Characterization of secretory mechanisms in lacrimal and salivary glands

Martin Dankis

Department of Pharmacology
Institute of Neuroscience and Physiology
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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martin@dankis.se

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To my wife Karin

"They're gonna know we
came through here"

Quincy Jones

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ABSTRACT

Dry mouth and dry eyes are multifactorial morbidities that can lead to a severely reduced quality of life. Approximately 20% of the population suffers from ocular or oral dryness. In the pursuit of pharmacological treatments of these troublesome symptoms, we sought to identify new targets and characterize underlying mechanisms that modulate lacrimal and salivary secretion.

Xerogenic and xerophthalmic effects of antidepressants were examined in a rat *in vivo* model. Centrally mediated secretion was stimulated by applying citric acid to the tongue or by administration of menthol to the surface of the eye, and peripherally induced secretion was mediated by *i.v.* injection of the muscarinic agonist methacholine. The xerogenic effects of the tricyclic antidepressant clomipramine, the selective serotonin reuptake inhibitor citalopram, and serotonin-noradrenaline reuptake inhibitor venlafaxine were shown to be centrally mediated. In contrast, only clomipramine attenuated the peripherally stimulated response, while it was ameliorated by citalopram and venlafaxine. Likewise, the xerophthalmic effects of clomipramine and citalopram were centrally mediated. Further, similar to what was displayed in the salivary investigation, clomipramine attenuated peripherally stimulated lacrimation. However, citalopram exhibited no peripheral hyposecretory effects. In conclusion, in contrast with the common perception, modern antidepressant compounds such as SSRIs do not feature peripherally mediated anticholinergic properties. These findings verify the more suitable therapeutic

profile of modern antidepressants and support the use of local parasympathomimetic treatment of drug induced dry mouth and dry eyes.

Lacrimal gland secretory mechanisms and the effects of cholinergic and purinergic mediators were studied in primary monocultures and co-cultures of rat lacrimal gland cells. The primary culture isolation procedure was validated by monitoring the cultures immunochemically. After four weeks, a monoculture of myoepithelial cells was established which was shown to be sustained throughout the six-week isolation process. Prior to this, at 2-3 weeks, a co-culture of acinar and myoepithelial cells was evident. In conjunction, lacrimal gland tissue and primary cell cultures were studied morphologically for identification of cholinergic receptors. Immunohistochemical investigation of both myoepithelial cells and lacrimal gland tissues showed expression of a heterogenous muscarinic receptor population, indicating a multifaceted presence of functional receptors. However, no alterations in intracellular calcium were observed in myoepithelial cells, following stimulation with cholinergic modulators. This finding indicated a functional cholinergic dependence on intercellular interactions with acinar cells and an alternative cholinergic signal transduction pathway that excludes calcium. Based on the monoculture results, we next established a primary co-culture of rat lacrimal gland acinar and myoepithelial cells. In these studies, myoepithelial cells displayed a latent calcium response to cholinergic stimuli. This response was attributed to purinergic intercellular interactions, likely via ATP released from acini cells.

In conclusion, the current findings show that antidepressant-induced hyposecretion is mainly centrally mediated. We established and validated sustainable isolation procedures for monocultures of primary myoepithelial cells, in which co-cultures of acinar and myoepithelial cells arise midway. Furthermore, we showed that lacrimal gland secretion can be multifaceted, highlighting the importance of investigating effects of selective muscarinic and purinergic modulatory compounds in the efforts of developing new treatments for dry eyes and dry mouth.

Keywords: *dry mouth, dry eyes, antidepressant, muscarinic receptor, lacrimal gland, primary cell culture*

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SAMMANFATTNING PÅ SVENSKA

Torr mun och torra ögon är besvärande symptom som påverkar livskvalité negativt. Symptomen kan uppstå på grund av virusinfektion, strålbehandling, kirurgiska ingrepp, den autoimmuna sjukdomen Sjögrens syndrom eller orsakas av läkemedelsbehandling, t ex med antidepressiva läkemedel. Idag lider ungefär 20% av befolkningen av ögontorrhet eller muntorrhet och behandlingsmöjligheterna är begränsade. I den här avhandlingen återfinns delstudier vars syfte var att identifiera nya målprotein för symptomlindrande läkemedelsbehandling av torr mun och torra ögon. I dessa studier undersöktes även bakomliggande mekanismer med avseende på hur symptomen uppstår i samband med antidepressiv läkemedelsbehandling.

För att undersöka hur mun- och ögontorrhet orsakas av olika typer av antidepressiva läkemedel utfördes experiment på sövda råttor vilka injicerades med moderna läkemedel mot depression eller äldre (tricykliska) antidepressiva läkemedel (delarbete I, och delarbete II). Arbetshypotesen var att torrhetsbiverkningar inte orsakas av effekter på lokal nivå, utan att det snarare är påverkan på det centrala nervsystemet som orsakar nedsatt utsöndring från körtlarna. För att undersöka effekterna i det centrala nervsystemet stimulerade vi reflexinducerad sekretion hos råttor antingen genom att droppa citronsyra på tungan eller genom att droppa mentol i ögonen. Därtill undersöktes lokala effekter genom att stimulera sekretion med substansen metakolin vars kemiska egenskaper gör att den inte kommer åt nervcellerna i hjärnan. Det experimentella förfarandet möjliggjorde därmed separata undersökningar av reflexutlöst och lokalt utlöst utsöndring från körtlarna. Studien på salivproduktion visade att samtliga studerade antidepressiva läkemedel orsakade hämmad utsöndring från salivkörteln genom att inhibera reflexinducerad salivering (delarbete I). I motsats till detta observerades en ökad salivutsöndring vid undersökningar med lokalt stimulerad salivsekretion efter behandling med de moderna antidepressiva substanserna citalopram och venlafaxin. Citalopram tillhör kategorin selektiva serotoninåterupptags-hämmare (SSRI) och venlafaxin tillhör gruppen serotonin-noradrenalin-återupptagshämmare (SNRI). Den äldre substansen klomipramin påvisade däremot en hämmande effekt på salivutsöndringen vid både centralt och lokalt medierad salivering.

Salivstudien föranledde en ny arbetshypotes där antidepressiva läkemedel antogs verka likadant på produktionen av tårar. För att undersöka detta utfördes nya experiment med liknande procedur som tidigare, fast där tårar mättes istället för saliv (delarbete II). Studien visade att effekterna av klomipramin

var analoga till de som hade observerats i salivstudien. Även citalopram visade liknande effekter som tidigare, med nedsatt reflexorsakad tårproduktion. Däremot orsakade citalopram ingen ökad tårsekretion vid lokal stimulering, utan produktionen förblev oförändrad. Som slutsats visar studierna att uppkomsten av muntorrhet eller ögontorrhet i samband med medicinering med moderna antidepressiva läkemedel inte orsakas av läkemedelsinteraktioner på lokal nivå i körtlarna utan genom inhibering av reflexbågen. Därmed bör behandling med SSRI vara fullt kompatibel med symptomlindrande läkemedelsbehandling med substanser som efterliknar den kroppsegna signalmolekylen acetylkolin.

För att lägga grunden för utveckling av läkemedelsbehandling mot torra ögon undersöktes så kallade muskarina receptorer i tårkörtel och celler från tårkörtel (delarbete III, och delarbete IV). Muskarina receptorer aktiveras av signalmolekylen acetylkolin. I tårkörteln är det känt att aktivering av muskarina receptorer leder till tårutsöndring från körtelcellerna, men det är inte känt vilka roller olika typer av muskarina receptorer spelar i utsöndringen. Arbetshypotesen var att uttrycket av muskarinreceptorer är mångfasetterat och att det i sin tur kan medföra interaktioner som måste beaktas i utvecklingen av nya läkemedel avsedda för att lindra torra ögon genom att stimulera produktion av tårar. För att undersöka uttryck hos och funktion av muskarina receptorer studerades tårkörtlar från råtta på tre olika sätt. Dels i isolerade cellkulturer av en typ av muskelceller vilka även är kända som myoepiteliala celler, dels i en kombinerad co-kultur bestående av muskelceller och tårutsöndrande aciniceller och dels genom studier av histologiska snitt från tårkörtelvävnad. Undersökningarna visade på ett heterogent uttryck av muskarina receptorer, vilket medför att effektiviteten hos läkemedelskandidater delvis avgörs av hur de interagerar med de olika typerna av muskarina receptorer. I undersökningar av biologiska svar från muskarina receptorer i muskelceller syntes inga svar från receptorerna. I studien undersöktes biologiska svar genom att mäta kalcium. Det uteblivna svaret innebär därmed möjligtvis att muskarina receptorer i myoepiteliala muskelceller signalerar via andra vägar som inte involverar kalcium (delarbete III). Vår bedömning var dock att det är mer troligt att aktivering av muskelcellerna sker först efter aktivering av en annan celltyp, nämligen tårutsöndrande aciniceller.

För att undersöka detta odlades en co-kultur av två celltyper, tårutsöndande aciniceller och myoepiteliala muskelceller (delarbete IV). I denna studie kunde en interaktion påvisas mellan cellerna där muskarinliknande läkemedel först orsakade ett exciterat tillstånd hos aciniceller, vilket i sin tur frisatte substanser som sedan stimulerade ett biologiskt svar i de myoepiteliala muskelcellerna. Vidare kunde isoleringsprocessen valideras genom att kulturen undersöktes

veckovis under fyra veckors tid. Cellerna undersöktes för uttryck av biomarkörer relevanta för celldelning, stamcellsliknande egenskaper, tårutsöndande acinicelluttryck, myoepitelialt muskelscelluttryck och uttryck av muskarina receptorer. Analysen visade att cellkulturen efter fyra veckor enbart bestod av myoepiteliala muskelceller och att uttrycket av muskarina receptorer var heterogent genom hela isoleringsprocessen.

Som slutsats visar avhandlingen att den muntorrhet och ögontorrhet som härstammar från behandling med moderna antidepressiva läkemedel i huvudsak är orsakad av inhibering av reflexbågen och att det därmed troligtvis går att behandla biverkningarna med läkemedel som efterliknar den kroppsegna substansen acetylkolin. Dessutom validerades isoleringsprocessen av myoepiteliala muskelceller, inklusive tidpunkten för när optimala co-kulturer av myoepiteliala- och aciniceller uppstår. Ett multifacetterat muskarint receptoruttryck påvisades, vilket styrker vikten av att undersöka effekterna av selektiva muskarinliknande läkemedel vid utveckling av ny läkemedelsbehandling mot torr mun och torra ögon.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Johnsson M.**, Winder M., Zawia H., Lödöen I., Tobin G. & Götrick B. In vivo studies of effects of antidepressants on parotid salivary secretion in the rat. *J Arch Oral Biol*. 2016 67:54-60.

II. **Dankis M.**, Aydogdu Ö., Tobin G. & Winder M.
Inhibitory effects of antidepressants on lacrimal gland secretion in the anaesthetized rat. *Submitted.*

III. **Dankis M.**, Carlsson T., Aronsson P., Tobin G. & Winder M.
Novel insights into the function of muscarinic and purinergic receptors in primary cultures of rat lacrimal gland myoepithelial cells. *Submitted.*

IV. **Dankis M.**, Aronsson P., Carlsson T., Tobin G. & Winder M.
Functional muscarinic and purinergic responses in primary co-cultures of rat lacrimal gland myoepithelial and acinar cells. *Manuscript.*

CONTENT

ABBREVIATIONS

4-DAMP	4-diphenylacetoxy-N-methylpiperidine
[^{3}H]QNB	3H-quinuclidinyl benzilate
A839977	1-(2,3-Dichlorophenyl)-N-[[2-(2-pyridinyloxy) phenyl]methyl]-1H-tetrazol-5-amine
AC	adenylate cyclase
ACh	acetylcholine
ANOVA	analysis of variance
AQP5	aquaporin-5
ATP	adenosine 5'-triphosphate
Ca^{2+}	calcium
cAMP	3',5'-cyclic adenosine monophosphate
CCh	carbachol
DAG	diacylglycerol
DAPI	4′,6-diamidino-2-phenylindole
DED	dry eye disease
EMA	European medical agency
ERK	extracellular signal-regulated kinase
FDA	U.S. food and drug administration
FLIPR	fluorometric Imaging Plate Reader
IP3	inositol triphosphate

MAOI	monoamine oxidase inhibitors
MAPK	mitogen-activated protein kinase
MeCh	methacholine
mRNA	messenger ribonucleic acid
NO	nitric oxide
NOS	nitric oxide synthase
PBS	phosphate buffered saline
PEST	penicillin/streptomycin
PFA	paraformaldehyde
pFHHSiD	4-fluoro-hexahydro-sila-diphenidol
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PLC	phospholipase c
PPADS	pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid
Pyk2	protein tyrosine kinase 2
qPCR	quantitative Polymerase Chain Reaction
RPMI	Roswell Park Memorial Institute
SNRI	serotonin noradrenaline reuptake inhibitors
SOX2	sex determining region Y-box transcription factor 2
Src	proto-oncogene tyrosine-protein kinase
SSRI	selective serotonin reuptake inhibitors

TCA	tricyclic antidepressants
TRPM	transient receptor potential melastatin
TRPV	transient receptor potential vanilloid
VAMP-8	vesicle associated membrane protein 8
VIP	vasoactive intestinal peptide
VIPAC	vasoactive intestinal peptide receptor
α-SMA	α-smooth muscle actin

1 INTRODUCTION

Dry eyes and dry mouth, also known as xerophthalmia and xerostomia respectively, are disorders that can result in a severely reduced quality of life [1-4]. Dry eye disease (DED) is defined as *"a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles"* and is classified into two subcategories, i.e. aqueous deficient dry eye and evaporative dry eye [5]. Regarding dry mouth, a definition has not been published in the same official manner as for dry eyes. However, the physiological factors affecting the subjective sensation of dry mouth have been reported to induce xerostomia following an attenuated salivary flow rate of 50 to 60% [6]. The symptoms can arise in conjunction with other disorders such as Sjögren's syndrome, which is a chronic inflammatory autoimmune rheumatic disorder. The primary form of Sjögren's syndrome is characterized by lymphocytic infiltration of salivary and lacrimal glands, which leads to progressive symptomatic xerostomia and xerophthalmia. Most affected by the disease are women between 40-50 years of age, with a female/male ratio of 9:1, indicatory of the significant role played by sex hormones. However, the prevalence of Sjögren's syndrome is only approximately 0.5% while the prevalence of dry mouth and dry eyes is approximately 21% and 20%, respectively [4,7,8]. Interestingly, medical treatment of other ailments represents the most common etiology of dry mouth and dry eyes, specifically pharmacotherapy, and both surgical therapy and radiation therapy can result in hyposecretory side effects [9-11]. Among pharmacotherapies associated with both dry mouth and dry eye, antidepressants, pharmaceutical therapies for benign prostate enlargement, and antihypertensive drugs, have been shown to be associated with an increased risk of DED [12-15]. Therefore, investigations of how these drugs can affect the induction of salivation and lacrimation are of high relevance in the endeavors of developing new pharmacological treatment strategies.

1.1 TREATMENT TODAY

Various drugs have been tested in search of symptomatic pharmacological treatment of DED, unfortunately without satisfying results. Due to scarcely documented effects of systemic parasympathomimetics such as pilocarpine and cevimeline, topically administered eye drops with non-pharmacological

attributes are presently the most commonly used [16]. Even though these eye drops provide improved quality of life for patients, they are nevertheless only effective for a short time, requiring patients to apply eye drops frequently throughout the day. In xerostomia however, the therapeutic effects of pilocarpine and cevimeline are well documented which induces the question why these treatments are unable to attenuate the symptoms of DED. Interestingly, cevimeline and pilocarpine have been shown to display rather unselective binding profiles to the five different muscarinic receptors where binding to the inhibitory receptors M2 and M4 has been shown to be more potent than to the excitatory receptor M3 [17,18]. This could be the cause for their ineffective properties in the treatment of DED.

The P2Y2 purinoreceptor agonist diquafosol was approved for dry eye treatment in Japan in 2010, making it the first approved topical tear stimulant treatment of dry eyes in the world. Diquafosol has not been shown to stimulate lacrimal secretion, but rather to induce secretion from goblet cells in the conjunctiva [19-21]. However, diquafosol did not meet primary or secondary endpoints in a clinical study performed in the USA, and consequently the manufacturer did not receive food and drug administration (FDA) approval [16].

It is also worth mentioning that there are immunomodulator eye drops available for treatment of DED, e.g. eye drops containing cyclosporine. Such eye drops are indicated to potentiate the tear production in patients with keratoconjunctivitis sicca, or chronic DED, but there are mixed opinions among physicians regarding their efficacy [22]. Whilst cyclosporine containing eye drops have been marketed with FDA approval since the late 1980s, it is only recently that they have been allowed European market entry by the European medical agency (EMA). Remarkably, a recent systematic literature review showed that out of more than one hundred multicentered clinical studies of topical eye treatment for dry eyes, none showed statistical significance on primary endpoints, compared to control treatment [23]. One possible cause for the limited results of the clinical studies of various candidate drugs is the lack of pathophysiological characterization of dry eye. In fact, it is only in the recent decades that the ophthalmological profession has made significant progress towards standardizing diagnosis and treatment of the disease. To this end, a decennial international workshop has been established, designated “the dry eye workshop” [24], in which substantial accomplishments have been made regarding

classification and etiology. With better understanding of the various subcategories of the disease and with a better qualification of clinically relevant quantitative measurements in diagnostics one could argue that it has been made easier to study the effect of novel drugs in clinical studies today.

1.2 AIMS

The project aims are to define possible targets for symptomatic pharmacological treatment of dry mouth and dry eyes. Characterization of the glandular secretory mechanisms from these studies will help build a foundation to be utilized in etiological investigations. So far, dysfunction of the autonomic nervous system has been indicated as a major factor in the pathogenesis of DED and dry mouth as Sjögren's syndrome mimics several symptoms of autonomic nervous system failure [25]. The potential treatment strategies that may be derived from this project could, in addition to monotherapy, be of a multitherapeutic approach where for instance two drugs, such as an adrenergic agonist and a muscarinic agonist, may be combined. The possible risks involved in combination treatment are an elevated probability of interaction-induced adverse effects from the simultaneous exposure to two substances. This needs to be taken into consideration in the future design of drug development strategies.

This thesis addresses the following aims, as listed below:

1. Identify underlying mechanisms relating to hyposecretory adverse effects in antidepressant pharmacotherapy (Paper I and Paper II).
    a. Investigation of drug induced hyposecretion from the most commonly subscribed tricyclic antidepressants, selective serotonin reuptake inhibitors and serotonin noradrenaline reuptake inhibitors.
    b. In vivo investigation of peripherally and centrally mediated modulation of secretory response in rat salivary parotid gland and rat tear production.
2. Identify muscarinic receptor expression and function in rat lacrimal gland.
    a. Morphological, protein expression and functional characterization of muscarinic receptors in rat lacrimal gland isolated primary monocultures of myoepithelial cells (Paper III).

   b. Morphological identification of muscarinic receptors in rat lacrimal gland tissue (Paper IV).
3. Identify functional intercellular interactions between acinar and myoepithelial cells in coculture.
   a. Functional characterization of lacrimal gland primary co-culture containing myoepithelial and acinar cells (Paper IV).

1.2.1 SIGNIFICANCE

The characterization of secretory mechanisms in salivary and lacrimal glands can lead to refined targets for drug treatment, not only for Sjögren's syndrome, but also for other dry mouth and dry eye etiologies, such as hyposecretion caused by surgical therapy or radiotherapy, as well as pharmacotherapies for other diseases. Conceivably, the project will build a foundation for the development of new strategies in the pharmacological treatment of glandular failure. Such novel treatments should preferably be applied topically, i.e. as eye drops and/or mouth wash. Thus, the treatments would be compliant with concomitant pharmacotherapies treating other ailments, thereby minimizing potential interactions. Any treatment that leads to significant reduction in suffering from xerostomia or xerophthalmia would be of great importance for a large number of patients and would greatly increase their quality of life.

1.3 GENERAL GLANDULAR ANATOMY AND PHYSIOLOGY

Saliva and tears are produced in glandular structures, either directly secreted over target tissue, or in the periphery, relocated through ductal systems running from exocrine glands. The secrete arises from acinar cells, that are held together by tight junctions, and assembled as a tubular structure in stacked form. If one were to do a cross section of this cluster of acinar cells the observed structure could be described as a circular shape composed by numerous acinar cells placed next to each other in a circular fashion with a large membrane exposed to the external region, called the basolateral membrane, and a small membrane exposed to the middle lumen cavity, also known as the apical membrane (Figure 1). The acinar cells secrete water, electrolytes, proteins and mucins into the lumen through the apical membrane [26]. The basolateral membranes, on the outer side of the circle, contain the receptors which interact with different signal substances, such as neurotransmitters and growth factors. Both the apical and basolateral membranes contain ion channels and ion transport proteins, which together with the tight junctions and gap junctions between the acinar cells maintain a

polarization between the outer and inner parts of the acinar cluster. By the net transport of various electrolytes over the membranes, a resulting physiological system is achieved which results in absorption of ions through the basolateral membrane and secretion through the apical membrane, into the lumen [26]. Through the gap junctions and aquaporins the acinus cluster forms a functional syncytium, where intercellular communication is mediated through ion transport [27,28]. Furthermore, proteins are synthesized in the endoplasmic reticulum (ER) and the Golgi apparatus of the acinar cells, and upon stimulation, these proteins are subsequently secreted through exocytosis via membrane-fused granules [29].

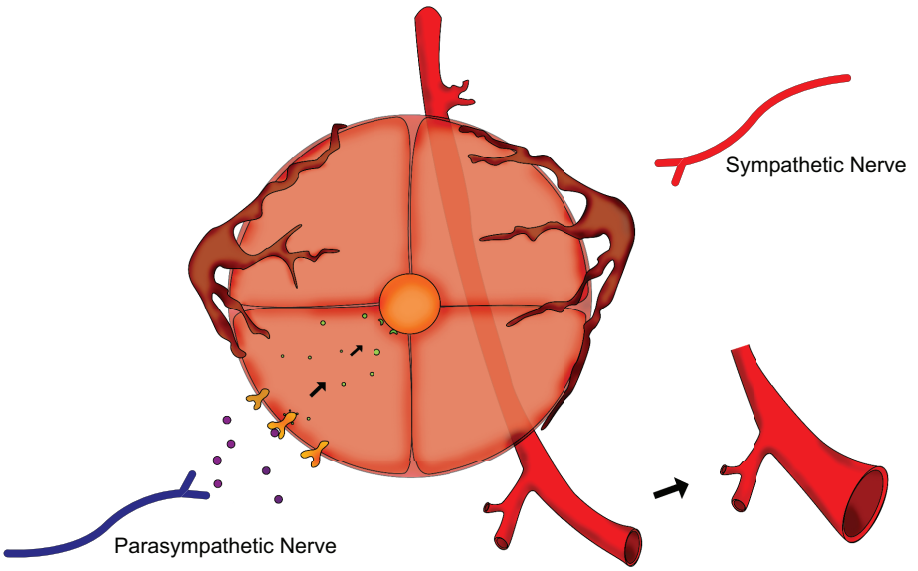


Figure 1. Schematic drawing depicting three major components involved in lacrimal and salivary secretion. In the lower left, an endogenous agonist (purple) is released from a parasympathetic nerve (blue). The agonist binds to receptors on the basolateral side of the acinar cell in the lower left corner of the circular structure (acinus) which contains four acinar cells. Through a G-protein cascade this eventually leads to exocytotic secretion in the lumen. Myoepithelial cells (brown) hold the acinus structure together. They contain α-SMA that contracts upon stimulation with endogenous agonists. In addition to the parasympathetic innervation, the acinus is also innervated by sympathetic nerves (red) which are represented in the top right corner of the drawing. The blood vessels also contain smooth muscle that relax upon stimulation resulting in increased blood flow.

The blood supply to the acini may be of significant importance for the secretory function over time (Figure 1) [30]. Through systemic circulation of blood, water, oxygen, nutrients and hormones are supplied to the exocrine glands. The blood pressure and blood flow are regulated by the function of bifurcated arteries and veins and have been shown to be directly correlated to the amount of secreted product from both salivary and lacrimal glands. The capillaries are attached to acinar cells and myoepithelial cells by pericytes [31]. Additionally, through the blood the salivary and lacrimal glands are immigrated by immunomodulating cells such as lymphocytes, mast cells, plasma cells, dendrite cells and macrophages. The immunoreceptive and responsive cells function as important barriers in the immune system, protecting the body from the continuous exposure to infectious microorganisms by sensing foreign biological expression and secreting antimicrobial enzymes and antibodies, such as lysozyme and IgA.

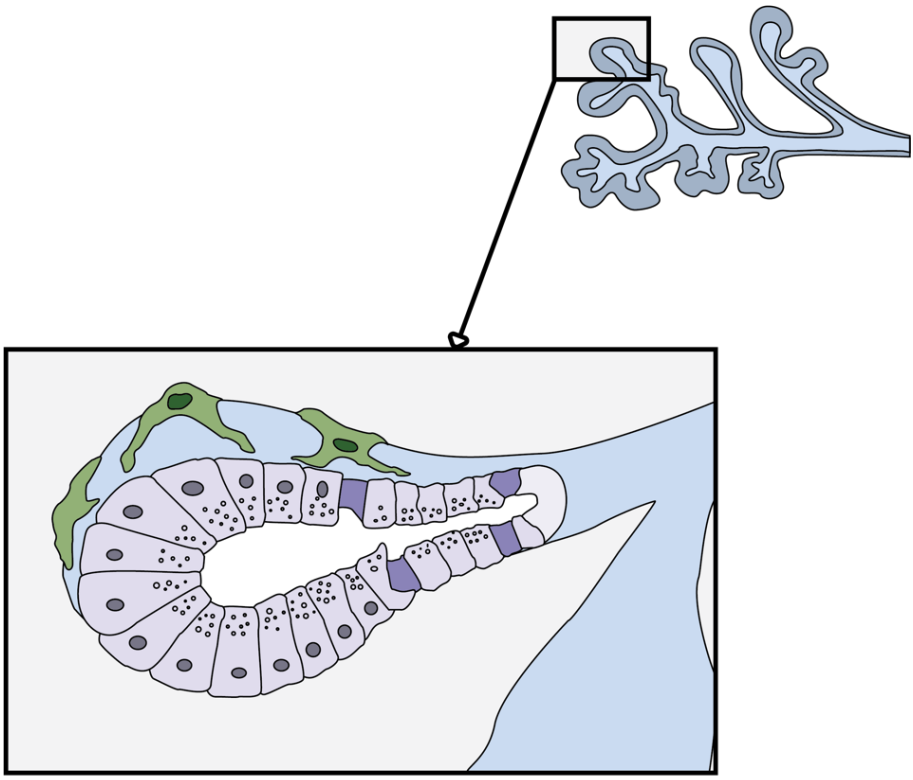


Figure 2. Schematic drawing depicting a cross-sectioned tubule where the acinus cluster, composed of acinar cells (purple with dark nuclei), connect to a terminal duct. The acinus is surrounded by myoepithelial cells (green).

Further down the tubule, the lumen converges into excretory ducts, in a fine branched system (Figure 2). Upon stimulation, the secreted product from the acini first enters the intralobular ducts, which in turn unite in the interlobular ducts. The duct branches then continue into the larger intralobar ducts which in turn congregate into the interlobar duct. The interlobar duct is connected to the main excretory duct where the secrete flows to the eye or mouth. The duct cells that constitute the ducts are joined by tight junctions forming a polarized structure, similar to the acinar cells in the acini. These duct cells are also linked by aquaporins and possibly by gap junctions. The presence of duct cell gap junctions has been indicated in salivary glands, but still remains to be shown in the lacrimal gland [32,33]. The duct cells secrete water, electrolytes, mucins and proteins, and can modify the secrete consistency and therefore express a slightly unique membrane setup as compared to the other glandular cells. In the submandibular gland the duct cells have been shown to play an important role in the physiochemical properties of the saliva. Namely, the saliva that is secreted from the acini is isotonic, but as the saliva is transported through the duct the concentration of electrolytes in the saliva is modified by the duct cells to become hypotonic. Thus, duct cells are relevant not only as a secretory conduit but also as potential modulators of the physiochemical properties of the secreted product.

The third major cell type in exocrine glands is part of the acinar cluster and surrounds the acinar cells. These cells are known as the myoepithelial cells and are located in close vicinity to the basolateral membranes of the acinar cells. The myoepithelial cells are also located near the duct cells. They are star-shaped with long slender processes forming around the acinar cluster and duct basal lamina (Figure 2). They contain α-smooth muscle actin (α-SMA) and myosin and they have been shown to support the secretory acinar cells in multiple exocrine glands, for instance salivary and mammary glands. Following stimulation, the myoepithelial cells contract, which forces the excretion of secretory product into the lumen of the acini [34]. However, this phenomenon has not yet been fully proven in the lacrimal gland. In the mammary gland, oxytocin-induced myoepithelial contraction has been shown to be central in the very brief burst of milk ejection following pup suckling in lactating rats [35]. Furthermore, the myoepithelial cells are progenitor-like and play significant roles in atrophy of glandular tissue [36,37]. In the development of treatments for glandular dysfunction induced by atrophic effects related to radiotherapy and Sjögren's syndrome the myoepithelial cells can therefore play a significant role as possible targets for cell proliferation and tissue regeneration. Also, in the search for symptomatic treatment of hyposecretion these cells should be incorporated in the risk assessment of adverse effects possibly resulting in tumorous growth.

1.4 THE EYE AND LACRIMATION

The afferent sensory nerves that affect lacrimation are branched in the ophthalmic nerve, which together with the maxillary nerve and the mandibular nerve branches out of the trigeminal nerve. In direction towards the periphery, the ophthalmic nerve is subsequently divided into three branches containing the lacrimal nerve which runs through the lacrimal gland and into certain areas of the bulbar conjunctiva of the eye and into the skin which covers the lateral upper part of the eyelid, the frontal nerve which innervates the skin in the forehead and in the scalp, and finally the nasociliary nerve which synapse in the ciliary ganglion and innervate the cornea of the eye through postganglionic ciliary nerves. In addition to the lacrimal nerve's sensory innervation of the conjunctiva, the infraorbital nerve branch of the maxillary nerve supplies supplementary sensory input from the remaining bulbar conjunctiva, the palpebral conjunctiva, and from the skin in the margins of the eyelid. Thus, the afferent signal from sensory neurons can be triggered by various sensory inputs from the surface of the eye and the skin within and surrounding the ocular orbit. Temperature sensing receptors such as the cold sensing transient receptor potential melastatin (TRPM) ion channels and heat sensing transient receptor potential vanilloid (TRPV) ion channels in the cornea have been shown to have tear evoking effects when stimulated [38-42]. In addition to cold and heat, changes in pH and osmolarity also trigger signaling from these receptors [43,44].

The lacrimal gland is dense in acinar cells and is located within the bony orbit of the eye, in the lateral region of the upper eyelid, also known as the superior tarsal plane. The acinar cells are bundled in acinus clusters which are surrounded by myoepithelial cells located in close vicinity to the basolateral membranes of the acinar cells. The apical membranes of the acinar cells are located in the center of the acinus cluster, forming the lumen cavity into which the secretory product is discharged.

The lacrimal fluid flows through ducts that run through the upper eyelid, and out over the eye where it congregates with lipids and mucins to form the tear film. This tear film is generally described to consist of three layers, the mucin layer, the aqueous layer, and the lipid layer. The mucin layer is located closest to the surface due to the coarse and viscoelastic properties of its main component, mucins, which are partly secreted from the lacrimal gland but are mainly the product of secretion from goblet cells located in the conjunctiva of the eye. In the tear film, the mucin layer interacts mostly with the aqueous layer which contains water, protein and electrolytes. The aqueous layer is by far the thickest layer out of the three and is primarily constituted by secretory products

from the main lacrimal gland. On top of the aqueous layer is the lipid layer which prevents evaporation of water. The lipid layer is a composite of polar lipids, such as phospholipids, and non-polar lipids, such as cholesterol esters and wax esters. The lipids are secreted from the meibomian glands which are tubuloacinar glands arranged perpendicular to the margin of the eyelid in both the superior and inferior tarsal plate.

1.4.1 PARASYMPATHETIC INNERVATION OF THE LACRIMAL GLAND

Widespread parasympathetic innervation has been shown in the lacrimal gland [45-47]. Furthermore, morphological characterization of the gland showed a presence of parasympathetic neurotransmitters together with related enzymes, such as choline acetyltransferase and acetylcholinesterase, which function as catalyzers of the synthesis and metabolic breakdown of the transmitters, respectively [48-50]. The functional significance of parasympathetic neurons has also been shown in denervation studies in the lacrimal gland [51,52]. In functional studies of rabbit and rat lacrimal glands, the aforementioned parasympathetic neurotransmitters acetylcholine and vasoactive intestinal peptide have been shown to play significant roles in lacrimal secretion [53,54].

The efferent parasympathetic innervation of the lacrimal gland is provided through the postganglionic fibers of the zygomatic nerve, which interacts with the maxillary nerve, which in turn synapses at the pterygopalatine ganglion where the vidian nerve terminates. The vidian nerve is a part of the superficial petrosal nerve and the deep petrosal nerve. The superficial petrosal nerve courses through the geniculate ganglion without synapsing to the superior salivary nucleus in the pontine tamentum [55,56]. Microarray analysis of genes in preganglionically denervated lacrimal gland has been shown to correlate with downregulation of genes for the ER and Golgi apparatus. Furthermore, there was an up-regulation of genes for cytoskeletal and extracellular matrix components, inflammation and apoptosis [57,58].

1.4.2 CHOLINERGIC TRANSMISSION

Acetylcholine is the key neurotransmitter of the cholinergic part of the parasympathetic regulation of the lacrimal gland. The known possible cholinergic receptor targets are the nicotinic acetylcholine receptors and the muscarinic acetylcholine receptor family, which is divided into five known subtypes, designated M1-M5. M1, M3, and M5 are excitatory receptors while M2 and M4 generally act in an inhibitory manner. In myoepithelial cells, the unselective muscarinic agonist carbachol (CCh) has been shown to cause an increase in intracellular calcium, leading to myoepithelial contraction [59].

Ishikawa et al showed that $G_{q/11}$ coupled receptor activation causes mobilization of the water channel protein AQP5 (aquaporin-5) from intracellular membranes of the acinar cells to the apical membrane [60]. Furthermore, studies of the lacrimal gland show that cholinergic agonists also activate pathways that attenuate secretion over time. Transcription factors known as p44-p42 mitogen-activated protein kinase (MAPK) or extracellular regulated kinase 1/2 (ERK) has also been shown to decrease lacrimal protein secretion [61]. The MAPK signal is transduced through protein kinase c (PKC), which activates non-receptor tyrosine kinases proto-oncogene tyrosine-protein kinase (Src) and protein tyrosine kinase 2 (Pyk2) [62]. It is hypothesized that this secretory attenuation could be the cells way of adjusting to the exhaustion that they undergo when they are excited by cholinergic agonist, possibly an adjustment in the form of mitogenesis or hypertrophy, which would explain the reduced protein secretion. Thus, the acinar cells are restraining protein production and therefore not releasing proteins.

The blood supply in the lacrimal gland might be regulated by muscarinic receptors in the lacrimal artery. Unfortunately, to date there are no studies published, to this authors knowledge, on cholinergic function in the lacrimal artery, specifically. However, investigations have been performed on the cholinergic function in the ophthalmic artery in knockout mice [63]. The ophthalmic artery which branches out into the lacrimal artery. Therein, it was shown that arterial vasodilation was predominantly regulated by M3 receptors. The authors partly strengthened their claim by studying vasodilatory effects from cholinergic agonism in M3 and M5 knockout mice. Their experiments showed almost completely abolished vasodilation in M3 knockout mice as compared to wild-type, while M5 knockout mice displayed no statistically significant difference from wild-type. Furthermore, qPCR investigations showed approximately one hundred times higher expression of mRNA for excitatory muscarinic receptors as compared to the inhibitory muscarinic receptors [63].

Generally, in the recent literature, the muscarinic receptors M1, M3, and M5 are regarded as excitatory $G_{q/11}$ coupled receptors. Interestingly, out of these three receptors the M3 receptor has previously been concluded to be the only muscarinic receptor subtype expressed in the lacrimal gland [64]. The inhibitory muscarinic receptors M2 and M4 have not yet been investigated in the lacrimal gland and are generally considered to be coupled to inhibitory G-protein, designated $G_{i/o}$. $G_{i/o}$ inhibits AC (adenylate cyclase) which catalyzes the conversion of ATP (adenosine-5'-triphosphate) to cAMP (3',5'-cyclic adenosine monophosphate) and pyrophosphate. The M4 receptor is also

considered to inhibit Ca^{2+} channels, while M2 inhibits both Ca^{2+}- and K^{+} channels [65].

1.4.3 VIP-ERGIC TRANSMISSION

The parasympathetic neurotransmitter vasoactive intestinal peptide (VIP) mainly interacts with two receptors: vasoactive intestinal peptide receptor 1 (VIPAC1), which is expressed in the basolateral membrane of the acinar cells, and vasoactive intestinal peptide receptor 2 VIPAC2, which is expressed in the myoepithelial cells. The VIP receptors are G-protein-coupled receptors, specifically coupled to G_s-subtypes of the G-protein family, in which the alpha subunit subsequently interacts with the enzyme adenylate cyclase, following G-protein excitation. The increased levels of cAMP that follow in turn phosphorylate protein kinase A (PKA), thus making it active. VIP causes secretion of protein into the lumen of the acinar cluster. This possibly occurs through phosphorylated PKA which is hypothesized to interact with unknown target proteins [66]. To provide clarity in this, Funaki et al investigated the role of cAMP in the MAPK pathway in lacrimal glands. The study showed that an increase in cAMP concentration significantly inhibited MAPK signaling. The authors hypothesized that this effect could account for the role of cAMP in lacrimal protein secretion [67]. The relevance of cAMP in the physiochemical properties of the secretory product stemming from lacrimal glands could thereby be of relevance for tear film stabilizing factors such as mucins. Thus, receptors that modulate the intracellular levels of cAMP, such as M2, M4 and beta-adrenergic receptors, can be of significant relevance in the development of a novel treatment for dry eyes.

1.4.4 SYMPATHETIC INNERVATION OF THE LACRIMAL GLAND

The lacrimal gland is innervated by sympathetic nerves that originate from the superior cervical ganglia. Their nerve fibers have been located in the vasculature, near lacrimal gland arteries and arterioles. So far, lacrimation has been shown to be affected by adrenergic signaling through the α1 receptor subtype, which is expressed in blood vessels, ducts and in the cytoplasm and basolateral membrane of acinar cells [68]. In functional investigations of lacrimal acini preparations, it was shown that α-adrenergic stimulation induces protein secretion [61,69]. Interestingly, muscarinic inhibition has been shown to follow α1 stimulation [70]. Furthermore, in a clinical study, lacrimal volume and tear flow were measured following topical administration of an ophthalmic preparation containing the α-adrenergic agonist oxymetazoline. The authors found that both the tear volume and tear secretion were significantly decreased for six hours post administration and concluded that ophthalmic preparations

containing α-adrenergic agonists, such as redness relief marketed eye drops Clear Eyes®, may cause DED following frequent application [71]. Moreover, adrenergic receptor subtype β1 has been identified in the cytoplasm of acinar cells and might also be relevant, if not central, in the exhibited adrenergic effects on protein secretion in adrenoceptor-induced lacrimation. Therefore, adrenergic activity in the lacrimal gland play dual in roles, where acinar secretion is induced from adrenergic stimuli, but with the concomitant arterial vasoconstriction and adrenoceptor-induced parasympathetic inhibition that results from adrenergic stimulation, the net effect is inhibited lacrimal secretion.

1.4.5 PURINERGIC CO-TRANSMISSION IN THE LACRIMAL GLAND

ATP is an integral component in synapse vesicle transmission and is thereby generally co-transmitted with corresponding neurotransmitters from both parasympathetic and sympathetic nerve terminals. ATP, alongside other purines such as adenosine, uridine triphosphate (UTP), diadenosine triphosphate (Ap3A), is a receptor modulator in the purinergic signaling. In humans there are many different subtypes of purinergic receptors, generally divided into two different categories. The category designated P1 are adenosine selective while the P2 category have a higher affinity for ATP. The P1 receptors are G-protein-coupled receptors while the P2 receptor family is vast and contains both ionotropic receptors (P2X) and G-protein-coupled receptors (P2Y). Moreover, purines have recently been suggested to play an important role in lacrimal gland secretion, thereby purinoceptors could be promising targets in the search for pharmacological treatment of dry eye symptoms [72]. Studies utilizing western blotting and immunofluorescent microscopy have shown that purinergic P2X receptors are expressed in the rat lacrimal gland, mainly in acini cells [73]. However, the expression of P2X5 purinoceptors seems to be modest [74]. To date, mainly the P2X7 purinoceptor has been identified as an important target, with expression in both rat lacrimal acini and isolated myoepithelial cells [75,76]. In studies on the P2Y receptor family, the majority of the subtypes have been identified in the rat lacrimal gland [76-78]. P1 purinoceptors have not yet been investigated in the rat lacrimal gland, but their expression has been reported in rabbit lacrimal gland acini [79,80]. In the literature, purines are proposed as possible mediators of synergistic effects, in combination with cholinergic stimuli. Purines are thereby interesting candidates for combinatorial treatment.

1.5 SALIVARY GLANDS

1.5.1 SALIVARY GLAND INNERVATION

Parasympathetic and sympathetic secretomotor and vascular nerves innervate the salivary gland, and the control of salivary secretion depends mainly on nerve reflex impulses that involve their efferent stimuli. The autonomic nerves reach most cell types in the salivary glands [81], and similar to the lacrimal gland, the salivary glands exhibit dense parasympathetic innervation which has been shown in close vicinity to the acinar-, duct-, and myoepithelial cells [82]. While the parasympathetic activity induces a profuse secretion that is relatively poor in protein, activity within the sympathetic innervation induces little but protein-rich saliva. Additionally, in blood vessels of salivary glands, electrical stimulation of parasympathetic glandular nerves has been shown to induce vasodilation. The vasodilatory response is mediated through nitric oxide (NO) which is synthesized by nitric oxide synthase (NOS) as a result of cholinergic and VIPergic stimulatory pathways [83,84]. Furthermore, 4-DAMP- and pirenzepine-sensitive cholinergically induced blood flow has been recorded in the rat submandibular gland [85]. Following further investigation into the blood supply of the rat submandibular glands, a cholinergically induced vasoconstriction in submandibular veins and contrasting vasodilation in submandibular arteries following administration of muscarinic agonist methacholine has been observed [86]. In the same study similarities were shown to be consistent in the submandibular vein and the jugular vein, as well as in the submandibular artery and carotid artery which indicates that the regulation of blood supply in the lacrimal gland might be analogous to that of the submandibular gland. Interestingly, a heterogeneity was shown, both functionally and morphologically, concerning the muscarinic receptors that mediated the cholinergic vasomodulatory responses in the carotid artery and jugular vein. Secretion and blood flow are thereby controlled by noradrenaline and acetylcholine but are also regulated by neuropeptides, like VIP [87].

The cholinergically induced secretion has generally been attributed solely to the activation of the muscarinic receptor subtype M3 [88]. This concept has been supported by results of functional studies on rat parotid glands and in the parotid cell line PAR-5 in addition to studies using subtype-specific antisera [89]. However, binding and immunochemical experiments on ovine, ferret, and rat submandibular glands reveal the expression of M1, sometimes accompanied by M5, in addition to the M3 receptors. Similar observations have been made in human labial glands [90]. The M1 receptor has been shown to play a significant role in the secretory response, in the rabbit and ovine submandibular gland, and in rat submandibular and sublingual glands [85]. Thus,

other subtypes than M3 contribute to the salivary response. In animal models, autoantibodies against muscarinic receptors have been shown to inhibit secretion, revealing a possible model for dry mouth. Furthermore, such antibodies have been suggested in the disease etiology, and in Sjögren's syndrome the acinar M3 receptors are up-regulated as a result of autoantibody receptor inhibition [91]. The up-regulation seems to include the expression of the muscarinic receptor subtypes M4 and, in particular, M5 [85]. Up-regulated M5 receptor expression has been observed in other states of inflammation and therein play an increased role in functional responses [92,93].

1.6 ANTIDEPRESSANTS AND HYPOSECRETION

Antidepressant drugs have been marketed for nearly 70 years, with many iterations of improvement along the way. It started with the introduction of the monoamine oxidase inhibitors (MAOIs) and the tricyclic antidepressants (TCAs) following close behind, which revolutionized the treatment of depression but held some issues regarding patient compliance due to a vast record of adverse effects. Then, thirty years later, in the late 1980s, came the modern selective serotonin reuptake inhibitors (SSRIs), which led to a significant improvement in mental health in the 1990s, mostly due to their mitigated adverse effect profile [94]. In general, antidepressants are categorized into two subcategories; the older antidepressants, containing the MAOIs and TCAs, with anecdotal reports of clinicians verifying achieved therapeutic effects when patients reported subjective symptoms of mouth dryness, and the newer generation consisting of the presently most commonly subscribed SSRIs and the later approved serotonin noradrenaline reuptake inhibitors (SNRIs). In some markets, such as the USA, noradrenaline reuptake inhibitors (NRIs) have also been approved for treatment of depression. Antidepressants have, alongside many other pharmacological therapies, been shown to induce xerostomia and xerophthalmia. Interestingly, xerophthalmia has not been reported in the clinical trials of recently tested SSRIs but post market approval epidemiological studies of adverse effects from these compounds have shown that dry eye is reported to a rather significant extent [3,95]. The reason for this can be due to that dry eyes was excluded from the adverse event questionnaire that was handed to the patients in the clinical trial. Surprisingly, in studies where blurred vision was included, it was reported among 11% of the patients receiving SSRI treatment [96]. Blurred vision is closely related to dry eye and it would be plausible to assume that the blurry vision experienced by the patients in the clinical trials was caused by antidepressant-induced hyposecretion from glands contributing to the components of the tear film. This in turn led to

destabilization of the tear film which led to what the patient experienced as optical aberrations or blurred vision. Today, antidepressants are one of the most commonly prescribed drugs to cause hyposecretion in lacrimal and salivary glands. Amongst many other improvements in the adverse effect profile, the new generation of antidepressants, i.e. SSRIs, a significant reduction in reports of xerostomia is reported [96]. Following the further developments of treatment with the new generation of antidepressants, an effect on noradrenergic uptake has been documented to be of significance in some patient groups [97]. Therefore, today the SNRI treatment complements or substitutes the treatment with SSRIs in patients where the antidepressant effect of said SSRIs is deemed unsatisfactory.

2 METHODOLOGY

In all four papers of this PhD-thesis adult male rats of the Sprague–Dawley strain were used which was approved by the local ethics committees at the University of Gothenburg and the University of Lund/Malmö (ethics number: 196/13 and 1794/18). The study design and experimentation followed local rules and regulations at the University of Gothenburg, the University of Malmö as well as the ARVO guidelines for Use of Animals in Ophthalmic and Vision Research.

2.1 IN VIVO INVESTIGATIONS INTO ANTIDEPRESSANT EFFECTS ON SALIVARY AND LACRIMAL SECRETION

Rats anaesthetized with pentobarbitone were prepared for acute experiments by surgical insertion of catheters into the femoral vein, enabling intravenous substance administration, and the femoral artery facilitating blood pressure surveillance (Figure 3). Also, trachea cannulation was performed to prevent stimulatory salivary-induced lung obstruction. The experiments that followed the preparations were designed to investigate antidepressant peripheral and central effects on salivary- and lacrimal secretion, respectively. Thereby, secretion was stimulated by two different strategies, one for peripheral stimuli utilizing the quaternary ammonium muscarinic agonist methacholine, which does not permeate the blood brain barrier, and one by reflex arch evoked centrally mediated stimuli using tongue administered citric acid or eye drop administered menthol (Figure 4). In investigations on the antidepressant effects on salivary secretion the parotid duct was cannulated which enabled collection of saliva. The modulatory effects of administered antidepressant were then investigated through collection of saliva and subsequent measurement of weight and analysis of protein content of said saliva or by filter paper absorption of tears followed by weight measurement of the filter papers. To investigate possible noradrenergic effects in antidepressants, the peripheral cholinergically induced response in the presence of antidepressant was subsequently repeated in the presence of an adrenergic antagonist cocktail.

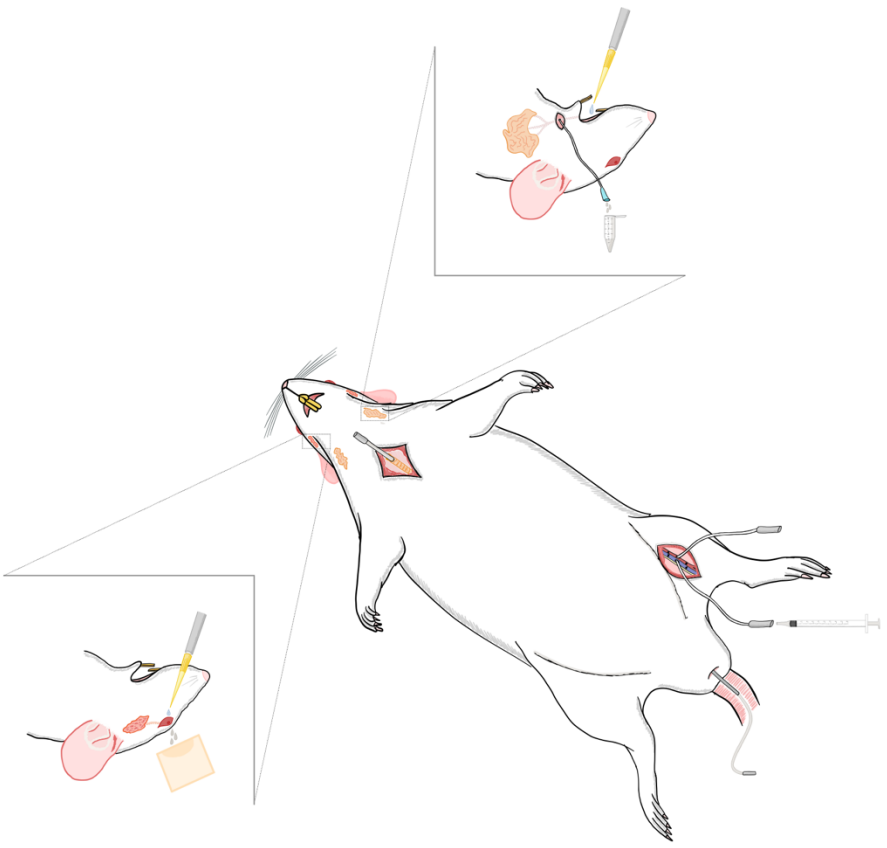


Figure 3. Schematic drawing of the in vivo experimental setup in anesthetized rats. Cannulated trachea for sustained airflow is depicted in the neck region, just above the chest. The femoral artery and the femoral vein were cannulated for monitoring blood pressure and to enable i.v. drug administration, respectively. The blood vessel cannulations are depicted in the left hind leg. Body temperature was monitored by an anal thermostat and maintained by a thermoregulated heating pad upon which the experiments were performed. In the tear secretion protocols, centrally mediated lacrimal secretion was induced by administering menthol to the surface of the eye with a pipette, shown in the lower left corner of the picture. Secreted tears were collected and weighed using filter paper. In the salivary secretion protocols, centrally mediated salivation was induced by administering citric acid to the tongue by using a pipette, shown in the upper right corner of the drawing. Saliva was collected from the cannulated parotid duct and subsequently weighed and stored for analysis of protein content. Illustration by Maria del Pilar Murillo Angarita.

2.2 ISOLATION OF PRIMARY CULTURED CELLS

Excised rat lacrimal glands were minced and digested in RPMI 1640 medium containing collagenase type I. The cells were subsequently centrifuged, the pellet was resuspended and thereafter seeded in culture dishes where they were isolated in supplemented RPMI 1640 medium for 4 weeks, eliminating all the other cell types. Medium was renewed every second day and the isolation procedure was performed in accordance with previously published isolation of myoepithelial cells from rat lacrimal gland [76]. The used medium has been shown to be crucial for myoepithelial cell differentiation [36]. Over the four weeks of isolation, the culture developed in manners similar to that documented in the literature. During the first week the culture proliferated and when confluency was reached nodules of a certain cell type emerged, which was presumed to be myoepithelial cells, and from these nodules these cells proliferated to eventually cover the entire culture dish during the following three weeks. When the proliferating cell type started reaching full confluency the culture started displaying apparent myoepithelial cell tendencies, with ruffled edges, with each cell covering a large surface area, and exhibiting parallel actin filaments. Co-cultures were harvested from primary cultured cells two weeks post primary cultivation. The timing for the harvest was chosen based on weekly morphological investigations in which the cultures were stained for markers relevant to proliferation (Ki-67), progenitor transcription (SOX2), acinar (VAMP-8) and myoepithelial expression (α-SMA) and muscarinic receptor expression (M1-M5). At the second week, the cultures displayed co-culture features with equal proportions of acinar and myoepithelial cells. The co-culture was sustained until the third week, thereafter the acinar cells gradually diminished.

2.3 IMMUNOCHEMISTRY

Expression of proteins was investigated utilizing immunological tools, i.e. antibodies which were manufactured with recombinant technologies, injecting host animals with antigen target protein emulating epitope peptides which in turn causes the host's immune system to produce antibodies aimed at said epitope. A common approach when using this technology is to first expose a sample assumed to contain the target protein to antibody containing solution and let a primary antibody interact with the target protein. The primary antibody is designed to have high, almost irreversible, affinity to the target protein due to the manufacturing procedure described above. After the sample has been exposed to the primary antibody, a secondary antibody is introduced.

This secondary antibody has been manufactured to detect the tail of the primary antibody, which is unique for each animal host, and the secondary antibody is furthermore conjugated to a marker. There are various types of markers but in fluorescent immunochemistry, fluorophores are used, which can fluoresce at different wavelengths and thereby can be used to show different types of proteins and biomolecules in a sample. This technology is used for many different analyses and herein they have been used to display protein expression in cells using fluorescent microscopy immunocytochemistry, together with immunohistochemistry. Furthermore, the technology was used in protein expression analysis, also known as western blot.

2.3.1 IMMUNOCYTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

Primary cultured cells were harvested and relocated to microscopy eligible dishes by using trypsin, a proteolytic enzyme which cleaves the protein structures through which the cells adhere to the surface of the plate, after which the cells were allowed to settle in their new environment for one to two days under observation. The cells were then fixated with paraformaldehyde, which crosslinks primary amino groups in proteins, after which the immunochemical reactants were introduced. In order to penetrate the cellular membranes of the fixated cells a detergent, Triton x-100, was introduced together with the primary antibodies. Normal serum from the host species from which the secondary antibodies were produced was also added to block non-specific interactions with proteins in the cells. The primary antibodies were added in a cocktail containing two primary antibodies, produced in different species, to avoid possible interactions in subsequent experimental procedures. Following the incubation with primary antibodies, the cells were exposed to a similar cocktail of secondary antibodies with conjugated fluorophores which fluoresce at different wavelengths, visualized as green (Alexa 488) and red (Texas Red) by fluorescent microscopy. After the incubation with antibody solutions the cells were successively incubated with solution containing nuclear reagent 4′,6-diamidino-2-phenylindole (DAPI), which binds to the adenine and thymine in DNA and fluoresces at a wavelength corresponding to blue following its binding to the nucleic acids. It thereby operates as a counter stain for nuclear staining.

The immunocytochemical procedures were very similar to the immunohistochemical, wherein 6 μm thick paraffinized histological slices of rat lacrimal glandular paraformaldehyde fixated tissue were used. The slices were prepared for immunochemical incubation by first being deparaffinized with xylene and then rehydrated in solutions of ethanol and subsequent increasing concentrations of water. The tissue slices were successively boiled

in citrate solution to retrieve protein structures, thereby providing protein access for the following antibody reagents. Prior to incubation with antibodies, the slices were exposed to ammonium copper sulphate which was used to reduce autofluorescence in the tissue and then incubated with normal serum from the species which the secondary antibody had been produced in. After this pre-incubation with normal serum the procedure was identical to that performed in the immunocytochemical procedures. Stained cells and slices were examined under a Nikon 90i brightfield and fluorescence microscope and micrographs were recorded utilizing a DS-Fi camera and analyzed with NIS Element Imaging Software v.4.40 (Nikon Corporation, Tokyo, Japan).

2.3.2 WESTERN BLOT

For protein expression analysis, a western blot protocol was used. Herein isolated rat lacrimal gland monocultures containing myoepithelial cells and co-cultures containing myoepithelial cells and acinar cells were lysated with buffer containing detergents, phosphatase and protease inhibitor cocktails in order to inhibit hydrolytic cleavage of proteins. The lysate was centrifuged and the protein containing supernatant was stored at -80°C. The protein samples were quantified for protein content using Lowry protein analysis and denaturated to allow for size-charge gel electrophoretic separation. The samples were loaded in wells on a sodium dodecyl sulphate–polyacrylamide gel and the proteins were separated by molecular size by application of electric voltage. Following successful protein separation, the proteins were blotted over to nitrocellulose membranes which were exposed to immunochemical reagents where antibodies were introduced to the membrane one by one and documented separately. Separate measurements were performed because the coloring component for the antibodies did not enable analysis on different signal channels.

2.4 INTRACELLULAR CALCIUM MEASUREMENTS

In functional investigations of the effects of muscarinic and purinergic stimuli in cells from primary cultured rat lacrimal gland, a calcium fluorescent reagent, FLIPR Calcium 6, was used. The reagent is absorbed by the cells and subsequently cleaved into its active form which fluoresces at a certain band of wavelengths when it is bound to Ca^{2+}. The intensity of the fluorescent signal can then be measured and the changes in signal intensity signifies changes in intracellular calcium concentration. One day before measurement, primary

cells were trypsinized from culturing dishes to microscopy dishes with suitable optical properties. The recordings were made using the plate reader SpectraMax i3x or a Nikon 90i brightfield and fluorescence microscope.

Pharmacological effects were measured in monocultures of myoepithelial cells (paper III). The cells were pre-incubated with calcium reagent containing antagonists or corresponding volume of vehicle solution. To investigate the muscarinic effects, the unselective agonist methacholine was administered to cells which were pre-incubated with either the M1/M3/M5-selective antagonist 4-DAMP, "M3-selective" antagonist pFHHSiD or M2/M4-selective antagonist methoctramine. To investigate purinergic effects, the unselective agonist ATP was administered to cells which were pre-incubated with either the P2-selective antagonists suramin or PPADS, or the L-type calcium channel blocker verapamil. Prior to administration of agonist, the baseline calcium levels were recorded. The subsequent response, which was recorded following agonist administration, was analyzed as maximum response divided by average of baseline. Agonist dose-response experiments were performed with injection of agonist solution or corresponding solution of vehicle for control measurements. Monoculture functional responses were recorded with the plate reader SpectraMax i3x.

The investigations of muscarinic modulation of calcium responses in rat lacrimal gland co-culture were performed in similar fashion as in the recordings in monocultures. In order to investigate responses from the different cell types that constituted the co-culture, the co-cultures were measured using a Nikon 90i brightfield and fluorescence microscope. To study the hypothesized purinergically induced release of acetylcholine, the cells were pre-incubated with the muscarinic antagonist atropine and recorded during administration of ATP. Similarly, the hypothesized muscarinically induced release of ATP was investigated in cells which were pre-incubated with the purinergic P2 antagonist suramin and subsequently recorded during administration of methacholine.

2.5 STATISTICAL ANALYSES

Statistical significance was determined by one-way or two-way analysis of variance (ANOVA) followed by either Bonferroni's, Dunett's, or Šídák's test for multiple comparisons. P-values <0.05 were regarded as statistically significant. Functional data are presented as mean ± S.E.M. Graphs were generated and statistical analyses were computed in the GraphPad Prism 9 software (GraphPad Software Inc, San Diego, USA).

In paper I, the difference between antidepressant treatments and the control group was analyzed by two-way ANOVA and the comparison between baseline secretion, antidepressant ameliorated secretion and adrenoceptor antagonist normalized secretion was analyzed by one-way ANOVA.

In paper I, both types of analyses were followed by Bonferroni's test for multiple comparisons. In paper II, the difference between antidepressant treatments and the control group was analyzed by two-way ANOVA followed by Dunnett's test for multiple comparisons.

In paper III, the differences between agonist induced responses and control responses, as well as the differences between antagonist inhibited response and agonist induced response, were analyzed by two-way ANOVA, followed by Šídák's test for multiple comparisons.

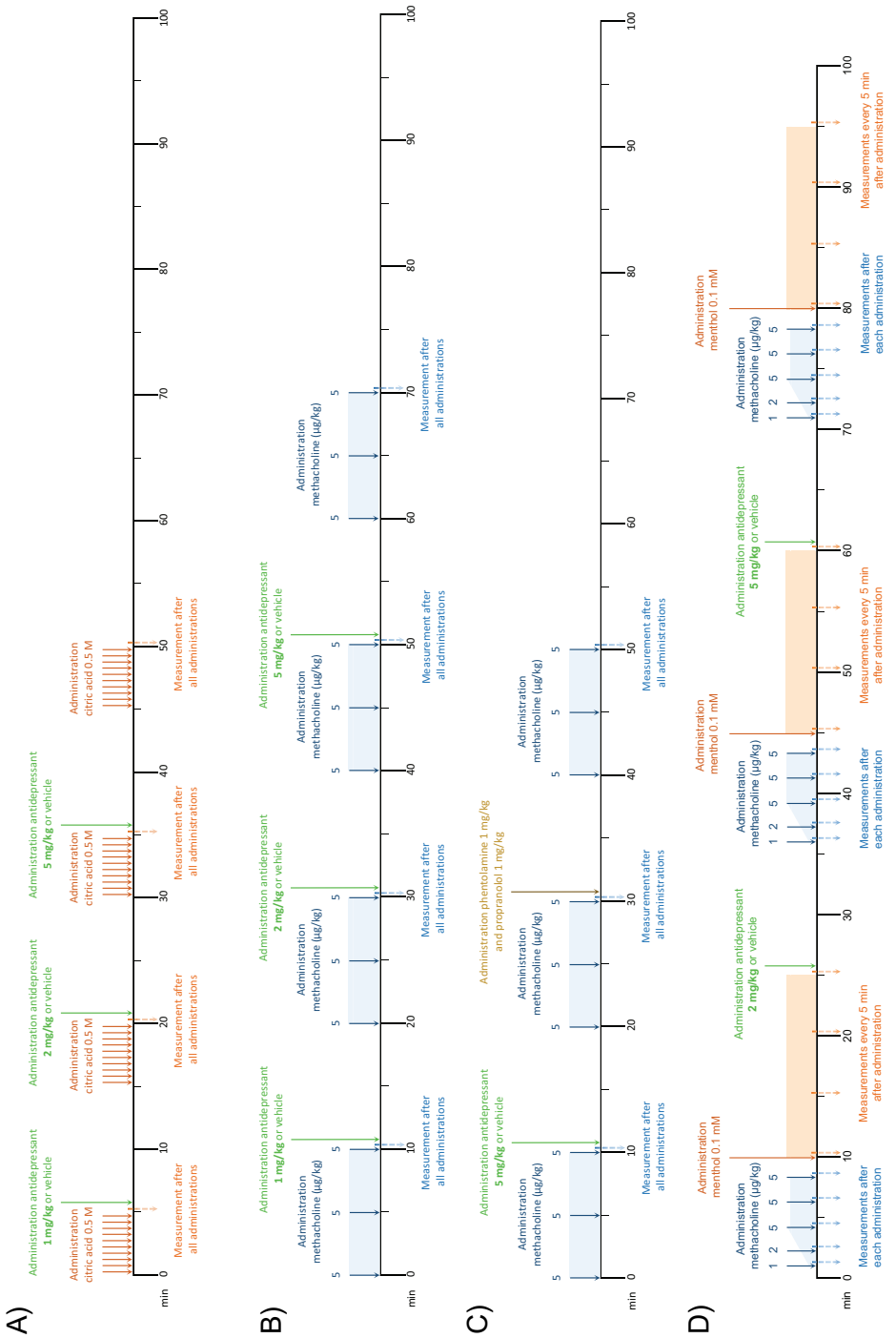
Statistical analysis is yet to be performed on the data in paper IV.

3 RESULTS

This thesis constitutes four papers and in general the project can be divided into two subprojects where the first pursuits an understanding of how antidepressants affect secretion from salivary and lacrimal glands. In the second project we focused on the expression and function of a possibly heterogenous muscarinic receptor population in the lacrimal gland.

3.1 THE EFFECTS OF ANTIDEPRESSANTS IN LACRIMAL AND SALIVARY SECRETION

In vivo experiments were performed in anaesthetized rats to investigate the effects of antidepressants on peripheral stimuli, induced by the quaternary ammonium compound methacholine, and centrally mediated stimuli evoked by administering menthol to the ocular surface or citric acid to the tongue. The main reason for these different strategies of secretory stimulation was to enable investigations of the claimed anticholinergic effects that antidepressants are attributed in the literature, an epithet sometimes interpreted as being of a pharmacodynamic nature, meaning that the substances act directly as antagonists on cholinergic receptors. The results of our studies in paper I and paper II showed that this was not the case.


A)
Administration antidepressant 1 mg/kg or vehicle
Administration citric acid 0.5 M
Measurement after all administrations
Administration antidepressant 2 mg/kg or vehicle
Administration citric acid 0.5 M
Measurement after all administrations
Administration antidepressant 5 mg/kg or vehicle
Administration citric acid 0.5 M
Measurement after all administrations
min
0
10
20
30
40
50
60
70
80
90
100
B)
Administration antidepressant 1 mg/kg or vehicle
Administration methacholine (µg/kg)
5
Measurement after all administrations
Administration antidepressant 2 mg/kg or vehicle
Administration methacholine (µg/kg)
5
Measurement after all administrations
Administration antidepressant 5 mg/kg or vehicle
Administration methacholine (µg/kg)
5
Measurement after all administrations
C)
Administration antidepressant 5 mg/kg or vehicle
Administration methacholine (µg/kg)
5
Measurement after all administrations
Administration phentolamine 1 mg/kg and propranolol 1 mg/kg
Administration methacholine (µg/kg)
5
Measurement after all administrations
Administration methacholine (µg/kg)
5
Measurement after all administrations
D)
Administration menthol 0.1 mM
Administration methacholine (µg/kg)
1 2 5 5 5
Measurements after each administration
Measurements every 5 min after administration
Administration antidepressant 2 mg/kg or vehicle
Administration menthol 0.1 mM
Administration methacholine (µg/kg)
1 2 5 5 5
Measurements after each administration
Measurements every 5 min after administration
Administration antidepressant 5 mg/kg or vehicle
Administration menthol 0.1 mM
Administration methacholine (µg/kg)
1 2 5 5 5
Measurements after each administration
Measurements every 5 min after administration

← *Figure 4. Flowchart representation of the protocol setup in investigations of antidepressant effects in lacrimal and salivary secretion. Three different series of experiments were performed in investigations of salivary secretion (A-C; Paper I). (A) Local citric acid stimulation was repeated every 30 s during 5 min periods in the absence and presence of increasing doses of antidepressant (clomipramine, citalopram or venlafaxine; 1-5 mg/kg i.v.) or vehicle (saline; i.v.). (B) Methacholine responses were repeatedly measured in the absence and presence of increasing doses of antidepressant (clomipramine, citalopram or venlafaxine; 1-5 mg/kg i.v.) or vehicle (saline; i.v.). (C) Methacholine responses were repeatedly measured in the absence and presence of antidepressant (citalopram or venlafaxine; 5 mg/kg i.v.) and, subsequently, in the presence of antidepressant and adrenoceptor antagonists (phentolamine and propranolol; 1 mg/kg i.v.). D) Tear secretion protocol (Paper II). Methacholine responses and responses to local application of menthol were repeatedly measured in the absence and presence of increasing doses of antidepressants (clomipramine or citalopram; 2-5 mg/kg i.v.) or vehicle (saline; i.v.). Illustration by Maria del Pilar Murillo Angarita modified from figures in Paper I and Paper II.*

3.1.1 CENTRALLY MEDIATED RESPONSES

In investigations of antidepressant modulation of secretion stemming from reflex evoked stimuli, the centrally mediated response was dose dependently attenuated on all accounts (Figure 5). In salivary glands, the tricyclic antidepressant clomipramine, the selective serotonin reuptake inhibitor citalopram and the serotonin noradrenaline reuptake inhibitor venlafaxine all caused a reduction of salivary secretion from the rat parotid gland in a very similar fashion. Following reflex evoked stimulation of secretion in the lacrimal gland, the tear volume was correspondingly lowered following administration of citalopram and clomipramine.

3.1.2 PERIPHERALLY MEDIATED RESPONSES

The peripheral stimulation of lacrimal and salivary secretion, caused by i.v. injection of methacholine, resulted in somewhat obscure responses in the presence of the antidepressants that were investigated. Peripherally stimulated salivation was attenuated following administration of clomipramine but rather intriguingly the salivary response was potentiated following administration of citalopram or venlafaxine (Figure 5). The induction was dose dependent with similar secretory effects from both substances. The secretory responses that resulted from glandular stimuli of tears showed analogous effects following administration of the tricyclic antidepressant clomipramine, but the tear volume was unaffected by citalopram as compared to control animals pretreated with saline.

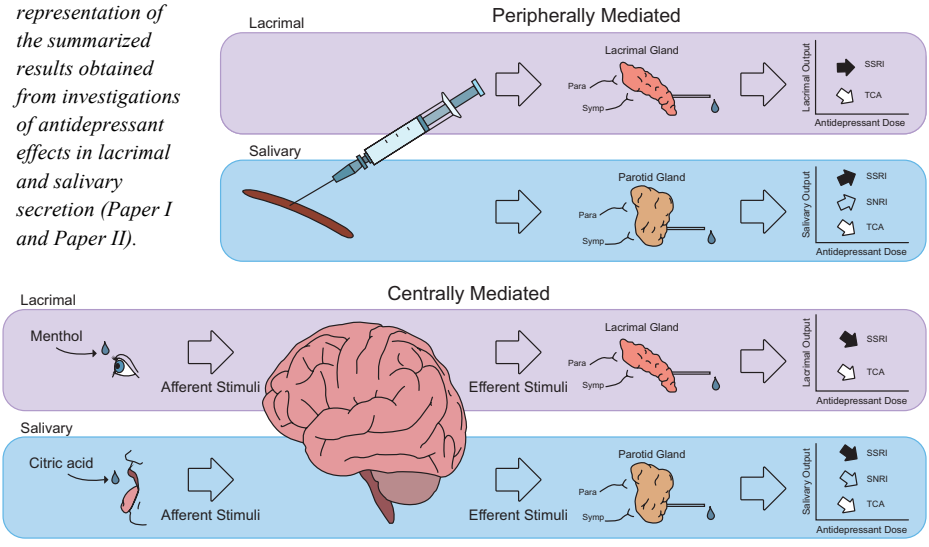


Figure 5. Schematic representation of the summarized results obtained from investigations of antidepressant effects in lacrimal and salivary secretion (Paper I and Paper II).

The ambiguous potentiation of salivary secretion following venlafaxine and citalopram administration was further investigated in an independent experimental setup where glandular methacholine-induced salivation was quantified in presence of the highest dose of antidepressant (5 mg/kg), the adrenergic α-adrenoreceptor antagonist phentolamine and β-adrenoreceptor antagonist propranolol. The combination of antidepressant and adrenoreceptor antagonists showed a normalized methacholine generated response compared to the response generated solely by methacholine. The collected saliva was consecutively analyzed for protein concentration and the protein output was more than four times larger in methacholine stimulated saliva after administration of citalopram compared to the output from methacholine before administration of citalopram. In the venlafaxine group the protein output was more than ten times higher compared to the protein output from methacholine before administration of the antidepressant. In presence of the adrenergic antagonists the protein outputs were attenuated but still seemed to be enlarged in comparison to the quantified protein output obtained before administration of antidepressant and adrenoreceptor antagonists.

3.2 CHARACTERIZATION OF MUSCARINIC RECEPTORS IN RAT LACRIMAL GLAND

The studies into lacrimal secretion first started with attempts to characterize muscarinic expressional pattern in lacrimal gland tissue. The analysis resulted in strong indications of a heterogenous muscarinic receptor population (Figure 6). While M1 stains were either weak or non-existent, the M2 stains marked what was interpreted as terminating neurons. As expected, M3 staining was strong in acinar cells but did not show any presence of M3 receptors in myoepithelial or neuronal cells. Intriguingly, the M4 and M5 muscarinic receptor expression was specific to myoepithelial cells.

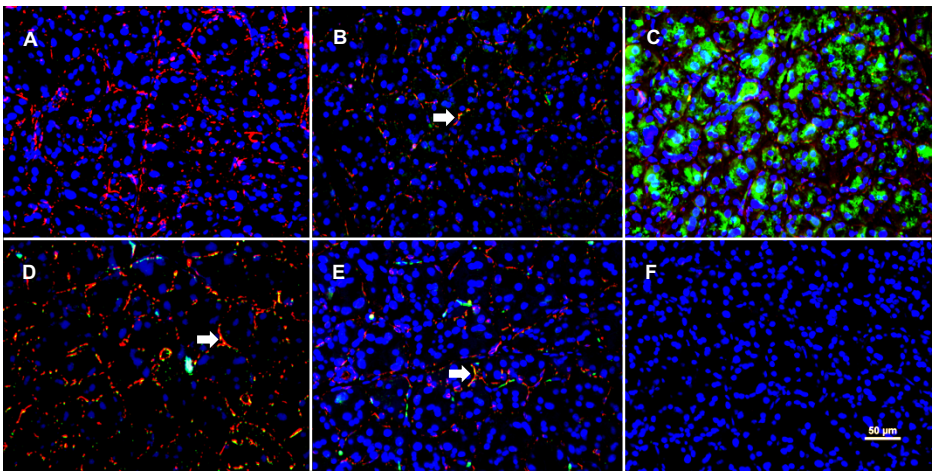


Figure 6. Micrographs from fluorescent immunohistochemical investigations of rat lacrimal gland muscarinic receptor expression (green; M1-M5), additionally stained for expression of α-SMA (red) and nuclear stain with DAPI (blue). (A) Absence of M1 expression. (B) Moderate M2 expression, only visible in myoepithelial cells (white arrow). (C) Abundant M3 expression, mainly in acinar cells. (D) M4 expression, only visible in myoepithelial cells, indicated by orange stain due to green and red overlap (white arrow). (E) M5 expression, moderately visible in myoepithelial cells, indicated by both green and orange stain (white arrow). (F) Corresponding negative control. Scale bar applies to all panels.

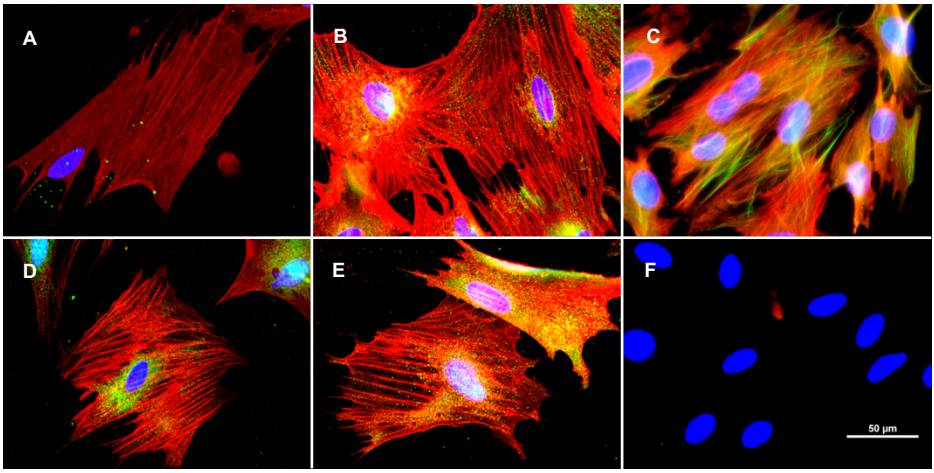


Figure 7. Micrographs from fluorescent immunocytochemical investigations of myoepithelial cell monocultures isolated from rat lacrimal gland. Muscarinic receptor expression (green; M1-M5) additionally stained for expression of α-SMA (red) and nuclear stain with DAPI (blue). (A) Absence of M1 expression. (B) Moderate nuclear adjacent M2 expression. (C) Abundant M3 expression with increased stain surrounding the nuclei as well as microfilament adjacency. (D) M4 expression, also displaying nuclear adjacency and moderate microfilament proximity. (E) M5 expression, nuclear proximal stain. (F) Corresponding negative control. Scale bar applies to all panels.

3.2.1 MUSCARINIC RECEPTORS IN MONOCULTURES OF MYOEPITHELIAL CELLS

Following the immunohistochemical results, efforts were made to characterize the myoepithelial cells functionally and morphologically by investigating isolated myoepithelial cells from primary cultured rat lacrimal glands (Paper III). The isolation procedure was performed in analogy with previously published research where myoepithelial cells in monoculture were characterized for purinergic function [76]. Comparably, the cells were isolated over four weeks post cultivation and were investigated immunocytochemically for markers indicating acinar, progenitor and myoepithelial cells. Following monoculture verification in the fifth week, muscarinic receptor function and expression were investigated (Figure 7). The immunocytochemical staining for muscarinic receptors showed expression of M2 receptors in close proximity to the nuclei. The M3, M4 and M5 were more abundantly expressed throughout the cell with M3 and M4 indicating filament patterns and with M5 having a stronger tendency of being positioned in close proximity to the nuclei. The M1 expression was absent. The cells were counterstained with α-SMA and cytokeratin 17 to verify myoepithelial cell expression.

Functional studies further validated the successful establishment of a myoepithelial monoculture by measuring intracellular calcium responses. Administration of increasing concentrations of the purinergic agonist ATP showed dose-dependent intracellular responses similar to what had been obtained by Ohtomo et al.. Further, purinergic dose-response causality was shown with inhibition studies in which ATP was administered to cultures which had been preincubated with increasing concentrations of the purinergic antagonists pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) (PPADS) or suramin (Figure 8). Curiously, investigations into muscarinic function exhibited an absence of intracellular calcium responses following administration of the unselective muscarinic agonist methacholine in increasing concentrations. To exclude the possibility of the absence in response being the net result of a composite inhibitory (M2/M4) and excitatory (M1/M3/M5) muscarinic receptor activity, the intracellular calcium response to methacholine was further investigated in monocultures which had been

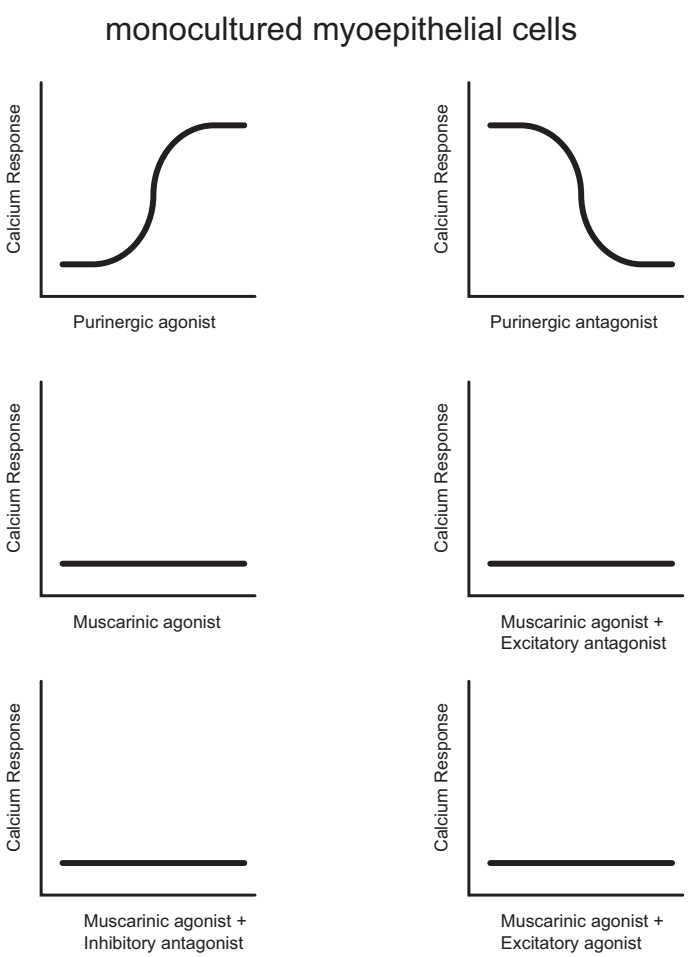


Figure 8. Schematic summarizing representation of functional dose-response xy-scatter plots obtained from measurements of intracellular calcium in myoepithelial monocultures (Paper III).

preincubated with increasing concentrations of muscarinic antagonists. Neither 4-diphenylacetoxy-N-methylpiperidine (4-DAMP; muscarinic M1/M3/M5-selective antagonist), methoctramine (muscarinic M2/M4-selective antagonist) or 4-fluoro-hexahydro-sila-diphenidol (pFHHSiD; muscarinic "M3-selective" antagonist) modulated the intracellular response (Figure 8).

3.2.2 DEVELOPMENT OF LACRIMAL GLAND CO-CULTURE

The study of the myoepithelial cell monoculture led us to investigate the effects of the isolation procedure by morphologically characterizing the muscarinic expression in the co-cultures that were developed during the isolation procedure. Given the anatomical expression of acinar cells in lacrimal gland tissue we stipulated that there should be an intermediate state where the culture consists of a composite of acinar and myoepithelial cells. In this state we sought to investigate possible intercellular interactions between the acinar and myoepithelial cells, hypothesizing that the cholinergically mediated myoepithelial calcium response, which had been documented from intact acini, might be mediated via acinar excitation, subsequently causing release of transmitter which in turn mediated an increase of intracellular calcium in the adjacent myoepithelial cells.

Development of co-cultures was investigated on a weekly basis, post cultivation, for four weeks. The culture was stained immunochemically with acinar selective antibodies with high affinity for vesicle associated membrane protein 8 (VAMP-8) in conjunction with myoepithelial selective antibodies aimed at α-SMA. The specificity of the stains was verified in lacrimal gland tissue and showed an equal expression of the two different cell types at the second week post cultivation, while acinar cell expression was absent at the fourth week post cultivation. Thereafter, functional studies and immunocytochemical studies were set up to investigate muscarinic receptor expression and function in acinar and myoepithelial co-cultures two weeks post cultivation. The co-culture displayed heterogenous muscarinic receptor expression with indications of all five muscarinic receptor subtypes. The M1 stain seemed to be more selective to cells which did not get counterstained by the myoepithelial marker α-SMA, indicating M1 expression in acinar cells. M2 had similar tendencies as M1 and thereby also seemed to be expressed in acinar cells. The M3 receptor was abundantly stained in both cell types and even though M4 was similarly expressed in both cell types, the intensity was not equally distributed throughout the co-culture. Staining for M5 receptors showed an abundant presence in all cells, with some cells only expressing intense stains in close vicinity to the nuclei.

3.2.3 FUNCTIONAL INVESTIGATIONS OF LACRIMAL GLAND CO-CULTURE

In order to investigate the cell specific intracellular calcium responses in co-cultured conditions, we sought to record these during live microscopy, enabling us to make distinctions between signals expressed in different cells. Following methacholine administration, a delayed response was seen in cells that showed myoepithelial morphology. In detail, first an immediate but yet faint response was recorded in all cells following methacholine administration. This signal subsequently dissipated during the one-minute recording. Between 10-20 s post administration, an intense response was recorded in cells which displayed myoepithelial features, i.e., ruffled edges, covering a large surface area, and exhibiting parallel actin filaments. Furthermore, this response was not observed in co-cultures which were preincubated with the purinergic antagonist suramin.

In the pursuit of investigating the purinergic function, and possible cholinergic interactions, in co-cultured cells the unselective purinergic agonist ATP was administered to the co-culture in conjunction with the muscarinic readings. An intense intracellular calcium response was recorded following ATP administration with no recorded differences following administration of ATP to co-cultures which had been preincubated with the muscarinic antagonist atropine.

4 DISCUSSION

4.1 ANTIDEPRESSANTS AND HYPOSECRETION IN LACRIMAL AND SALIVARY GLANDS

The anticholinergic effects that have been attributed to antidepressants have possibly led to great clinical and economical costs. The term is derived from evident muscarinic antagonist properties in the older generation of antidepressants, i.e. the TCAs. Mitigating muscarinic receptor affinity was one of the integral components in the development of the new generation of antidepressants. As a result, the adverse effects attributed to anticholinergic properties were observed with significantly reduced occurrence among patients treated for depression [98]. Nevertheless, even though the remaining side effects showed no indication of being caused by pharmacodynamic interactions with muscarinic receptors, the anticholinergic epithet remained. Remarkably, a recent literature review of the clinical efficacy of muscarinic agonist treatment of antidepressant induced dry mouth and dry eyes concluded that there were no relevant evidence-based guidelines or studies published on the matter [99]. In an effort to investigate aspects of available treatments and to build a platform for future developments in the treatment of dry mouth and, especially, dry eyes for patients undergoing psychopharmacological treatment, we sought to characterize the underlying mechanisms of the hyposecretory effects found during pharmacological treatment of depression.

In the preclinical studies of the xerogenic and xerophthalmic effects of various types of antidepressants, presented herein in paper I and paper II, we showed that the hyposecretory response which was generated from the treatment was mainly mediated via inhibition of the reflex arc. In peripherally mediated stimulation of glandular secretion, only the tricyclic antidepressant clomipramine resulted in a significantly attenuated response. This secretory attenuation is most likely attributed to the binding profile of the tricyclic substance, being the only one out of the three investigated antidepressants rendering high affinity for muscarinic receptors. Intriguingly, in parotid gland, both the SSRI citalopram and the SNRI venlafaxine caused an augmented salivary response, thus displaying cholinergic potentiation rather than anticholinergic tendencies. The potentiation was normalized following administration of adrenoreceptor antagonists, indicating a noradrenergic component in both compounds.

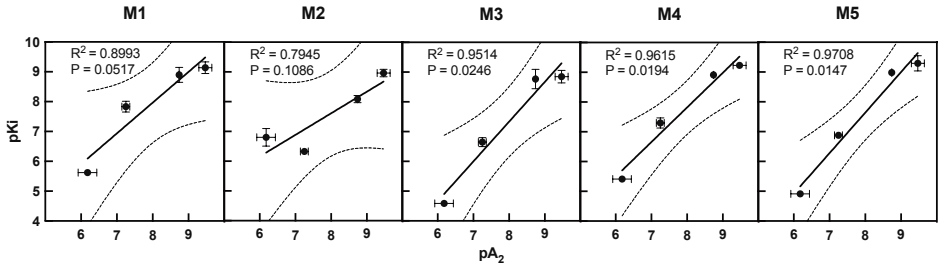
Given the noradrenergic component, which was exhibited following the peripherally induced stimulation in the presence of the modern antidepressant compounds, it was hypothesized that such a component could have a contrasting inhibitory effect in lacrimal secretion. This was based on the physiological difference between the parotid and lacrimal gland. The reason why we chose the parotid gland for investigation of the effects of antidepressant modulatory effects on salivary response was mainly due to the ease of quantifying the saliva secreted from this gland. In comparison to the other major salivary glands, the parotid gland only produces saliva following stimulation. In contrast, the sublingual gland exhibits spontaneous salivation which would have to be quantified continuously in order to compare the sialometric changes in the salivary flow[100]. The non-spontaneous properties of the parotid gland are relevant in noradrenergic modulation when considering the significance of the blood supply on the proteinaceous secretion from the parotid gland, as compared to the other salivary glands and in regard to the lacrimal gland. Similar to the sublingual gland the lacrimal gland exhibits spontaneous secretion [101]. In order to sustain a spontaneous flow of tears, the acinar cells must be replenished with new aqueous liquid, which is provided via the arteries [30]. Thus, the arterial blood flow should be a crucial component in lacrimal secretion and is limited by arterial vasoconstriction induced by noradrenergic stimulation. Contrarily, the parotid gland is not as dependent on the blood supply as it does not secrete spontaneously and that could represent a significant component in the measured increase in peripherally induced salivation following administration of citalopram and venlafaxine. But nevertheless, the quantification of tears showed no change compared to the control group, thus falsifying the hypothesis of noradrenergic lacrimal inhibition being exhibited in peripheral stimulation following citalopram administration. The absence of modulation could possibly be a composite of the amelioration seen in the parotid salivation and an inhibited blood supply. More importantly though, the peripheral stimulation following citalopram administration exhibited no inhibitory effects on lacrimal secretion. Thus, the compound manifested no anticholinergic effects. Leaving the question why the response was potentiated in the parotid gland. Adrenergic stimuli have been shown to mediate and potentiate secretion from acinar cells and it is thereby possible that the noradrenergic component exhibited in the presence of citalopram and venlafaxine potentiated the methacholine induced peripheral response. This was further supported by the measured drastic increase in the protein output, as β-adrenergic acinar stimulation has been shown to facilitate protein exocytosis in the parotid gland [102]. Even though reduced, the protein output was not normalized in the presence of adrenoreceptor antagonists, leaving the question of the exact mechanism of antidepressant-augmented cholinergic induced production of saliva unanswered.

4.2 CHARACTERIZATION OF MUSCARINIC RECEPTORS IN THE RAT LACRIMAL GLAND

There is a strong consensus in the literature regarding the muscarinic receptor population in the rat lacrimal gland being homogenously expressed and functionally dominated by the muscarinic receptor subtype M3. If an M3 receptor population were to dominate in the lacrimal gland then the pursuit of developing a symptomatic pharmacological treatment of DED should be quite effortless, as it thereby should be possible to stimulate lacrimal secretion with any unselective muscarinic agonist. Unfortunately, this has not been the case. Marketed systemically administered treatments for dry mouth consisting of muscarinic agonists such as cevimeline or pilocarpine show little effect on lacrimal secretion and alleviation of dry eye [16].

Herein, the rat lacrimal gland muscarinic receptor expression was characterized on three different levels, in tissue (paper III and IV), in primary cultured isolated myoepithelial cells (paper III) and in primary co-culture containing myoepithelial cells and acinar cells (paper IV). Morphologically, a heterogenous muscarinic receptor population was observed on all occasions with a consistent expression of M3, M4 and M5 receptors. In concordance with the literature, lacrimal gland tissue displayed an intense expression of M3 receptors in acinar cells. However, M4 and M5 receptor staining was observed to overlap with a counterstain for alpha smooth muscle actin in myoepithelial cells. In contrast to this, binding studies have often been concluded to indicate a homogenous muscarinic receptor population in lacrimal glands [103,104]. However, comparisons of pA_2 values attained in functional studies [64] correlate with binding data in cloned M3, M4 and M5 receptor populations (M3, R^2=0.9514, p=0.0246; M4, R^2=0.9615, p=0.0194; M5, R^2=0.9708, p=0.0147), obtained from competitive binding experiments (Figure 9). This correlation analysis shows that the data, which the assumed M3 receptor homogeneity was based upon, actually supports the findings presented herein, i.e. that the lacrimal gland expresses a heterogenous receptor population consisting of M3, M4 and M5. Furthermore, analysis of muscarinic receptor expression in which lacrimal gland tissue homogenate was separated by polyacrylamide gel electrophoresis, resulted in three different peaks, which correlate with the expression of at least two different subtypes of the muscarinic receptor family[105]. None of these findings exclude the possibility of M3 receptors being the functionally most important subtype, but they clearly show that also M4 and M5 receptors need to be incorporated as a possible part of the cholinergic function in parasympathetic regulation of the lacrimal gland. As for the M1

receptor, morphological studies showed its expression in the acinar and myoepithelial cell co-culture with selective staining in acinar cells. The M2 stain showed similar tendencies. Thereby, the expression of M1 and M2 in the rat lacrimal gland cannot be excluded. In fact, northern blot analysis in porcine tissue has shown lacrimal gland expression of both M1 and M3 receptors [106], leaving only the absence of findings supporting M2 lacrimal gland expression. The M2 receptor should still not be excluded as a possible target for lacrimal secretion, especially since it probably has a significant relevance in modulation of endogenous cholinergic transmission from parasympathetic nerves and is possibly operative in the regulation of blood flow in the gland.



Antagonist	pA_2	M1	M2	M3	M4	M5
Atropine	9.5	9.1	9.0	8.8	9.2	9.3
Pirenzepine	7.3	7.8	6.3	6.6	7.3	6.9
4-DAMP	8.7	8.9	8.1	8.8	8.9	9.0
AFDX 116	6.2	5.6	6.8	4.6	5.4	4.9

Figure 9. Linear regression analyses of atropine, pirenzepine, 4-DAMP, and AFDX 116 pKi values (y-axis) from [^{3}H]QNB competitive binding experiments in Sf9 cells expressing cloned rat and human muscarinic receptors M1-M5 [89,107-120]. The binding data is correlated to functional pA_2 values (x-axis) obtained from digested lacrimal gland acini (N=3) where functional responses were quantified by measuring [^{3}H]inositol phosphate production [64]. Points represent the mean value ± standard deviation, the slope represents linear regression analysis with dashed line depicting slope 95% confidence interval. Corresponding goodness of fit is displayed as R^2 and slope being statistically significant compared to R^2=0 displayed as p-value. Graphed pA_2 values and pKi values are listed in the table.

The presently observed absence of intracellular calcium response following muscarinic stimulation in isolated myoepithelial cells is somewhat obscure. In contrast to our findings, a calcium mediated myoepithelial response following muscarinic stimuli has been shown in primary cultured acini and in what is claimed to be isolated myoepithelial cells [59,78]. In the primary cultured acini, the data indicates an 8 second delay in the response expressed in acinar and myoepithelial cells. This possibly indicates an intercellular interaction where

muscarinically stimulated acinar cells first are excited, which in turn could cause release of an intermediary transmitter and thereby stimulation of the myoepithelial cells. The longevity of the delay indicates that the myoepithelial excitation is mediated via release of transmitter rather than the response being propagated via electrolytical exchange through gap junctions. Additionally, in the paper presented by García-Posadas et al. (2020), a muscarinic agonist was shown to cause an increase in the concentration of intracellular calcium in monocultures which had been isolated for two to three weeks. In our experience such an isolation procedure cannot lead one to exclude the presence of acinar cells. Furthermore, according to García-Posadas et al., the response measured following ATP administration did not exhibit similar characteristics as previously reported in the literature and the recorded contractions showed strong indications of heterogeneity in the primary culture following administration of both CCh and ATP, in regard to fraction contracting cells and expression of alpha smooth muscle actin.

The functional results presented in paper III could possibly indicate that the muscarinic receptor response is mediated via second messengers, other than calcium. In fact, the inhibitory receptor M4 is generally known to inhibit the intracellular synthesis of cAMP [65], a second messenger which is synthesized from ATP and which is known to mediate smooth muscle relaxation [121]. Thereby, one can hypothesize that the inhibition of cAMP could attenuate a state of relaxed basal tonus, causing inhibition of relaxation, and subsequent amelioration in smooth muscle tonus. Over time this could be perceived as a contractile response to cholinergic stimuli. Furthermore, if cAMP synthesis is inhibited, that could lead to accumulations in substrate concentrations, i.e. increase in concentration of intracellular ATP. Speculatively, the increase in ATP might cause ATP exocytosis and in turn the released ATP could stimulate purinergic receptors which has been shown, herein and in the literature, to induce an intracellular calcium response. The functional recordings presented in paper III were all obtained with a duration of 20 seconds post agonist administration. Recordings with longer duration would have to be made in order to falsify a hypothesized delayed response.

The herein hypothesized intercellular interaction was further investigated in primary co-cultures containing acinar and myoepithelial cells (paper IV). The delayed calcium response, which was recorded 10-20 seconds following administration of the muscarinic agonist methacholine, was consistently recorded in cells which displayed myoepithelial features and the delayed response was not observed in cells which were preincubated with purinergic antagonist suramin, indicating that ATP plays a crucial role in the observed delayed response. The homogenous and intense response measured following ATP administration was sustained in atropine preincubated cells, which

indicates that the role of acetylcholine was not as integral or at least did not modulate the ATP induced response. Effects similar to what was shown herein, have also been shown in the parotid gland isolated acini where purinergic antagonist A839977 significantly diminished the calcium response obtained following administration of muscarinic agonist [122]. Additionally, ATP has been shown to be absorbed in, and released from, zymogen granules in exocrine pancreas during exocytotic activity [122,123]. More relevant, Novak et al. studied cholinergic effects on ATP release from acini in pancreas, parotid gland and in lacrimal gland. Therein, lacrimal acini labeled with quinacrine showed intense labeling, indicating ATP storage in acinar cells, and somewhat faint labeling in duct cells. In additional support of a hypothesized ATP involvement in cholinergic stimulation of myoepithelial cells, ATP was shown to be released from acinar cells following administration of the cholinergic agonist CCh in pancreas and the parotid and lacrimal gland [124].

Taken together, these findings strongly indicate an intercellular mediation of cholinergic stimulation, where stimulated acinar cells in turn release ATP which subsequently stimulates myoepithelial cells. This leaves the question why the myoepithelial cells display heterogenic expression of muscarinic receptor M2, M3, M4 and M5. If the receptors are expressed in myoepithelial cells, then that should enable calcium mediated stimulation in these cells. Hypothetically, besides the possibility of a receptor signal being mediated via other second messengers, it could be possible that the receptors exhibit a cytosolic expressional pattern. In fact, none of the micrographs which were recorded in our investigations into the muscarinic receptor expression in myoepithelial cells exhibited any indication of membranal localization. On the contrary, M2 and M5 staining strongly indicated localization near the nuclei, possibly in the ER or Golgi apparatus, and the M3 and M4 stains showed similar expression in the vicinity of nuclei in conjunction with filament adjacency. This could indicate that none of the muscarinic receptors are located in the outer membrane of myoepithelial cells. Hypothetically, the internalized receptor expression can serve as a sequestering function for the accumulation of acetylcholine, if acetylcholine were to be released from parasympathetic neurons in toxic concentrations. Following this hypothesis, the myoepithelial cells would serve as a form of glial cell, facilitating acetylcholine uptake, synthesis and metabolism. Intriguingly, in the studies of parasympathetic innervation of the lacrimal gland, immunohistochemical staining for acetylcholine esterase displayed stains with patterns resembling myoepithelial cells [48,49]. This could explain the obscure results obtained regarding muscarinic receptor expression and function in monocultured myoepithelial cells. In future studies, immunocytochemical staining of monocultured myoepithelial cells with acetylcholine metabolizing enzyme antibodies could be a good starting point for testing this hypothesis.

5 CONCLUDING REMARKS

In this thesis findings are presented which show that the claimed anticholinergic properties of antidepressants are only attributed to the older generation, i.e. tricyclic antidepressants, and that supplementary treatment with cholinergic agonists potentially can alleviate the adverse effects of dry mouth and dry eyes experienced by some patients undergoing pharmacological treatment for depression. This proclamation rests firmly on our findings, indicating that modern antidepressants, i.e. SSRIs and SNRIs, only displayed reflex arc mediated xerogenic and xerophthalmic effects, and not local. The observed centrally mediated effects are contrasted by the augmented peripherally induced salivation seen in the presence of the modern antidepressants citalopram and venlafaxine, and the unaffected peripherally induced tear secretion following administration of the selective serotonin reuptake inhibitor citalopram. Thus, local administration of muscarinic compounds, e.g. through eye drop application or mouthwash, should effectively alleviate adverse effects of antidepressants.

To functionally characterize the rat lacrimal gland on a cellular level a sustainable isolation procedure for monocultures of primary myoepithelial cells was validated, in which co-cultures of acinar and myoepithelial cells which can be used for investigations of intercellular interactions arise midway. The investigations into muscarinic expression and function in lacrimal glands resulted in findings supporting a hypothesized heterogenous muscarinic receptor population. The potential effects of M4 and M5 stimulation should therefore be considered in the development of novel parasympathomimetic drugs for symptomatic treatment of DED. In addition, it was shown that purinergic responses follow in conjunction with cholinergic stimulation. Thereby, purinergic interactions should be incorporated in the risk and benefit analysis of pharmacological treatment of DED with muscarinic agonists.

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