The Neurobiology of the Meibomian Glands

Stephanie M. Cox, OD, and Jason J. Nichols, OD, MPH, PhD

ABSTRACT This article compiles research regarding the neuroanatomy of the meibomian glands and their associated blood vessels. After a review of meibomian gland morphology and regulation via hormones, a case for innervation is made based on anatomical findings whereby the nerves lack a myelin sheath and Schwann cells. The localization and co-localization of dopamine beta-hydroxylase, tyrosine hydroxylase, neuropeptide Y, vasoactive intestinal polypeptide, calcitonin gene-related peptide, and substance P are explored with emphasis on differences that exist between species. The presence of the various neuropeptides/neurotransmitters adjacent to the meibomian gland versus the vasculature associated with the meibomian gland is documented so that conclusions can be made with regard to direct and indirect effects. Research regarding the presence of receptors and receptor proteins for these neuropeptides is documented. Evidence supporting the influence of certain neurotransmitters and/or neuropeptides on the meibomian gland is given based on research that correlates changes in meibomian gland morphology and/or tear film with changes in neurotransmitter and/or neuropeptide presence. Conclusions are drawn related to direct and indirect regulation and differences between the various nervous systems.

KEY WORDS calcitonin gene-related peptide, dopamine beta-hydroxylase, innervations, meibomian gland, neuroanatomy, neuropeptide Y, substance P, tyrosine hydroxylase, vasoactive intestinal polypeptide

I. INTRODUCTION

The meibomian glands secrete meibum, which ultimately makes its way to the lipid layer of the tear film. This superficial lipid layer prevents evaporation of the aqueous tear film from the ocular surface; therefore, a loss of integrity to these glands would be a cause of dry eye. The Definition and Classification Subcommittee of the 2011 Tear Film and Ocular Surface Society (**TFOS**) International Workshop on Meibomian Gland Dysfunction established a definition of meibomian gland dyfunction (**MGD**), a cause of dry eye. One component of the definition is "…a chronic, diffuse abnormality of the meibomian glands…,"[1] and a loss of meibomian gland integrity would classify as such an abnormality. The epidemiology subcommittee report of the Workshop highlighted the fact that epidemiological statistics for MGD vary with age, race/ethnicity, and parameters used within studies to define MGD; however, it also pointed out that the prevalence of MGD has been documented to be as high as 69.3%.[2] Dry eye can severely compromise patients' quality of life with annoying symptoms and inconvenient treatment options (instilling artificial tears during work and other daily activities).[2] Dry eye patients often spend hundreds of dollars per year on over-the-counter or prescription lubricating drops and doctor visits.[3] Understanding the cause and pathophysiology behind dry eye can help to develop effective treatments for these patients. The purpose of this review is to enhance such understanding and, in particular, to focus on the neurobiological regulatory control of the glands in health and disease.

II. MEIBOMIAN GLAND ANATOMY AND MEIBUM PRODUCTION

The meibomian glands are modified sebaceous glands found within the upper and lower eyelids. They consist of a duct whose orifice opens at the lid margin and travels distally from the lid margin in a direction perpendicular to the lid margin. In humans and many other mammals, several ductules divert laterally from the central duct and open into acini.[4-8] The signal from peroxisome proliferator-activated receptor (**PPAR**) gamma is known to affect the development of the meibomian gland.[4]

Each acinus has a basement membrane, which forms the boundary between it and the surrounding connective tissue. The connective tissue contains fibroblasts, collagen,

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From the College of Optometry, University of Houston, Houston, TX, USA.

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Single-copy reprint requests to Dr. Jason J. Nichols (address below).

Corresponding author: Jason J. Nichols, OD, MPH, PhD, The University of Houston, The Ocular Surface Institute, 505 J. Davis Armistead Building, Houston, TX 77204. Tel. 713-743-2471. Fax: 832-842-5233. E-mail address: jnichols@optometry.uh.edu

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capillaries, and nerves. Basal cells are found adjacent to the basement membrane. The basal cells mature and migrate toward the center of the acini and are then called *meibocytes*.[4,5]

As meibocytes develop and migrate, vesicles containing a substance called meibum increase in number and size. This increase in meibum-containing vesicles within the meibocytes occurs concurrently with other changes to cellular organelles, such as the reduction in size of mitochondria. The meibocytes' membranes eventually degenerate at the acinus center and the released meibum and fractioned cellular components move through the ductules of the meibomian glands toward the main glandular duct.[4,8,9]

III. SEX STEROID REGULATION

Regulation of the meibomian glands via androgens and estrogens have been investigated extensively. This review provides only an abbreviated version of this information.

A. Androgens

Analysis of mRNA and protein expression within meibomian glands of rats, rabbit, and humans suggest the presence of androgen receptors.[10,11] Enzymes involved in the activation and inactivation of androgens have also been identified within the human meibomian gland.[12] Further, testosterone or androgen exposure in mice has been shown to increase expression of mRNA and genes that encode for enzymes involved in fatty acid and cholesterol synthesis and other genes important in meibum production and metabolism.[13-15] Similar upregulations were also observed in immortalized human meibocytes treated with dihydrotestosterone. In addition, proteins related to keratinization, which have been shown to be upregulated in patients with MGD, were downregulated in these treated cells.[16]

B. Estrogen

Immunohistochemical staining and mRNA expression suggest the presence of estrogen receptors within the meibomian glands of rabbits and humans.[11,17] These receptors have been shown to be more prominent at the basal cell layer than in the more mature meibocytes at the center of the acini. Further, no receptors were localized to the ducts.[18] Enzymes involved in estrogen production, activation, and inactivation have been identified in human meibomian glands.[12] Research also suggests that treatment with 17β-estradiol within ovariectomized mice caused an overall downregulation of genes involved in lipid production.[18,19]

C. Functional Effects

Changes in dry eye symptoms and clinical signs with sex steroid treatments vary. The presence of neutral lipids within the castrated rabbit meibomian gland was decreased, and the fraction of long-chain fatty acids expressed by the meibomian glands of castrated rabbits treated with 19-nortestosterone were more similar to intact male rabbits compared to placebo-treated castrated rabbits. However, this study also showed no reduction in interferometry measures, tear film changes, or changes in the morphology of the meibomian gland.[10] This finding was also found in humans when no significant correlation was found between clinical signs or symptoms of dry eye and estrogen expression.[18]

Antiandrogen treatments in humans have been correlated with altered meibum components.[20,21] In addition, some symptoms and clinical signs are increased in patients taking antiandrogen treatments or who have been diagnosed as having androgen insensitivity.[21,22] Based on survey responses and/or a clinical diagnosis of dry eye, patients being treated with estrogen hormone replacement therapy had a higher prevalence of dry eye than patients not taking hormone replacements.[23]

IV. CHANGES WITH MEIBOMIAN GLAND DYSFUNCTION

Changes observed clinically in association with MGD are well documented in the literature. For example, tear film breakup time (**TFBUT**) decreases while lid margin abnormalities and corneal/conjunctival staining increase. In addition, symptoms of burning, itching and grittiness increase with MGD.[24] Specific morphological changes have

also been documented to occur in association with MGD, as described below.

A. Duct/Ductules

Exposure to topical epinephrine in a rabbit model yielded meibomian glands with narrowed orifices and lumen filled with debris. Some glands were found to have dilated ducts and cystic formations of desquamated cornified cells lined by hyperkeratinized ductal epithelium.[25] Similar changes have also been found in the rhino mouse[26] and in the monkey when exposed to polychlorinated biphenyl poisioning.[27] Ductule dilation and cyst formation has been noted in older individuals, which would be expected given that age is a risk factor for MGD.[2,28]

B. Acini

Atrophic changes with age resulting in decreased number and size of acini have been observed in mice and humans.[28,29] This atrophy has been associated with changes in gene expression of gamma subtype PPAR. PPARγ is expressed in the cytoplasm and nuclei of meibocytes of the meibomian gland acini of young mice. However, this expression was not found in the cytoplasm of meibocytes in older mice. Older mice not only show atrophic morphological changes, but also decreased proliferation in the meibomian gland acini.[30]

Atrophic changes have been suggested to manifest as areas of dropout in meibography images.[31] Meibography uses an infrared light to image the meibomian glands through the palpebral conjunctiva. Areas where meibomian glands cannot be visualized are considered areas of meibomian gland dropout, and increased dropout has been correlated with increased dry eye symptoms as assessed using the Ocular Surface Disease Index (**OSDI**).[31]

Confocal microscropy studies have suggested that MGD, which was diagnosed based on the appearance of meibomian gland orifices, gland secretion, and displacement of the mucocutaneous junction, was associated with increased acinar diameters and decreased acinar density.[32] Contact lens wear, a risk factor for MGD, causes a reduction in acinar diameter and an increase in acinar unit density.[2,33] Atopic keratoconjunctivitis is another risk factor for MGD, and in affected eyes the acinar diameters and areas and acinar unit density was decreased.[2,34] These changes in acinar size and density indicate compromise to the meibomian glands.[35] Signs of inflammatory cells were observed in all confocal microscopy studies, and patients with atopic keratoconjunctivitis showed fibrotic tissue development with atrophy of the meibomian gland acini extending to the nearby conjunctiva.[31-36]

C. Meibum

Subjects with MGD have increased meibum viscosity, which is thought to increase tear osmolarity.[9] Increased meibum viscosity manifested with meibomian gland morphological changes was seen in monkeys with polychlorinated biphenyl poisoning[27] and in an epinephrine-induced MGD rabbit model.[37] Decreased meibum production with age is shown to accompany morphological changes with age in mice. This is suspected to also be true in humans, given the similarities observed between histology of mice and humans.[29] Meibum analysis has shown that patients with MGD have decreased levels of cholesterol esters compared to normals.[9]

Although the contribution of sex steroids to regulation of the meibomian glands is well recognized, it is of interest that the meibomian glands are unique from other sebaceous glands by the presence of nerves.[38] This characteristic difference suggests that the nerves play a role in meibomian gland regulation. Thus, evidence-based knowledge regarding the neurobiology of the meibomian glands is reviewed below.

V. NERVOUS SYSTEM STRUCTURE

Before considering the specific type of nervous innervation of the meibomian gland, it is important to understand the parts of the nervous system and their various neurotransmitters. The peripheral nervous system consists of autonomic and somatic branches. In very general terms, the autonomic system is not within cognitive control while the somatic branch is within cognitive control. Therefore, it is not surprising that the meibomian glands would have autonomic innervations. Preganglionic autonomic fibers synapse with postganglion fibers at ganglion structures. The postganglionic fibers then project to the meibomian glands. Within the autonomic nervous system are parasympathetic and sympathetic nerve fibers. The parasympathetic postganglionic fibers mainly utilize acetylcholine as a neurotransmitter, while the sympathetics utilize catecholamines.[39] The parasympathetic and sympathetic nerve fibers also use different neuropeptides to modulate the actions of these main neurotransmitters. In addition to the autonomic innervations, the eyelids and meibomian glands contain sensory innervations. Throughout the body, the sensory nerves relay messages regarding pain, pressure, temperature, etc., from the area of sensation to the central nervous system and/or other structures. The neuropeptides associated with this branch of the nervous system include substance P and calcitonin- gene-related-peptide (**CGRP**).[40]

Differences in neurotransmitters and neuropeptides between the branches of the nervous systems have allowed a deeper understanding of the neural structure surrounding the meibomian glands. In most experiments, antibodies are used to indicate that a nerve is reactive for a specific neuropeptide. The characterization of the neuropeptide being tested by the antibody indicates the parasympathetic, sympathetic, or sensory nature of the nerve. Some evidence suggests that these distinct systems may have more overlap and complexities than originally thought.[39,41] This is discussed in greater depth later in this review.

VI. METHOD OF LITERATURE SEARCH

To investigate the neurobiology of the meibomian glands, we performed a search in PubMed and Web of Knowledge.

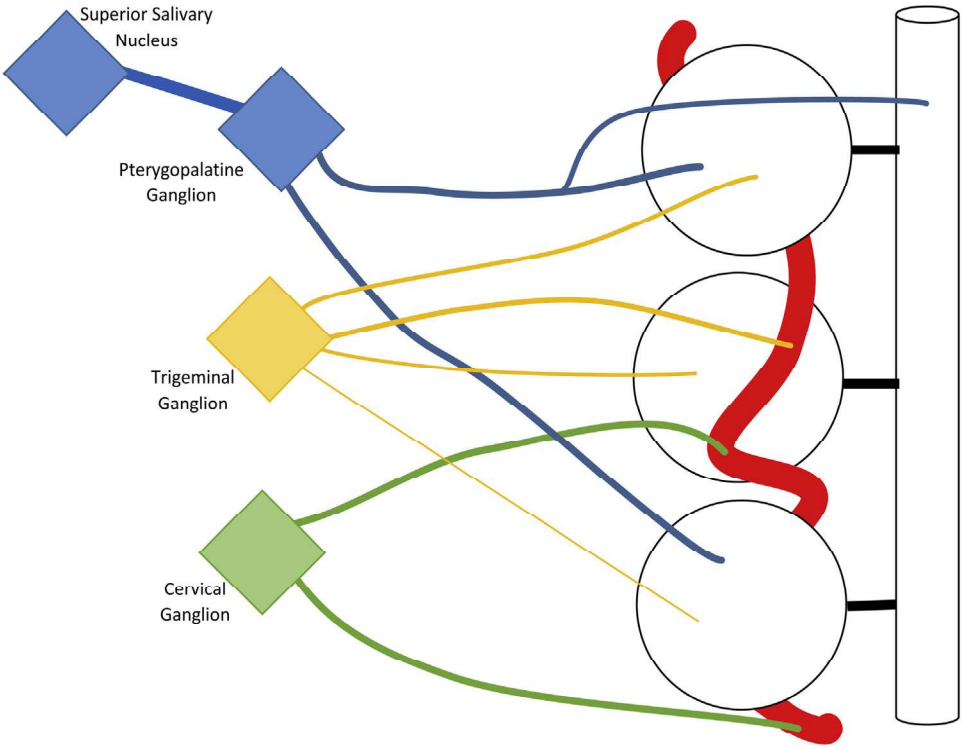

Figure 1. Illustration of a meibomian gland of the inferior eyelid. The orifice is represented at the top of the main duct (at the far right) by a circular opening. The circles represent acini, and ductules connect the acini and the central duct. The red line represents the vasculature associated with the meibomian gland. The parasympathetic system is represented in blue, the sensory system represented in yellow, and the sympathetic system represented in green. The ganglions and nucleus of note are labelled. Connections between the ganglions, nucleus, meibomian gland, and vasculature represent nerves. Thicker connecting lines represent a stronger association based on the density of neuropeptide appearance.

No languages were excluded in the search parameters. Two separate searches were conducted within PubMed with keywords of "meibomian gland AND nerve" and "meibomian gland AND neuron." A total of 46 results were found. Nineteen articles were excluded: 8 of 16 articles that were found in both searches; 5 that were deemed not applicable upon review; 5 that were review articles related to dry eye; and one that was in Russian. This yielded 27 total references for review.

A topical search for "meibomian gland" was conducted within the Web of Knowledge search engine, which yielded 2102 results. A topical search for "nerve" was then conducted within these results, which yielded 45 results. Twenty-four were duplicates from the PubMed search, and three search results were duplicates within the search. Twelve were found to be not applicable upon review, and one was found to be an abstract only. This yielded a total of five additional articles.

Additional articles not listed in these search engines were obtained via secondary literature searches using the bibliographies and references from the primary search articles attained as described above.

VIII. NEURONAL REGULATION

A. Evidence of Nervous Innervations

The close relationship between nerves and the meibomian glands has been observed in the guinea pig,[6,42] rat,[7] rabbit,[42] cat,[42,43] dog,[42] horse,[42] sheep,[44] nonhuman primates,[8,42] such as the cynomolgus[45,46] and rhesus[46] monkeys, and the human.[6,42,47] Some studies have been based on appearance and proximity; however, other studies have noted unmyelinated nerve fibers making contact with the basal lamina of the meibomian gland acini directly and/or with the associated vasculature. In addition, axons may lack a Schwann cell covering within that area of contact with the basal lamina.[6,45,48] These areas are very specific, as evidenced by the finding of Schwann cells on the nerves on the opposite side of contact,[45] and vesicles containing neurotransmitters and neuropeptides have been visualized at these areas.[6,48] This selective lack of perineural epithelium and presence of selective vesicles suggests that the meibocytes are the target tissue of these nerves. The association of nerves with the vasculature of the meibomian gland[6,45] suggests a possible indirect effect on meibomian gland structure and function.[46]

B. Sympathetic Innervations

Retrograde staining from the rat eyelid shows nerve fiber projections from the superior cervical, trigeminal, and pterygopalatine ganglions. Staining specific for sympathetic neuropeptides within these ganglia and denervation experiments suggest that the majority of the sympathetic nerves come from the superior cervical ganglion.[40] The distributions of sympathetic neuropeptides are discussed below. Figure 1 represents many of the findings regarding the sympathetic nervous system.

1. Dopamine Beta-Hydroxylase

Dopamine Beta-hydroxylase (**DBH**) is an enzyme known to be involved in catecholamine synthesis within adrenergic nerves of the sympathetic nervous system. The soluble portion of the enzyme is known to be released with norepinephrine into the synaptic cleft.[49] DBH immunoreactive nerves are mainly associated with the vascular elements of

Table 1. Neuropeptides identified in each species

Species	DBH		TH		NPY		VIP		CGRP		SP	
	Vasc	Gland	Vasc	Gland	Vasc	Gland	Vasc	Gland	Vasc	Gland	Vasc	Gland
Rat	✓+	✓			✓	✓	✓	✓+	✓+	✓	✓	✓+
	39	39			40,49	40,49	39,40	39,40	39,40,65	39,40,65	40,64	40,64
Guinea pig			✓+	✓	✓	✓	✓	✓	✓+	✓	✓	✓
			6	6	6	6	6	6	6	6	6	6
Cat							✓	✓				
							42,53	42,53				
Dog							✓	✓				
							53	53				
Pig							✓	✓				
							53	53				
Cynomolgus monkey	✓+	✓	✓+	✓	✓	✓	✓	✓+	✓+	✓	✓	✓
	44	44	44,45	44,45	44,45	44,45	44,45	44,45	44,45	44,45	44,45	44,45
Rhesus monkey			✓+	✓	✓	✓	✓	✓+	✓+	✓	✓	✓
			45	45	45	45	45	45	45	45	45	45
Human							✓	✓+	✓	✓+	✓+	✓
							47,53	47,53	6	6	54	6

DBH, dopamine beta-hydroxylase; TH, tyrosine hydroxylase; NPY, neuropeptide Y; VIP, vasoactive intestinal polypeptide; CGRP, calcitonin gene-related peptide; SP, substance P; Vasc, meibomian gland associated vasculature. The presence of nerves immunoreactive for each peptide in a species' meibomian gland or meibomian gland associated vasculature is indicated by a check. A check with a plus indicates the area (meibomian gland or meibomian gland associated vasculature) with a higher density of nerves immunoreactive for that specific neuropeptide. The number(s) beneath each check or check plus indicate(s) the reference(s) that provided the information.

the meibomian gland in the rat[40] and cynomolgus monkey,[45] and a small portion is associated with the acini.

2. Tyrosine Hydroxylase

Tyrosine hydroxylase (**TH**) is another enzyme known to be involved in catecholamine synthesis within adrenergic nerves of the sympathetic nervous system.[49] Like DBH, TH immunoreactive nerves showed greater density around the meibomian gland vasculature than meibocytes.[6,46] This distribution has been seen in the guinea pig,[6] the cynomolgus monkey,[45,46] and rhesus monkey.[46] This unequal distribution of nerves immunoreactive for DBH and TH is indicated by the presence of a check versus a check plus in the columns labeled DBH and TH in Table 1, which shows species differences of this and other neuropeptides.

3. Neuropeptide Y

Nerve fibers immunoreactive for neuropeptide Y (**NPY**) have been observed around the blood vessels and meibomian gland acini of the rat[41,50] and guinea pig.[6] In the cynomolgus and rhesus monkey, NPY immunoreactive fibers have been observed within the walls of the small blood vessels near the acini and near the meibomian gland.[45,46]

NPY has traditionally been associated with the sympathetic branch of the nervous system. However, removal of the sympathetic superior cervical ganglion did not eliminate all of the NPY-stained nerves, which would be expected if NPY were isolated to the sympathetic branch. Electrocoagulation of the pterygopalantine ganglion, the largely parasympathetic ganglion, eliminated a portion of NPY-stained fibers in the rat eyelid.[41] NPY's association with both a parasympathetic and sympathetic ganglion is one reason for the association of NPY with both branches.

NPY immmunoreactive nerves often show co-localization, which may help to better understand areas of parasympathetic and sympathetic innervations. It has been suggested that localization with enzymes associated with the sympathetic system (DBH and TH) would indicate

sympathetic origin. This co-localization has been seen in the rat and monkey.[45,50] The nerves with this co-localization in the cynomolgus monkey have been associated with the vasculature near the meibomian glands.[45] Another study noted that very few nerves are immunoreactive for TH near the meibocytes, which would suggest that the NPY immunoreactive fibers near these cells are parasympathetic.[46] These facts led several authors to conclude that the NPY immunoreactive fibers near the vasculature of the meibomian gland are sympathetic.[45,46] This conclusion is illustrated in Figure 1 as sympathetic nerves innervating the vascular supply surrounding the meibomian glands.

4. Receptor Expression

Immunolabeling techniques show that NPY receptor 1 is localized to the nuclear membrane of the acinar cells and to the cell membrane of the meibomian gland ductal and acinar cells in the mouse. In addition, genes related to NPY receptor 1 expression increased within meibocytes compared to brain tissue, although there was no significant difference in protein expression.[51]

5. Functional Effects

In the 1980s, topical epinephrine began to be used to create animal models of MGD based on the morphological changes in the meibomian glands described earlier (cyst formation within the meibomian glands and keratinization of the meibomian gland orifices).[25] Clinical compromise was also observed in the form of mild conjunctival erythema, engorgement of the meibomian glands, and thickened meibomian gland secretions.[37] It is important to note that epinephrine acts as a neurotransmitter within the sympathetic nervous system, which means that the epinephrine-induced effects are linked to its action, either directly on the meibomian glands or indirectly by acting on the vasculature.

In addition to clinically apparent changes, immunoblot techniques comparing the meibum of epinephrine-treated rabbits to that of normal rabbits showed differences when specific keratin monoclonal antibodies (**mAb**) were used. Meibum of epinephrine-treated rabbits with MGD expressed the acidic 56.5 kD AE1-positive and the basic 65-67 kD AE3-positive keratin proteins, which are also found in keratinized epithelia. In addition, the acidic 50 kD AE1-positive and basic 58 kD AE3-positive keratin proteins were resolved. Finally, the basic 56 kD AE3-positive keratin protein was resolved. This keratin has been associated with callouses, keratoacanthoma, and other disorders that cause hyperproliferation of epithelial cells.[37]

The meibomian glands of treated rabbits also showed changes in the localization of staining of monoclonal antibodies AE1, AE2, and AE3, which are associated with keratinization. An increase in basic mAb AE3 and basic mAb AE2 staining was observed in the acini and ducts of treated rabbits, and a reduction of acidic mAb AE1 stain was observed in their acini. The rabbits showed hyperkeratinization of the orifices and keratinized cysts in deeper portions of their meibomian glands. The appearance of keratinized cysts without orifice plugging suggests that orifice plugging is not necessary for other morphological changes. The epinephrine treatment could be causing cellular changes due to its action on the meibocytes directly or indirectly via the vasculature.[37]

C. Parasympathetic Innervations

Viral-based retrograde staining within the ganglions of rats suggest that most parasympathetic fibers project from the pterygopalatine ganglion[40,43,52,53] and originate at the superior salivary nucleus.[52]

The appearance of acetylcholinesterase, an enzyme that breaks down the parasympathetic neurotransmitter acetylcholine, within the nerves near the meibomian glands would indicate parasympathetic innervation. This has been found in primates, rabbit, guinea pig, cat, dog, and horse.[42] The appearance of other neuropeptides associated with the parasympathetic system are outlined below and are illustrated in Figure 1.

1. Vasoactive Intestinal Polypeptide

Vasoactive intestinal polypeptide (**VIP**) is very closely associated with the parasympathetic branch of the nervous system. One reason for this strong association is the presence of VIP immunoreactive fibers within the pterygopalatine ganglion and the lack of VIP immunoreactive fibers within other ganglia.[45]

In the rat, VIP immunoreactive fibers were found to be associated with almost every meibomian gland acini and occasionally with the associated vasculature.[40,41] This dense distribution was not noted within the rat tarsal muscle or palpebral conjunctiva, which suggests that VIP may provide some innervation to the meibomian gland acini.[40] VIP immunoreactive nerves have also been found associated with the meibocytes and meibomian gland-associated vasculature of the of the guinea pig,[6] cat,[43,54] dog,[54] and pig.[54] The lack of comparative distribution information in these species is reflected in Table 1 by the absence of a check versus a check plus.

Investigations in primate species have found a strong association of VIP with the meibomian glands. VIP immunoreactive fibers have been localized around the acini and central duct of the meibomian gland of the rhesus[46] and cynomolgus monkeys.[45,46] There appear to be some conflicting reports regarding the presence of VIP immunoreactive neurons around the small blood vessels associated with the meibomian glands of primates.[45,46,54,55] Therefore, no relationship of the parasympathetic system and the vasculature is illustrated in Figure 1. Recent work that specifically looks at the meibomain glands suggests that VIP is more closely related to the acini.[46] The association between the meibomian gland and nerves immunoreactive for VIP has been confirmed in the human.[48,54] An example of the staining from previous work on the human is shown in Figure 2. Notice the close proximity between the acini and the nerves and the distribution between the acini.

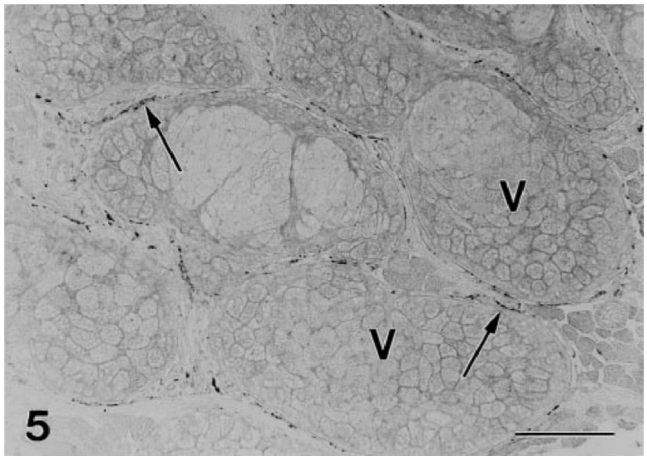

Figure 2. This figure shows VIP immunoreactive nerve fibers and their association with the meibomian gland acini. The arrows point to examples of nerves that were immunoreactive for VIP. The structures labeled V are acini. Bar = 50 microns. Reprinted from *Experimental Eye Research*, Volume 68, Seifert P and Spitznas M, Vasoactive intestinal polypeptide (VIP) innervation of the human eyelid glands, page 687, Copyright 1999, with permission from Elsevier.

2. Neuropeptide Y

NPY immunoreactive fibers have been found around vessels and the meibomian gland of the rat,[41] the guinea pig,[6] and rhesus[46] and cynomolgous monkeys[45,46] The co-localization of NPY with the sympathetic enzymes was described previously, but this neuropeptide was also co-localized with VIP. The co-localization with VIP was mostly associated with the meibomian gland acini and ducts of the cynomolgus monkey.[45] This co-localization with a neuropeptide very strongly associated with the parasympathetic branch of the nervous system suggests that these nerves are parasympathetic. The presence of immunoreactive nerve fibers are indicated in Table 1 across species. Clarification of distribution is not indicated due to the complex nature of the information on this particular neuropeptide.

3. Receptor Expression

In addition to the NPY receptor 1 expression already discussed, mice express VIP-1 receptor protein within the cell membranes of meibomian gland acini and ductal cells, as seen by immunolabeling.[51] The expression of mRNA for VIP 1 and 2 receptors has been shown within human meibocytes and immortalized human meiboytes.[56] Protein expression for VIP receptors 1 and 2 have been demonstrated within immortalized human meibocytes.[56] The expression of genes and proteins for VIP-1 receptor was found to be statistically significant when compared to brain cells in mice.[51]

The mouse also expresses proteins for the muscarinic receptors M1, M2, M3, M4, and M5 within the cell membranes of meibomian gland acini and ductal cells. Localization of receptors to the nuclear membranes was limited to M1, M2, M3, and M5 within the acinar cells and M2 within the ductal cells.[51] In the monkey, M3 receptors were localized to the basal epithelium of the meibomian gland acini, and M4 receptors were localized to the ductal epithelium.[57] mRNA expression of M2 and a species similar to M3 has been identified within human meibocytes and immortalized human meibocytes.[56] Like the VIP receptor 1 gene expression, the expression of genes for the muscarinic receptors was increased compared to brain cells in mice.[51] The expression of proteins for muscarinic receptors 2 and 3 has been demonstrated within human meibocytes.[56] The expression of proteins for M2 and M3 receptors was increased with the M2 protein expression reaching statistical significance compared to brain cells. M1, M4 and M5 showed decreased protein expression compared to brain cells.[51]

4. Functional Effects

Retinoic acid is known to negatively affect the meibomian gland morphology and/or tear film integrity in many species, including human. Changes include increased atrophic appearance of meibomian glands, increased meibum thickness, decreased meibum volume, and increased osmolarity.[58-60] Using nicotinamide adenine dinucleotide diaphorase staining, the presence of nitric oxide synthase, the enzyme that generates the neurotransmitter nitric oxide, was observed in nerves surrounding and between the meibomian gland acini.[61] Rats exposed to retinoic acid prenatally developed meibomian glands that were smaller than those that were not exposed. In addition, nerves were displaced from between the meibomian gland acini.[62] While this observation does not fully illustrate the relationship between the meibomian glands and the nerves, it does show a concurrent effect, which suggests a relationship between meibomian gland morphology and the surrounding nerves.

Ovariectomized rats showed increased NPY staining density and intensity and decreased VIP staining density and intensity compared to sham and untreated controls.[63] This study showed an important association with decreased serum estradiol levels, which is common in post-menopausal women.[63] The increase in the prevalence of dry eye in post-menopausal women has caused much dry eye research to center around changes in sex-steroids; however, this study's findings suggests a possible neurological change associated with menopause. It is unclear how this neurological association is related to dry eye symptoms or the changes observed with sex steroids.

The concentration of neuropeptide Y has been found to be lower in subjects with decreased TFBUT.[64] TFBUT is a measure of tear film stability. Therefore, a decreased TFBUT suggests that the patient has evaporative dry eye, which can be caused by MGD.

Cellular changes have been noted using immortalized human meibocytes.[56] One example, would be a change in the concentration of intracellular cyclic adenosine monophosphate (**cAMP**), which is the end product of the adenylyl cyclase pathway. Forskolin and 3-isobutyl-1-methylxanthine are known to increase intracellular cAMP, but their impact on intracellular cAMP was altered by the addition of VIP or carbachol, a drug that mimics the effects of parasympathetic

neuropeptides. Changes were also noted in calcium ion concentration in cells exposed to VIP or carbachol. Calcium ions are released during G-coupled second messenger reactions. Changes in calcium ion and cAMP concentrations indicate changes in the cellular pathways and reactions within the immortalized human meibocytes. The same study also found that the number of immortalized human meibocytes increased with exposure to VIP compared to untreated cells. This could indicate an increase of meibocyte proliferation and increased meibum production with VIP exposure. Changes to the cells with exposure to parasympathetic agents suggest that the muscarinic and VIP receptors are functional.[56]

D. Sensory Innervations

Sensory neuropeptide staining of ganglia has been found within the pterygopalatine[65] and trigeminal[40,41,45,65,66] ganglia. However, it is suspected that the staining within the pterygopalatine ganglion could be due to co-localization (discussed below). Therefore, studies suggest that most of the sensory nerves projecting to the meibomian glands come from the trigeminal ganglion in the rat and monkey.[40,41,45,65,66]

1. Calcitonin Gene-Related Peptide

Nerves immunoreactive for calcitonin gene-related peptide (**CGRP**) were localized to the vascular elements associated with the meibomian glands of the rat[40,41,66] and guinea pig.[6] The rat also showed CGRP immunoreactivity at the anterior segments of the ducts.[40] Within the palpebral conjunctiva of the rat, this peptide showed decreased immunoreactivity density with increased distance from the mucocutaneous junction.[66]

In the rhesus and cynomolgous monkeys, CGRP immunoreactive fibers have been associated with the meibomian gland; however, their association was more prominent within the meibomian gland associated vasculature.[45,46] This is represented in Figure 1 by a thicker connection between the trigeminal ganglion and the vasculature compared to the acini. In human, CGRP immunoreactive fibers were associated with the meibomian gland,[6] which is denser at the lid margin conjunctiva.[46] This is also represented in Figure 1; increased thickness of the connections to the acinus at the lid margin from the trigeminal ganglion compared to the other acini has been illustrated. Table 1 reflects the presence of CGRP in the rat, guinea pig, nonhuman primates, and humans. The absence of CGRP immunoreactive nerves associated with several of these species in the vasculature or gland is reflected by an empty box.

2. Substance P

Substance P is a neurotransmitter or modulator that affects sensory innervations.[39] Nerves immunoreactive for substance P have been identified near or between the acini of rat meibomian glands, but in a sparse density.[41,65] CGRP shows a much denser distribution comparatively in the rat,[41,65] and many CGRP immunoreactive fibers show co-localization with substance P in the rat.[66] In the guinea pig, the substance P immunoreactive fibers are associated with the meibomian gland and the associated vasculature.[6]

The presence of substance P immunoreactive nerves surrounding the meibomian gland acini, central duct, and associated vasculature of the cynomolgus[45,46] and rhesus[46] monkeys has been observed. However, the density is less than that for CGRP.[46] Most, but not all, of these nerves also stained for CGRP. Because both of these neuropeptides are associated with the sensory branch, it was concluded that these nerves were sensory. There was modest co-localization with VIP and rare co-localization with NPY and VIP in the cynomolgus monkey.[45] Due to the strong association of VIP with the parasympathetic branch discussed previously, the substance P immunoreactive nerve fibers with this co-localization are suspected to be parasympathetic.[45] The human meibomian gland shows sparse association with substance P immunoreactive fibers,[6] but some association has been found with the vasculature associated with the meibomian gland.[55] Table 1 reflects the presence of substance P. As noted previously, the lack of comparative distribution information in some species is reflected by the use of checks only in Table 1.

3. Receptor Expression

Zhu et al localized the substance P receptor to the nuclear membrane of the meibomian gland acini cells and to the cell membrane of the meibomian gland duct and acini cells in meibomian gland of the mouse.[51]

4. Functional Effects

The sensory nerves are suspected to be the receptor organ for the sensations of temperature, dryness, etc. The lid margin has increased sensation in relation to the palpebral conjunctiva,[67,68] and conjunctival sensitivity has been shown to increase in human palpebral conjunctiva with decreased proximity from the mucocutaneous junction.[69] These findings agree with the distribution of CGRP immunoreactive nerve fibers within the rat conjunctiva.[66]

Sensation decreases with age until 60 years of age in the lid margin and conjunctiva and across all ages for the cornea. This negative correlation of sensation with age corresponds to the tendency of MGD to occur later in life. In addition, studies suggest that decreased conjunctival sensitivity is associated with MGD and dry eye.[70,71]

The tears of non-Sjögren syndrome dry eye have been found to have significantly reduced levels of CGRP.[64] Within the non-Sjögren syndrome dry eye group could have been several patients with MGD, which would indicate that the decreased sensory neuropeptide CGRP is associated with MGD.

VIII. CONCLUSIONS

The true effect of the nerves on the meibomian glands is unclear; however, the presence of nerves suggests that they affect the function and/or regulation of the meibomian glands. Nerves have been found to change in pathological

conditions of other sebaceous glands. In sebaceous glands of the skin, an increase in nerve growth factor and the appearance of nervous tissue within and near the gland was observed in patients with acne but was absent in those without acne.[72] This finding suggests that nerves can have an impact on sebaceous glands, especially under pathological conditions such as MGD.

Nerve growth factor has also been shown to have an increased concentration in patients with non-Sjögren syndrome dry eye.[64] Nerve growth factor modulates the remodeling of nerves and has been found to be increased in association with an increase in inflammatory mediators. The presence of inflammatory mediators observed with MGD provides a possible link between nerve growth factor and MGD. This possibility is also supported by improved tear quality of dogs treated with nerve growth factor. While the results specifically addressed improvement in goblet cell density, measures of mucin quality, and Schirmer test results, improvements to the other components of the tear film means that improvements in the third major component of the tear film would follow.[73]

Cranial nerve VII or the facial nerve provides efferent fibers for the sympathetic, parasympathetic, and sensory branches. The facial nerve is known to innervate the lacrimal gland, but innervation to the meibomian glands by this cranial nerve has not been explored, although the path of the parasympathetic fibers to the meibomian glands is very similar to that of the facial nerve.[52] Compromise to the corneal surface associated with facial nerve palsy could cause the patient's inability to blink. However, recent studies have shown that patients with unilateral cranial nerve VII palsy show decreased TFBUT, increased eyelid abnormalities, and compromised meibum expression compared to the contralateral unaffected eye.[74] Two studies showed increased meibomian gland dropout, which was more significant for the inferior lid than the superior lid.[74,75] These studies give credence to the possibility that the ocular surface compromise in these cases may be related to the nervous innervation of the meibomian gland or associated vasculature.

Changes to the nerves with MGD would provide much information regarding the influence of these nerves on the meibomian glands. It has been noted that sympathetic denervation was followed by changes within the ptygopalantine ganglion, which, as described previously, is largely parasympathetic. While the number of fibers within the ganglion did not change, the immunoreactivity of VIP within this ganglion was reduced, and DBH immunoreactive fibers were increased. In addition, immunoreactivity of nerves associated with the meibomian gland showed a reduction of VIP. This suggests that the nerves did not degenerate, but were able to adapt to the sympathetic denervation.[53] The observations made during this study illustrate the complicated nature of these nerves and suggests many possible changes that could occur with MGD.

Overall, the evidence suggests that the parasympathetic fibers may have a direct action on the meibocytes and the meibomian gland ductal cells, while the sympathetic fibers affect the vasculature of the meibomian glands and impact the meibomian glands indirectly. It is possible that these nerves modulate the permeability of the vasculature, which then impacts the amount of hormone exposure. This could provide a link between the presence of nerves and the research related to sex steroids and meibomian glands. The sensory branch may influence the performance of the meibomian gland in a manner similar to the way that the sensory nerves impact the lacrimal gland. How the nerves change with MGD could provide clues into how the nerves regulate these glands.

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