels, myoepithelial cells and ducts in addition to the secretory acinar cells (Garrett, 1999). The location of the extra-otic relay between pre- and postganglionic nerves was not located in the facial nerve distal to the level of the stylomastoid foramen. Moreover, the parotid gland is known to lack ganglia (Garrett, 1999). Where fibres to innervate the parotid gland join the facial nerve, if they do not originate from the nerve itself, is presently unknown. A secretory role for the facial nerve in the parotid gland may be a general phenomenon among the various species. In the dog, the facial nerve contributes to a small proportion of the acetylcholine synthesizing capacity of the gland and

to a minute cholinergic secretory response (Ekström & Holmberg, 1972). A brief report states that in cats the facial nerve trunk (at the level of the stylomastoid foramen) carries impulses evoking secretion from the parotid gland evoked by stimulation of the inferior salivatory nucleus (Porto *et al.,* 1981). In humans suffering from unilateral facial nerve paresis, parotid secretion on the parethetic side upon reflex secretion is reduced (Vollrath *et al.,* 1980).

The denervation technique applied was evidently of importance not only with respect to the inclusion of various pathways but also whether the approach of avulsion or cutting the respective nerve was chosen. Avulsion of a nerve is likely to cause more damage than just cutting the nerve (1,4). Avulsion of the auriculo-temporal nerve was more effective than otic ganglionectomy with respect to the decrease in the acetylcholine synthesizing capacity in the gland (99% versus 88%) and as effective as otic ganglionectomy with respect to abolishing reflex secretion (99% versus 98%). Avulsion of the auriculo-temporal nerve was also used to denervate the rabbit parotid gland (Nordenfelt, 1964) and presently, the ferret parotid gland, and also here, the acetylcholine synthesis was almost abolished (97% and 96%, respectively).

Regenerating cholinergic nerve fibres of an intentionally or unintentionally damaged auriculo-temporal nerve are no doubt the main cause to the re-innervation of the sympathetically denervated sweat glands and skin vessels (normally under the influence of a cholinergic transmission mechanism and possible, peptidergic transmission mechanisms such as that using vasoactive intestinal peptide, Drummond, 2002) and, consequently, the development of gustatory sweating and flushing (Glaister *et al.,* 1958; Dunbar *et al.,* 2002; Tugnolgi *et al.,* 2002). Regenerating cholinergic somato-motor nerve fibres only exceptionally establish functional contact with salivary gland cells (Emmelin *et al.,* 1960). With the present knowledge of a facial nerve that carries secretory parasympathetic nerve fibres to the parotid gland (1,2), a damaged facial nerve

may be regarded as a potential contributor to the development of Frey´s syndrome.

Loss of most of the cholinergic innervation of the parotid gland, by division of the auriculo-temporal, is followed by a gradual restoration of the acetylcholine synthesizing capacity and a gradual fall in the level of sensitivity of the secretory cells of the parotid gland over several months and concomitant with increased reflex responses, as shown in the cat (Ekström & Emmelin, 1974a). In the present *Thesis*, the decrease in the acetylcholine synthesizing capacity and the slight increase in the sensitivity to methacholine in the parotid gland, following the division of the facial nerve, were very short-lasting events and might easily go unnoticed (2). Similar transient changes in the level of sensitivity and acetylcholine synthesizing capacity occur in the partially denervated urinary bladder and further, the persisting cholinergic axons of the sprout (Alm & Ekström, 1981). Collateral sprouting from the fibres of the auriculo-temporal nerve and increased traffic of impulses in that nerve are likely causes to the rapid recovery after damage to the facial nerve.

The sensory periductal and perivascular nerve fibres of salivary glands, containing both substance P and calcitonin gene related peptide, disappear in response to capsaicin treatment showing that these fibres are of sensory origin (Ekström *et al.,* 1988a, 1989). The gland contents of substance P and calcitonin gene-related peptide are reduced by the capsaicin treatment, by 11% and 36%, respectively. For comparison, the same capsaicin treatment reduces the contents of substance P and calcitonin gene related peptide by 90% in the urinary bladder (Ekström & Ekman, 2005). A likely origin of these fibres is the trigeminal ganglion. In this ganglion, the two peptides are co-localized but, in addition, calcitonin gene related peptide occurs in neurons of the Aδ-fiber type, devoid of substance P (Ma *et al.,* 2001), and not likely to be sensitive to capsaicin. Thus, there is the possibility that also capsaicin-nonsensitive sensory nerves exist in salivary glands. Moreover, capsaicin-nonsensitive effector responses can be

elicited by the electrical stimulation of the trigeminal ganglion as shown by menigeal vasodilatation (Peitl *et al.,* 1999). Tentatively, the sensory nerves of salivary glands may exert protective functions by mobilizing defensive agents of the ductal cells, e.g. β-defensins (Darnell *et al.,* 2006), or they may stimulate the myopithelial cells to increase the intra-ductal pressure to overcome distension of the duct-system or to eject noxious substances. Interestingly, the substance P-relative non-mammalian tachykinin physalaemin contracts the myoepithelial cells (Thulin, 1976b). The perivascular localization may suggest that they may be associated with the inflammatory response. Both substance P and calcitonin gene-related peptide are involved in protein extravasation and oedema formation in the salivary glands (Asztély *et al.,* 1998). In recent years, neurogenic inflammation has come into focus, e.g. in connection with asthma (Barnes *et al.,* 1990). The afferent sensory nerves are thought to exert efferent functions through local axonal reflexes and may maintain and prolong the inflammatory response. Hypothetically, sensory nerves may be involved in chronic salivary gland inflammation. Moreover, they may be involved in the postsympathectomy pain in the parotid gland upon eating (Schon, 1985).

Evidently, the trigeminal ganglion did not seem to contribute with substance P and calcitonin gene-related peptide via the auriculo-temporal nerve trunk (3). A previous report (Sharkey & Templeton, 1984), showed that a portion of True Blue retrogradely labeled nerve cell bodies of the trigeminal ganglion, following injection of the dye in the rat parotid gland, contained immunoreactivity for substance P. This made the authors to conclude that substance P-containing neurons of the trigeminal ganglion innervate the parotid gland via the auriculo-temporal nerve trunk; however, compelling evidence for the auriculo-temporal pathway was not presented. Unfortunately, the dye was not injected in the parotid gland subjected to division of the auriculo-temporal nerve in advance to verify the auriculo-temporal nerve as the route for those neurons of the trigeminal ganglion that contained substance P. The great auricular nerve, like the facial nerve, penetrates the parotid gland (Zohar *et al.,* 2002), and both nerves are

likely pathways for both substance P and calcitonin gene realated peptide as shown in denervation experiments (Ekström *et al.,* 1988a). The great auricular nerve does also supply the parotid fascia (Zohar *et al.,* 2002). Stretching of the fascial layer encapsulating the glands, as for instance in response to gland swelling, is thought to give rise to pain (Shapiro 1973; Leipzig & Obert, 1979). A large number of calcitonin gene-related peptide-containing nerve fibres is presently not accounted for. They do not reach the gland via the sympathetic innervation (Ekström *et al.,* 1988a).

The zygomatic gland, located in a space in the anterio-lateral part of the soft palate in the infratemporal fossa less than one mm beneath the mucosa, is a well defined gland with two ducts opening on the mucosal ridge posterio-medial to the parotid gland. The lateral duct drains 80% of the gland and is easily accessible for cannulation (Shackleford & Wilborn, 1968; Ekström & Helander, 2002). Likewise, the molar gland, located opposite the lower molar teeth, is well defined but less in size and with several excretory ducts, emptying in relation to the same teeth. The zygomatic gland of the ferret (4) was found to be yet another gland to be added to the list of salivary glands that respond with a flow of saliva upon stimulation of the parasympathetic nerve in the presence of atropine (and adrenoceptor blockers). The concentrations of the neuropeptides in the five types of glands did not reveal any conspicuous pattern with respect to mucous glands *versus* serous/seromucous glands. Though, the muscarinic receptor subtype M1 usually is thought to be associated with mucous cells (Watson & Culp, 1994), no preponderance for the expression of the M1 receptor type in the mucous glands was found in the ferret glands. In fact, immunochemistry of the major human salivary glands shows the M1 receptor type to be confined to the serous cells and not to the mucous cells (Ekström, Helander, Godoy, Grunditz and Riva, to be published).

Apart from an early observation of the sublingual gland of the rat, the acetylcholine synthesis of mucous glands does not seem to have been studied

previously. In the rat, the acetylcholine synthesis, expressed in terms of concentration, was comparable to that of the parotid gland but higher than that of the submandibular gland (Ekström, 1972). Comparisons with respect to the acetylcholine synthesis, expressed as concentration, revealed a striking difference in the ferret between, on one hand, the mucous sublingual, zygomatic and molar glands and, on the other hand, the serous parotid gland and the seromucous submandibular gland, since the acetylcholine synthesis (per unit weight) was 3-4 times greater in the mucin-producing glands than in the parotid and submandibular glands (4). The zygomatic gland of the anaesthetized ferret, as also shown in the cat (Al-Gailani *et al.,* 1981), was found to secrete continuously at a low rate. At first sight this continuous secretion may be thought to be associated with the high acetylcholine synthesis. However, the resting secretion persists in the presence of atropine (Ekström & Helander, 2002). No support for the idea of an extra-neuronal glandular synthesis of acetylcholine of any significance was found in the glands, and this was regardless of whether the acetylcholine synthesis was high or low. Division of the buccal nerve, a branch of the mandibular nerve and thought to originate from the otic ganglion (Kuchiiwa & Kuchiiwa, 1996), reduced the acetylcholine synthesis (expressed per gland) by 95%, while avulsion of the auriculo-temporal nerve reduced the synthesis by 96% in the parotid gland. In analogy with the rat parotid gland (see above) nerves may escape the denervation procedure. With respect to the molar gland, this gland seems to have a dual innervation, the buccal nerve and, in addition, the mylohyoid nerve (Kuchiiwa & Kuchiiwa, 1996); in the molar gland the acetylcholine synthesis decreased by 85% in response to the division of the buccal nerve. The high acetylcholine synthesis, in terms of concentration, in the sublingual, zygomatic and molar glands is thought to reflect a high reflexly elicited parasympathetic tone over time. The fact that the parasympathetic postganglionic denervation of the zygomatic gland caused a much more pronounced fall in weight than that of the parotid gland supports such an assumption. Presently, nothing is known about the acetylcholine synthesizing capacity of human salivary glands.

In humans, several hundreds of minor glands are found in the mouth and named after their location, i.e., lingual, labial, buccal, palatinal, pharyngeal and uvulal glands. Only the gingiva and the midline and anterior part of the hard palate lack the minor glands. The glands are located in the submucosa surrounded by connective tissue or muscle fibres. In contrast to the major glands, the minor glands are not encased in a fibrous capsule. Apart from the posterior deep lingual gland (von Ebner´s), the minor human salivary glands are mucous in character but do also, to varying extent, contain seromucous cells usually capping the end of mucous acini and tubules (Hand *et al.,* 1999; Riva *et al.,* 1999). Secretory cells, classified as seromucous, show a cell morphology intermediate between true serous cells and mucous cells; the seromucous cells have granules with low electron density. Interestingly, the seromucous cells have been suggested to reflect maturative stages of mucous cells (Riva *et al.,* 1999). The minor glands are located just under the mucosal epithelium. The ductal system is less well-developed as compared to those of the major gland. The excretory duct of individual glands open directly onto the mucosa. The saliva produced, is likely to be responsible for the local environment of the mucosal surface. The buccal and lingual glands display the highest salivary flow rate, the labial gland an intermediate and the palatal gland the lowest flow rate (calculated per area of mucosal surface per min) (Riva *et al.,* 1999). As judged by the labial glands, the salivary secretion from minor glands in humans can be evoked reflexly by strong gustatory stimulants and mechanical stimulations such as chewing and speaking (Speirs, 1984; Gandara *et al.,* 1985; Boros *et al.,* 1999). The acinar cells of the labial glands are not only innervated by cholinergic nerves (Rossini *et al.,* 1979) but also by vasoactive intestinal peptide-containing nerves (Fehér *et al.*, 1999); the acinar cells lack adrenergic innervation (Rossini *et al.,* 1979). Local application of physostigmine evokes secretion from labial glands (Hedner *et al.,* 2001) and further, isolated pieces of labial glands respond to muscarinic agonists but poorly to adrenergic receptor agonists (Turner *et al.,*

1999). The contribution of the minor glands to the whole amount of saliva produced is calculated to 7-8% (Dawes & Wood, 1973)

Saliva forms a thin film that coats the oral structures. Marked regional differences in the thickness of the fluid layer on the oral mucosa exist, the highest figure (70 µm) is from the posterior dorsum of the tongue and the lowest figure (10 µm) is from the hard palate (DiSabato-Mordaski & Kleinberg, 1996; Wolff & Kleinberg, 1998). The volume of saliva in the oral cavity is not only depending on the secretion of saliva but also of evaporation, absorption through the oral mucosa and swallowing. Evaporative loss of fluid is mainly due to mouth breathing and speaking. The hard palate with its thin fluid layer will be particularly exposed to the effect of evaporation, since it is directly exposed to the flow of inspired air (Thelin *et al.,* 2008). The oral mucosa is permeable for water. Movement of water across the oral mucosa occurs due to an osmotic gradient (resting saliva being hypotonic) and to an active ion transport of sodium. Under normal conditions, the volume of saliva entering the mouth exceeds the loss of volume by evaporation and absorption. Excess of saliva initiates a swallowing reflex. The volume that is swallowed amounts to about 0.3 ml per swallow (Lagerlöf & Dawes, 1984).

Though the figure for a mean flow rate of resting whole saliva is about 0.3 ml per min in healthy subjects (Dawes, 1987), large inter-individual differences exist without complains of xerostomia: from 0.008 to 1.85 ml per min in one study (Becks & Wainwright, 1943) and from 0.25 to 5.58 ml per min in another study (Heintze *et al.,* 1983), and also illustrated in the present *Thesis.* Xerostomia is not a reliable predictor of salivary gland hypofunction. In large study groups, where all subjects complained of xerostomia, only 54-58% of them showed low flow rates (Field *et al.,* 1997; Longman *et al.,* 1995). Despite wide differences between healthy subjects in resting secretion, a parasympatholytic-induced decrease by about 50% of an individual's resting secretion gives rise to the feeling of oral dryness (Dawes, 1987; Wolff & Kleinberg, 1999). Under this circumstance, the thickness of the saliva film of the anterior dorsum of the tongue

and the hard palate is less than 10 µm, and from these locations the subjects experience the most pronounced symptoms of xerostomia (Wolff & Kleinberg, 1999). Interestingly, a decrease in the labial fluid output of 21% is correlated to the feeling of dryness (Eliasson *et al.,* 1996).

Physostigmine was locally applied onto the non-keratinized mucosa (5,6), which is permeable for drugs, and in a region where the density of minor glands is high: in a spray formulation onto the inside of the lower lip and the buccal mucosa in healthy subjects or in a gel formulation onto the inside of the lower and upper lip and then, distributed in the mouth with the tongue. The amount of saliva collected was in both types of experiments elevated over a relatively long period of time (in response to the same dose level), 90-120 min. In the group of subjects showing salivary gland hypofunction, the secretion was about twice that of baseline during the first 60 min. In these dry mouth-patients, the mean AUC-value for the volume response over 180 min to physostigmine was five times higher than that to placebo.

It cannot be excluded that the “grapefruit-like” taste of the drug gave rise to a certain reflex secretion initially, as reflected by the placebo-response. In addition, a transient osmotic attraction of fluid from the interstitium of the mucosa to the mucosal surface may have occurred, since in animal experiments, using the ferret as model, the osmolality of both the placebo and the physostigmine solutions is higher than in the body fluids (Ekström & Helander, 2002).

The local application of physostigmine in humans aimed at selectively activating the underlying submucosal minor glands to secretion of saliva, while at the same time cholinergic systemic effects would be avoided or minimized (5,6). Support for the assumption that physostigmine acts locally by diffusing through the mucosal barrier and causing secretion without systemic effects was initially gained from observations in the ferret (Ekström & Helander, 2002). In this preparation, the flow of saliva from the parotid, submandibular and sublingual

glands, on both sides, was diverted from the mouth. Local application of physostigmine on the buccal and labial mucosa on one side increased the amount of saliva on the exposed side but not on the contralateral side. Likewise, by duct-cannulation of the zygomatic gland, an increase in the resting flow rate of saliva was revealed in response to physostigmine applied on the overlying mucosa but no increase in the flow rate of saliva from the contralateral gland occurred. Moreover, there was no secretion from duct-cannulated parotid and submandibular glands providing further evidence for a local effect of physostigmine. The increase in secretory rate to physostigmine did not involve central mechanisms. It was due to a muscarinic action on the secretory cells. However, by increasing the concentration of the locally applied physostigmine a small secretory response of the contralateral side (and the contralateral zygomatic gland) as well as from the duct-cannulated major glands occurred, blockable by atropine, signalling systemic cholinergic effects. Subsequently, a direct evidence for local activation of labial glands in humans was provided by the topical application of physostigmine on an overlying small mucosal area (Hedner *et al.,* 2001).

In analogy, the secretion of saliva in response to physostigmine in the present studies is assumed to be preferentially due to the local action of physostigmine, by causing acetylcholine to accumulate in the neuro-glandular junction. The rigid protocol presently applied in the human studies did, unfortunately not, include the possibility to consider a systemic physostigmine- induced secretory contribution, for instance, by applying the Lashley-Crittenden cup over the orifice of the parotid gland to record the flow of saliva, if any, in a separate set of tests. The fact that systemic side effects, such as gastro-intestinal discomfort, appeared in respone to increasing doses of physostigmine shows that physostigmine reaches the circulation.

Though, certain drawbacks with the use of the Visual Analogue Scale to score the feeling of dryness and the possible relief in response to physostigmine, such

as not applying the scale at the screening process, not exposing all subjects to placebo, not allowing the subjects to see their immediate preceding score and difficulties in compliance, a significant effect of physostigmine was revealed (6). Intra-individual comparisons (with baseline before administration of physostigmine) showed long-lasting decreases in the feeling of dryness with respect to the lips and the whole mouth for up to 120-180 min, the reduction in scores being 25% to a dose of physostigmine of 1.8 mg, i.e the same dose level that was used for the objective measurement of the saliva secreted (see above). According to mean AUC value (over 180 min), the improvement was six times greater than that produced by placebo at this dose level. Though, the volume responses, to higher doses, of physostigmine are expected to increase, the relief in oral dryness, as judged by the Visual Analogue Scale, decreased instead. This inverse response at higher doses is probably due to the fact that feelings associated with side effects, such as nausea, got the upper hand and shifted the focus from oral sensations. The overwhelming type of systemic side-effects of the drug, in the doses tested, was gastro-intestinal discomfort, usually classified as mild to moderate. Compared to muscarinic receptor agonists on the market for systemic treatment of dry mouth, the frequency of side-effects is less with the local administration of physostigmine presently tested (Wiseman & Faulds, 1995; Wynn *et al.,* 2004). The therapeutic window for physostigmine, in its present formulation, may seem narrow as for all drugs with cholinergic effects.

The long duration of the effect of a single dose of physostigmine in terms of the secretion of saliva and the relief of the feeling of dryness was surprising, since the half-life of the drug is 15-30 min in plasma (Nordberg & Svensson, 1998). It is presently not known whether the long-lasting effect is due to the drug being retained in oral tissues, including the glands. Interindividually, large variations exist with respect to the pharmacokinetics of physostigmine (Sharpless & Thal, 1985; Thal *et al.,* 1986; Hartwig *et al.,* 1990; Asthana *et al.,* 1995). Physostigmine levels, the volume of distribution, and the clearance may vary threefold between patients receiving identical intravenous doses. The bioavailibilty of the drug after

swallowing (Nordberg & Svensson, 1998; Hartwig *et al.,* 1990) is usually considered low (<10%), but higher values have been reported (23%). The local treatment of dry mouth with physostigmine, as explored in this *Thesis*, is a novel approach that may turn out to be a therapeutic option. Since subjects may be more or less sensitive to the local application of physostigmine with respect to secretion and adverse effects based on pharmacokinetic causes, both the dose and the interval of administration may need to be individualized.

Main Conclusions

Almost all of the parotid cholinergic innervation *in the rat* is derived from the otic ganglion. Cholinergic nerve fibres from the ganglion reach the gland not only via the auriculo-temporal nerve but also via the facial nerve and, in addition, some other route(s). The reflexly elicited cholinergic (as well as the non-adrenergic, non-cholinergic) secretion is relayed via the otic ganglion.

The facial nerve supplies the parotid gland with cholinergic secreto-motor fibres and takes part in the reflex secretion.The nerve exerts a trophic influence on the secretory cells of the parotid gland. It is a potential contributor to the development of Frey´s syndrome.

Almost all substance P-containing nerve fibres innervating the parotid gland originate from the otic ganglion *in the rat.* Only a minor part of the calcitonin gene-related peptide-innervation of the gland is derived from the otic ganglion. The trigeminal ganglion does not contribute to the sensory substance P- and calcitonin gene-related peptide-innervation of the parotid gland *via* the auriculo-temporal nerve trunk.

The acetylcholine synthesis, expressed per gland weight, was three to four times higher in the mucin-producing sublingual, zygomatic and molar glands than in the

serous parotid and sero-mucous submandibular glands *in the ferret*. The high acetylcholine synthesizing capacity in the mucous glands reflects a high cholinergic tone over time. Muscarinic receptor subtypes M1-M5 were detected in all five glands but there was no preponderance for M1-subtype in the mucin-producing glands. A part of the parasympathetic secretory response of the zygomatic gland depended on non-adrenergic, non-cholinergic transmission mechanisms probably involving substance P and vasoactive intestinal peptide and possibly calcitonin gene-related peptide.

The activity of choline acetyltransferase was responsible for the acetylcholine synthesis in the glands. Denervation experiments showed virtually all of the acetylcholine synthesis, due to the activity of choline acetyltransferase, to be of nervous origin (and not of non-nervous origin).

The cholinesterase inhibitor physostigmine topically applied on the oral mucosa evoked a long-lasting secretion of saliva *in healthy subjects* and *in patients suffering from salivary gland hypofunction.* A long-lasting relief in the patients suffering from dry mouth was revealed by the use of a Visual Analogue Scale. It was possible to find a dose of physostigmine that both objectively and subjectively was of effect with cholinergic systemic effects at a minimum, if any. Local treatment aiming at activating hundreds of minor salivary glands, situated just below the oral epithelium, to secrete, while at the same time minimising systemic side-effects is a novel and promising approach to the treatment of dry mouth.

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