Published in final edited form as:
Physiol Genomics. 2004 June 17; 18(1): 108–118. doi:10.1152/physiolgenomics.00011.2004.

Microarray analysis of the rat lacrimal gland following the loss of parasympathetic control of secretion

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Abstract

Previous studies showed that loss of muscarinic parasympathetic input to the lacrimal gland (LG) leads to a dramatic reduction in tear secretion and profound changes to LG structure. In this study, we used DNA microarrays to examine the regulation of the gene expression of the genes for secretory function and organization of the LG. Long-Evans rats anesthetized with a mixture of ketamine/xylazine (80:10 mg/kg) underwent unilateral sectioning of the greater superficial petrosal nerve, the input to the pterygopalatine ganglion. After 7 days, tear secretion was measured, the animals were killed, and structural changes in the LG were examined by light microscopy. Total RNA from control and experimental LGs ($n = 5$) was used for DNA microarray analysis employing the U34A GeneChip. Three statistical algorithms (detection, change call, and signal log ratio) were used to determine differential gene expression using the Microarray Suite (5.0) and Data Mining Tools (3.0). Tear secretion was significantly reduced and corneal ulcers developed in all experimental eyes. Light microscopy showed breakdown of the acinar structure of the LG. DNA microarray analysis showed downregulation of genes associated with the endoplasmic reticulum and Golgi, including genes involved in protein folding and processing. Conversely, transcripts for cytoskeleton and extracellular matrix components, inflammation, and apoptosis were upregulated. The number of significantly upregulated genes (116) was substantially greater than the number of downregulated genes (49). Removal of the main secretory input to the rat LG resulted in clinical symptoms associated with severe dry eye. Components of the secretory pathway were negatively affected, and the increase in cell proliferation and inflammation may lead to loss of organization in the parasympathectomized lacrimal gland.

Keywords

muscarinic; dry eye

The exocrine lacrimal gland (LG) is the major secretory source of the tear proteins and electrolytes that are essential for the health of the cornea and ocular surface. The cornea and LG, together with other secretory tissues of the surface of the eye and the associated sensory, sympathetic, and parasympathetic nerves, form a functional unit to maintain the health of the ocular surface and the transparency of the cornea (64,65). Conceptually, this model suggests

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that neural systems, linked through the central nervous system (CNS), provide homeostatic control of the ocular surface. In response to environmental signals, sensory nerves relay environmental status information from the surface of the eye to the CNS. In the brain stem, neural information is processed by preganglionic parasympathetic neurons and finally relayed to the peripheral parasympathetic ganglia whose axons supply the secretory drive to the LG. Acetylcholine, released nonsynaptically within the LG from parasympathetic nerves, is acknowledged to be the principal route for stimulation of the largely M_3 muscarinic cholinergic receptors located on the membranes of the LG acinar cells (42,45,75,76).

We have previously shown that sensory innervation plays a role in this functional unit in the rabbit (41,57,71) and rat (50). In the rabbit, sensory input was eliminated by ablating a portion of the trigeminal ganglion containing the cell bodies whose axons receive sensory input from the surface of the eye. This resulted in structural changes in the organization of the LG and increased secretory responsiveness to in vitro stimulation of the LG acinar cells with carbachol, a cholinergic agonist (41,57). Interrupting the neural outflow of the functional unit by permanently severing the preganglionic parasympathetic outflow from the CNS produced more severe structural alterations of the LG, as well as significantly decreased tear flow within 24 h and pathological changes to the ocular surface by 7 days (71).

In the rat, the overall effect of the functional unit was tested by sectioning the preganglionic nerve, which resulted in reduced tear flow and clinical symptoms including disruption of the integrity of the surface of the eye (50). LG structure was also affected.

In our model of ocular surface homeostasis, constant muscarinic stimulation of LG secretion was suggested by regulation of the physiological activity of the parasympathetic ganglion from the CNS. It was also found that, without constant stimulation, the LG had no intrinsic ability to secrete (71). Interruption of neural outflow for tear secretion from the LG also affected components of translation and could lead to alteration in protein synthesis and processing (49). Thus we hypothesized that muscarinic stimulation controls a network of genes responsible for maintaining the secretory function of the LG epithelial cell.

Tissues are known to show adaptive changes in response to innervational status. Our model of the relationship between the innervation of the ocular surface and the target/secretory tissues suggests that this type of relationship may also hold true for the expression of genes. Modulation of gene expression in skeletal systems is probably the best-studied tissue model, and neural influences of both normal and aged muscle have been described (16,24,30). More recent studies on the autonomic innervation of the salivary and other glands have shown a significant but diverse range of effects on function and morphology (20,21,60,69). Rossi et al. (55) reported reduced tear production with dry eye-like symptoms in neurturin-related neurotrophic factor-deficient mice lacking parasympathetic innervation to the harderian glands and LG. Additionally, in mice lacking the M_3 muscarinic receptor (M3R), the salivary secretory response to cholinergic stimulation was reduced (40). The functional importance of muscarinic parasympathetic input to lacrimal secretion is well established; however, the role of muscarinic parasympathetic innervation in the regulation of genes associated with secretory function in exocrine tissues is unclear.

With DNA microarray technology, it is possible to examine the conditional regulation of many genes simultaneously, thus gaining a better understanding of gene function, interactions, and regulation. Because there is no functional cross-innervation between the two LGs of an individual animal and both exist under the same physiological conditions, gene expression in the contralateral LG provides an appropriate baseline level for determining muscarinic parasympathetic control of gene expression in the LG (28). DNA microarrays have been used

in the LG to profile gene expression patterns associated with gender and Sjögren syndrome (4,54).

In this study, we carried out gene expression profiling in the rat LG after removal of the preganglionic parasympathetic control of secretion. The identification of patterns of gene expression provides insights into genes that are conditionally linked to parasympathetic secretory control.

MATERIALS AND METHODS

Preganglionic parasympathetic denervation

Male Long-Evans rats (200–250 g) were purchased from Harlan Laboratories (Bethesda, MD). All animal studies were carried out in accordance with the ARVO Statement on the Use of Animals in Research in Vision and Ophthalmology and with approval from the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee (IACUC). Controlled parasympathetic denervation was carried out in the same manner as previously developed for the rabbit by sectioning and removing several millimeters of the greater superficial petrosal nerve (GSPN) using a middle ear approach (71). All animals undergoing surgery were anesthetized with a mixture of ketamine/ xylazine (80:10 mg/kg) delivered intramuscularly. One sham-operated rat was prepared. The GSPN was identified but not sectioned. The cornea and conjunctiva were evaluated by slit-lamp examination using instillations of fluorescein and rose bengal; these dyes are used clinically to disclose disruptions in the cell layers covering the surface of the eye. Tear secretion was measured from both the parasympathectomized (Px) and normally innervated, contralateral control (Ctla) sides using the Schirmer tear test, which was carried out by placing a 1-mm strip of Schirmer test paper in the cul-de-sac of the eye for 5 min.

Animals were killed with an overdose of pentobarbital sodium, and the LG was removed through a skin incision from the Ctla and Px sides. A 2 mm × 2 mm fragment of LG tissue was fixed for light microscopy; the remaining tissue was frozen immediately in liquid nitrogen. For structural analysis, the tissues from the Ctla and Px sides were placed in a mixed aldehyde solution containing 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) containing 0.2% picric acid for 4 h, and washed overnight in buffer. Plastic sections (1-μm thick) were prepared and stained with toluidine blue and photographed using a Nikon E600 microscope (41).

RNA extraction and microarray analysis

Total RNA was extracted from the experimental ($n = 5$) and control ($n = 5$) LGs [contralateral control ($n = 3$), normal unoperated control ($n = 1$), sham-operated control ($n = 1$)] using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA integrity was determined by spectrophotometry and by formaldehyde gel electrophoresis. Procedures for cDNA synthesis, labeling, and hybridization were carried out as described at **http://www.affymetrix.com/support/technical/manual/expression_manual.affx** (Affymetrix, Santa Clara, CA). All experiments were performed using Affymetrix RG U34A GeneChips as described at **http://www.affymetrix.com/products/arrays/specific/rgu34.affx**. Briefly, 8 μg of total RNA was used for first-strand synthesis using HPLC-purified T7-$(dT)_{24}$ primer. Synthesis of biotinylated-labeled cRNA was carried out using the ENZO RNA transcript labeling kit (Affymetrix) and processed for hybridization. For overnight hybridization, 15 μg of fragmented cDNA was used in an Affymetrix GeneChips Hybridization Oven 640, washed, stained with streptavidin-phycoerythrin using a microfluidics workstation (Affymetrix), and scanned with a confocal laser scanner (Agilent Technologies, Palo Alto, CA). Further

description of the methodology according to MIAME ("minimum information about a microarray experiment") guidelines (**http://www.mged.org/Workgroups/MIAME/miame.html**) is provided in the Supplementary Material (available at the *Physiological Genomics* web site).[1]

Microarray quantification and statistical analysis

Signal and background intensities were quantitated by pixel intensity, and expression signals were analyzed using Affymetrix Microarray Suite 5.0 (MAS 5.0). All array images and quality control measurements were within acceptable limits. Details of quality control measurements are provided in the Supplementary Material. Absolute expression transcript levels were normalized for each array by globally scaling all probe sets to a target signal intensity of 2,500. Three statistical algorithms [detection, change call, signal log ratio (SLR)] were then used to identify differential gene expression in control and experimental samples. The detection metric (present, marginal, or absent) was assigned to each transcript using default parameters in MAS 5.0. For comparison expression analysis, the control samples were used as a baseline, and batch analyses were performed in MAS 5.0, in which pair-wise comparisons between individual experimental and control arrays were made to generate an SLR value for each transcript.

Data and statistical analysis and data visualization were performed with LIMS 3.0 and Data Mining Tools (DMT) 3.0 (Affymetrix). Transcripts that were found absent in three of five experiments in both the control and experimental groups were eliminated from further analysis. Significant gene expression was analyzed using the Mann-Whitney test to compare the signal intensity between the Px LG and the Ctla LG. Two criteria were used to group significant changes in gene expression. First, we defined a positive change call as one in which more than 50% of the transcripts had a call of "increased" (I) or "marginally increased" (MI) for upregulated genes, and "decreased" (D) or "marginally decreased" (MD) for downregulated genes. The median value of the SLR from each comparison file was calculated using DMT 3.0. Second, genes with statistically significant changes were compared based on a percentage of present detection calls (>50%) in the five control or experimental LGs. Genes with median SLR values of >1 or less than −1 were grouped as upregulated and downregulated genes, respectively. Finally, genes were grouped based on their biological functions using Affymetrix Net-Affx, NCBI UniGene, and LocusLink. Complete microarray expression data are available at NCBI Gene Expression Omnibus (GEO) database (GEO submissions GSM12935 through GSM12953, GSE844, NCBI tracking system 15029722) at **http://www.ncbi.nlm.nih.gov/geo**.

RESULTS AND DISCUSSION

Ocular surface and tear secretion

Seven days after removal of parasympathetic neural control of the secretory drive to the rat LG, slit-lamp microscopy of the surface of the eyes revealed obvious pathological changes to the ocular surface. Corneal ulcers developed on the Px side in all animals (Fig. 1). The ocular surfaces of all Ctla eyes (Fig. 1) and all shamoperated eyes (not shown) appeared normal. Tear secretion significantly decreased in all Px eyes compared with the Ctla eyes, indicating a deficiency in tear flow, a consequence of the loss of muscarinic stimulation of acinar cells (Fig. 2). The loss of fluid secretion from the LG onto the ocular surface resulted in drying, abnormal cell sloughing, and loss of the epithelial barrier function as shown by fluorescein staining (71).

[1]The Supplementary Material for this article is available online at **http://physiolgenomics.physiology.org/cgi/content/full/00011.2004/DC1**.

Structural changes in the rat LG

Light microscopy of the LG showed obvious and consistent morphologic departures from the normal structure (Fig. 3). The Ctla LG revealed the lobular organization of the secretory epithelial cells that form the acini embedded in a connective tissue parenchyma characteristic of normal rat LG (59). The Px LG were disorganized and showed a loss of acinar structure, compared with the Ctla LG. Additionally, vesicles more common to endocytic or phagocytic processes were more prominent in the Px LG. The interstitium was more abundant in the Px LG, suggesting a loss or dissolution of the acinar structure. These changes are consistent with those previously reported in the rabbit; however, they were more pronounced in the rat than in the rabbit (41,71).

In our earlier rabbit studies, we found that secretory granules accumulated in the LG after sensory denervation (41). These results suggested that without functional muscarinic parasympathetic innervation, release of secretory proteins is reduced, leading to accumulation of secretory granules. These changes may also be related to the negative effect of removal of parasympathetic regulation of translation and secretory apparatus in the LG acinar cells, leading to reduction in the synthesis and maturation of secretory proteins. Moreover, parasympathetic innervation may provide an as-yet-unidentified type of trophic support to help maintain acinar structure and function of lacrimal acinar cells. In our studies, this trophic support was not a factor, as the experimental procedures did not alter the status of the innervation between the pterygopalatine ganglion and the LG, nor was there any intervention within the LG. In fact, the procedure did not involve any manipulation to ocular structures.

The dramatic decline in tear secretion clearly points to the absence of the muscarinic stimulation necessary to elicit tear flow. Thus, although the innervation is intact from the pterygopalatine ganglion to the LG, the results indicate that, as hypothesized, the neural signals required for the release of neurotransmitter are not available. As shown below, the results of DNA expression profiling suggest that the absence of muscarinic stimulation leads to substantial changes in levels of expression among networks of acinar cell genes.

Microarray data analysis

Based on the detection call comparison between the control and experimental LG, the number of genes expressed in the Px LG (2,792 present genes) was greater than the number in the Ctla LG (2,272 present genes). The greater numbers of expressed genes in the Px LG suggest an elevated gene transcription program in response to alterations in the organization and structure of the LG. The upregulated gene expression pattern may be a compensatory or stress response by acinar cells, which make up 80% of the LG cellular mass. The loss of the major parasympathetic innervation may have an impact on LG tissue homeostasis and trigger transcriptional responses from ductal and interstitial cells.

On the basis of present to absent detection calls for comparison between the Ctla and Px LG, 18 genes were found to be significantly downregulated, only one of which was not detected in any of the experimental LG samples. However, only five genes had a median SLR value less than −1 (Table 1). Conversely, 78 genes were found to be significantly upregulated and 41 of these 78 genes were not detected in any of the five Ctla LG samples, a substantially greater number of genes than was found in the downregulated group. Twenty of the upregulated genes had a median SLR value >1 (Table 2). Given the known biological functions associated with these upregulated genes, it appeared that, without parasympathetic input to the LG, expression of proinflammatory and proapoptotic transcripts increases. Experimental comparison of statistically significant genes based on change call criteria found 45 downregulated genes and 97 upregulated genes in the Px LG (Table 3 and Table 4).

Based on the ocular surface changes seen in both rat and rabbit models, it was hypothesized that the expression and synthesis of secretory proteins would be adversely affected after removal of the parasympathetic control (71). Analysis of the list of downregulated transcripts for putative secretory proteins found only a few such genes (Table 1 and Table 3). Since the messages for secretory proteins have long half-lives (>6 h) and are often found associated with ribonucleoprotein complexes, the transcriptional regulation may not change rapidly after the loss of stimulation. On the other hand, loss of muscarinic stimulation may have a nondestabilizing effect on secretory protein mRNAs, as was found for amylase mRNA in the parotid gland after cholinergic stimulation (37).

Cellular mechanisms controlling mRNA translation operate most efficiently at the initiation step of protein synthesis. The gene for PHAS-I, a heat- and acid-stable protein also known as eIF4E-binding protein 1, was significantly downregulated (Table 3). PHAS-I is a regulator of the initiation step of protein translation (51). However, regulation of secretory proteins is thought to occur at the elongation step of protein synthesis and involve the Ca^{2+}/calmodulin-dependent elongation factor-2 kinase, which was not found to be significantly changed in the Px LG (31). These results suggest that either the initiation step of translation was generally downregulated and/or that selective translation of mRNA is occurring in the Px LG. Accordingly, decreased expression of the L-type amino acid transporter 1 (LAT1) and 4F2hc surface antigen suggests that protein synthesis is decreased in the Px LG (Table 3). As such, it is possible that downstream pathways for secretory protein maturation involving protein folding, processing, and modification in the endoplasmic reticulum (ER) and Golgi may be affected by the lack of muscarinic cholinergic stimulation.

As shown in Table 3, 17 genes associated with protein translocation, folding, and glycosylation in the ER and Golgi were found to have significant changes, and 9 of these 17 genes were downregulated more than twofold. The majority of these downregulated genes are ER-resident proteins, which suggests that protein folding and processing are severely affected by removal of the secretory input.

Furin is a serine endopeptidase localized mainly in the trans-Golgi network. It is associated with proteolytic processing and maturation of precursor proteins into their bioactive form and may also play a role in protein processing of improperly folded membrane proteins in the ER (5,48). Some of the substrates for furin include transforming growth factor-β1 (TGF-β1), matrix metalloproteinase 1 (MMP1), β-nerve growth factor, and insulin-like growth factor (48,72). Another ER-Golgi protein processing activity that may be affected is the UDP-galactose transporter. This Golgi-associated protein is involved in the posttranslational modification of proteoglycan, a critical and rate-limiting step necessary for proper localization and insertion into the plasma membrane and extracellular matrix (62,70).

A number of other genes involved in protein translocation, folding and processing, and transport were found to be significantly downregulated, but less than twofold, including ribophorin II, translocon-associated protein (TRAP), p34, sec61, dolichyl-di-phosphooligosaccharide-protein glycotransferase, and rab26 (Table 3). Overall, based on the biological processes associated with these genes, synthesis of secretory proteins does not appear to be affected, probably because translation of mRNA for secretory proteins is regulated at the elongation step (31).

By contrast, protein quality control and translocation and transport through the lumen of the ER and Golgi do appear to be adversely affected by the lack of muscarinic stimulation, which reduces the capacity for regulated secretion of protein in the LG acinar cells. However, expression of ribosomal protein was upregulated in the Px LG, suggesting an increase in transcription of ribosomal DNA and ribosomal protein genes (Table 4). This situation is

counterintuitive, as protein synthesis is directly related to the status of ribosomal genesis. It is more likely that the expression pattern of upregulated ribosomal protein genes found in the Px LG is a consequence of posttranslational stabilization of the messages that may be correlated with a reduction in protein synthesis.

The development of denervation-induced supersensitivity in target tissues has been reported to involve changes in receptor number in the target tissues, including the rat salivary gland (67). In the rabbit LG, supersensitivity was seen following preganglionic parasympathetic and sensory denervation. Stimulation of LG fragments in vitro with carbachol (a cholinergic agonist) resulted in a significant increase in the amount of protein released (41,49). In the present study, however, the expression of M3R found predominantly in acinar cells was not significantly different 7 days after the GSPN was severed. Similarly, no significant change in the vasoactive intestinal peptide receptor (VIP-R) was found, even though VIP-R colocalizes with acetylcholine in the parasympathetic nerve terminal. Real-time RT-PCR also confirmed that the change in M3R expression was not statistically significant. (Details of M3R expression are available in the Supplementary Materials.) The absence of an increase in the expression of M3R in the parotid gland after unilateral postganglionic parasympathetic denervation has been reported, suggesting that other changes in the parotid gland may contribute to the supersensitivity response (2,67). The reported experimental procedure was somewhat different from that of the present study in that the nerves to the LG remained intact. Since the expression of these receptors was not changed in the present study, it is possible that components of the intracellular signaling pathways associated with either muscarinic or VIP receptor activation may become more sensitized or coupled more efficiently to downstream targets. On the other hand, as in the case of the Ins(1,4,5)P_3/Ca^{2+} and protein kinase C signal cascade, one branch may be preferentially activated after the loss of muscarinic stimulation.

Calcium released from intracellular stores stimulates secretion and serves as an important signaling and sensor molecule in the cytosol (8). Depletion of Ca^{2+} from the ER leads to accumulation of misfolded proteins, activates expression of ER chaperones, and inhibits protein translation (9). Removal of parasympathetic control, which would have the effect of decreasing muscarinic parasympathetic stimulation, may in fact serve as a trigger to mediate a cellular stress response. Thus one would expect that without stimulation-induced Ca^{2+} release, secretion would be reduced and expression of cytoplasmic and ER chaperones would not be altered. We found that mRNAs for protein disulfide isomerase (PDI), calreticulin, immunoglobulin heavy chain binding protein (BiP) [also known as the glucose-regulated protein-78 (GRP78)], and FK506 binding protein were significantly decreased, most greater than twofold. Cyclophilin B, a peptidyl prolyl isomerase that is also found in the ER, was also significantly decreased (Table 3). PDI catalyzes disulfide formation critical for folding and assembly of secretory proteins (19,26,43). In the absence of muscarinic activation, mobilization and depletion of Ca^{2+} stores in the ER do not take place, and the molecular chaperones and disulfide isomerases necessary for proper maturation of secretory and membrane proteins are not needed.

In the LG, as well as in the salivary gland and pancreas, Ca^{2+} is released from the ER store by activation of the Ins(1,4,5)P3 receptors and then taken up by SERCA, a Ca^{2+}-ATPase of the ER, after the secretory signal ceases (33). The Ca^{2+} storage capacity in the ER is modulated and enhanced by Ca^{2+}-binding proteins to maintain cellular Ca^{2+} homeostasis. Several Ca^{2+}-binding proteins were found to be significantly downregulated, including calreticulin, oncomodulin (also known as parvalbumin-β), and calmodulin (Table 3). These Ca^{2+}-binding proteins also serve as modulators of Ca^{2+}-mobilizing stimuli and as regulators of the cytoskeletal contractile apparatus (8,44). Additionally, calreticulin has been shown to modulate transcriptional activity of nuclear hormone receptors such as the androgen receptor and retinoic acid receptor, which are known to have a profound effect in maintaining LG function (15,

46). Calmodulin, an effector of Ca^{2+}-mediated secretion of the Ins(1,4,5)P_3/Ca^{2+} pathway, is also a member of this superfamily; its expression was also significantly decreased. The downregulation of these Ca^{2+}-binding proteins and effectors affects the cell's ability to respond to agonist-mediated inositol-dependent Ca^{2+} from the ER, with limited consequences on Ca^{2+} storage capacity in the ER (15,36). Additionally, expression of a Ca^{2+}-independent phospholipase A_2 was significantly downregulated in the Px LG (Table 1), which may affect membrane lipid hydrolysis and transmembrane ion flux (38). The expression of the Ca^{2+}-binding proteins may be under parasympathetic control, and the decreased expression of these proteins resulting from the lack of parasympathetic input may lead to the inability or reduced capacity of the Px LG to respond to hormonal stimulation.

Annexin represents a class of Ca^{2+}-binding proteins also known as the lipocortin protein family (8). In this study, expression of annexin I and annexin II were both upregulated (Table 4). Annexins are cytosolic and associated with the membrane or the cytoskeleton, binding phospholipid in a calcium-dependent manner. The annexin family of proteins plays a role in mediation of the steroid anti-inflammatory response, membrane aggregation and fusion, endocytosis and exocytosis, and apoptosis (3,7,52). Since these proteins bind cellular phospholipids, it is plausible that there may be an increase in membrane phospholipid metabolism, thereby affecting exocytotic and endocytotic processes (7). Similarly, increased expression of annexin I has been implicated in the membrane clustering mediation of the phagocytosis of apoptotic cells (3). In cells where annexin I was overexpressed, calcium release was abrogated on stimulation of purinergic or bradykinin receptors. However, basal calcium and calcium stores in the ER and mitochondria were not affected (27). The increase in annexin I expression and activity may affect Ca^{2+} release from Ins$(P)_3$-sensitive stores, an impairment that may be attributed to the lack of phospholipase C activity from loss of muscarinic cholinergic stimulation in the Px LG.

Initially, the LG acinar cell secretion is an NaCl-rich, plasma-like fluid. This fluid is modified by LG ductal cells, primarily by NaCl absorption, to produce a final KCl-rich fluid (47). Accordingly, stimulation via Ca^{2+} agonists enhances fluid exchange and transport by upregulating acinar NKCC1 cotransporter and Na^+/K^+ exchange activity (22). This cotransporter has been found in both acinar cells and duct cells and plays an important role in volume regulation. Treatment with a specific blocker of NKCC1 has been shown to reduce LG fluid secretion in response to carbachol (72). In the salivary glands of mice deficient in NKCC1, a severe deficit in saliva secretion in vivo was reported (23). This may be the result of a deficiency in Cl^- uptake as acinar Cl^- influx was reduced in vitro. However, in the present study, NKCC1 expression was upregulated in the absence of parasympathetic activation. This contradicts the expectation that downregulation of NKCC1 would occur in the face of diminished tear secretion in the Px LG as measured by the Schirmer tear test (71). It is possible that this is a compensatory mechanism responding to osmotic challenge and that other compensatory mechanisms may also exist and be activated in response to a decreased muscarinic signaling system.

The hormonal milieu of the LG also plays an important role in modulating LG secretion and function. Melanocortin-5 receptor (MC5R) is abundantly expressed in exocrine tissues, specifically in the acinar cells of the LG (10,34). In this study, expression of MC5R was significantly downregulated in the Px LG. Melanocortin-stimulating hormone (MSH) and adrenocorticotropic thyroid hormone (ACTH) increase cAMP level in the LG and peroxidase secretion (13,32). However, in mice deficient in MC5R, stimulation with physiological levels of MSH and ACTH did not stimulate protein secretion (10).

Another pituitary-derived peptide hormone with an important modulating role in the LG is prolactin. Prolactin has been shown to increase expression of muscarinic receptors, maintain

acinar cell morphology, and affect secretion (46). Prolactin and prolactin receptors are also synthesized in the LG, and prolactin is a secretory product of acinar cells (74). Expression of prolactin receptor and MC5R was significantly decreased, with a greater than twofold reduction, in the Px LG (Table 1 and Table 3). The decreased expression of the prolactin receptor may compromise prolactin-mediated modulation of LG prolactin levels and muscarinic receptors. Previous studies suggest that this may alter the immunoregulatory environment of the LG (46).

Pathological changes in the ocular surface and LG with and without associated autoimmune components correlate with increased lymphocytic infiltration, cytokine synthesis, and apoptosis (25). In the present study, expression of many genes coding proinflammatory cytokines, complements, and proteolytic enzymes, as well as apoptotic-related gene expression, was found to be upregulated in the Px LG (Table 1 and Table 4). Interestingly, a considerable number of genes coding for the major histocompatibility complex (MHC) class II were also upregulated (Table 4). Generally, normal LG acinar cells do not express MHC-related genes in vivo; however, carbachol stimulation can induce expression of MHC class II genes in the LG in NZB/W mice at the age of onset of autoimmune disease and in acinar cells grown in culture (46). It is plausible that removal or loss of extracellular matrix attachment, perturbation of normal cytosolic and ER Ca^{2+} homeostasis, or a dramatic change in transcriptional activity may induce MHC gene expression, thus transforming normal cells into antigen-presenting cells. Moreover, protein fragments derived from lysosomal cathepsin proteolysis and engulfed cellular contents that have entered the endosomal sorting may be bound by MHC class II molecules and presented at the cell surface as antigens (68). Expression of proinflammatory and apoptotic caspases 1, 2, and 6 was significantly upregulated (Table 2). Caspase 6 is the major caspase in apoptotic cells and has been found to cleave poly(ADP-ribose) polymerase and nuclear lamin A (12). DNA microarray analysis of MRL/lpr mice, one type of model of Sjögren syndrome, also showed increased expression of inflammation- and apoptosis-associated genes. However, the LGs of the mice used in that study had severe lymphocytic infiltration and represented a late stage of the disease (4). The proinflammatory and proapoptotic genes found in our study suggest an early event in the inflammatory process, in that light microscopy results did not reveal any lymphocytic infiltration.

Extracellular matrix proteins and regulatory mediators of synthesis, maintenance, and degradation were generally found to be upregulated in the LG after loss of muscarinic stimulation. Upregulated extracellular matrix proteins and secreted proteins included fibronectin, decorin, secreted acidic cysteine-rich glycoprotein (SPARC), amyloidogenic glycoprotein, and pulmonary surfactant protein (SP-D) (Table 4). SP-D, also called collectin-7, was abundantly present in the luminal contents of secretory cells, including the LG. SP-D binds carbohydrates and lipids in a calcium-dependent manner and may have a role in the innate host defense against pathogens at sites of entry into various organs (63). Cystatin C, another lipid-binding protein found to be upregulated, may be related to the lipocalin family of lipid-binding proteins and may have anti-cysteine protease properties (1,17,53,56). In our study, the common salivary protein 1 (CSP1) was the most highly expressed gene found in the Px LG (SLR = 3.51). CSP1 is secreted by the salivary gland and has been localized to the intercalated duct cells of the parotid, the submandibular glands, and demilune cells of the sublingual gland (29,61). As CSP1 had not been previously detected in the LG (29), the identification and differential expression of CSP1 found in this study may be the result of a difference in the sensitivity of the molecular technical procedure. Ebnerin, another gene that was upregulated in this experiment, is homologous to the deleted gene in malignant brain tumor 1 (DMBT1), CRT-ductin, and Hensin (6,11,35,66). It has been found in association with SP-D and is also found in tear fluid (39,58). Ebnerin's function in the LG is also unclear, but it may, like SP-D, affect the innate immune response and, possibly, mediation of cell proliferation (6,11,58).

In the present study, an interesting though not surprising finding was the increased expression of genes associated with cell structure and tissue remodeling. Expression of structural proteins, including smooth muscle SM22, myosin regulatory light chain isoform C, tropomyosin, actin binding protein coronin 1A, and fibronectin, suggests increased myoepithelial and parenchymal cell activity (47). Coronin regulates non-muscle cell cytoskeleton remodeling, as well as cell adhesion and migration (14,18). The conditions for normal tissue remodeling are favorable when the expression and activity of proteases, protease inhibitors, and extracellular matrix-associated proteins are coordinately regulated. Three genes were significantly upregulated more than twofold in the Px LG: SPARC, tissue inhibitor of metalloproteinase type 2 (TIMP-2), and plasma protease C1 inhibitor precursor (similar to α-1 antitrypsin). This suggests a shift in the balance of protease and protease inhibitors to a more inhibitory condition that may lead to abnormal tissue maintenance. Finally, intracellularly, expression of lysosomal membrane glycoprotein 1, lysosomal acid lipase, and proteasome subunit RC1 was upregulated, suggesting an increase in lysosomal activity and protein proteolysis.

Overall, DNA microarray analysis showed gene expression patterns shifted toward greater gene activation, possibly as part of the survival genetic response. In the absence of activation of the M_3 muscarinic receptor, genes coding for components of the M_3 muscarinic receptor signal transduction cascade were repressed, as were genes associated with protein synthesis and posttranslational processing. This illustrates the importance of the main neural input in protein synthesis and directly links quality control to protein processing (protein folding and glycosylation) in a secretory tissue. Precursor proteins that transit through the secretory pathway often require proteolytic cleavage to release their bioactive entities. A loss of or reduction in the capacity for this process may negatively affect the regulated secretory pathway and the proper expression of specific proteins and secretory granule genesis. Perturbation of Ca^{2+} homeostasis decreases the capacity of acinar cells to respond to stimuli, affects quality control of protein processing and maturation, and represents a form of stress that may trigger apoptosis. Additionally, by modulating the expression of receptors for hormones, parasympathetic innervation affects the responsiveness of acinar cells to circulating hormones such as ACTH and prolactin. The MC5R may have a direct, trophic role in maintaining lacrimal function and secretion as the MC5R–deficient rat develops alacrimia; this dysfunction was hypothesized to be the result of impairment of MC5R–coupled signal transduction pathways (34). On the other hand, parasympathetic input may be required for the maintenance of acinar epithelial differentiation and overall tissue organization, in that the current study demonstrated an increase in interstitial content including extracellular matrix production, inflammatory mediators, induction of immunity-associated genes, and proteolytic activation. Loss of parasympathetic activation of acinar cells may trigger glandular atrophy, followed by induction of apoptosis and targeting of acinar cells for destruction by infiltrating lymphocytes.

In conclusion, parasympathetic innervation and, by extension, muscarinic cholinergic stimulation are critical elements in maintaining LG structure and cellular organization. The consequences of loss of innervation may be an early step in the pathogenesis of dry eye in the clinical setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Juan Reynaud and Lia Pedroza-Schmidt of the LSU Eye Center for their invaluable assistance with photography and light microscopy.

GRANTS

This work was supported in part by National Eye Institute Grants R01-EY-012416 (to R. W. Beuerman) and P30-EY-002377 (departmental core grant) and by an unrestricted departmental grant from Research to Prevent Blindness, Inc., New York, NY.

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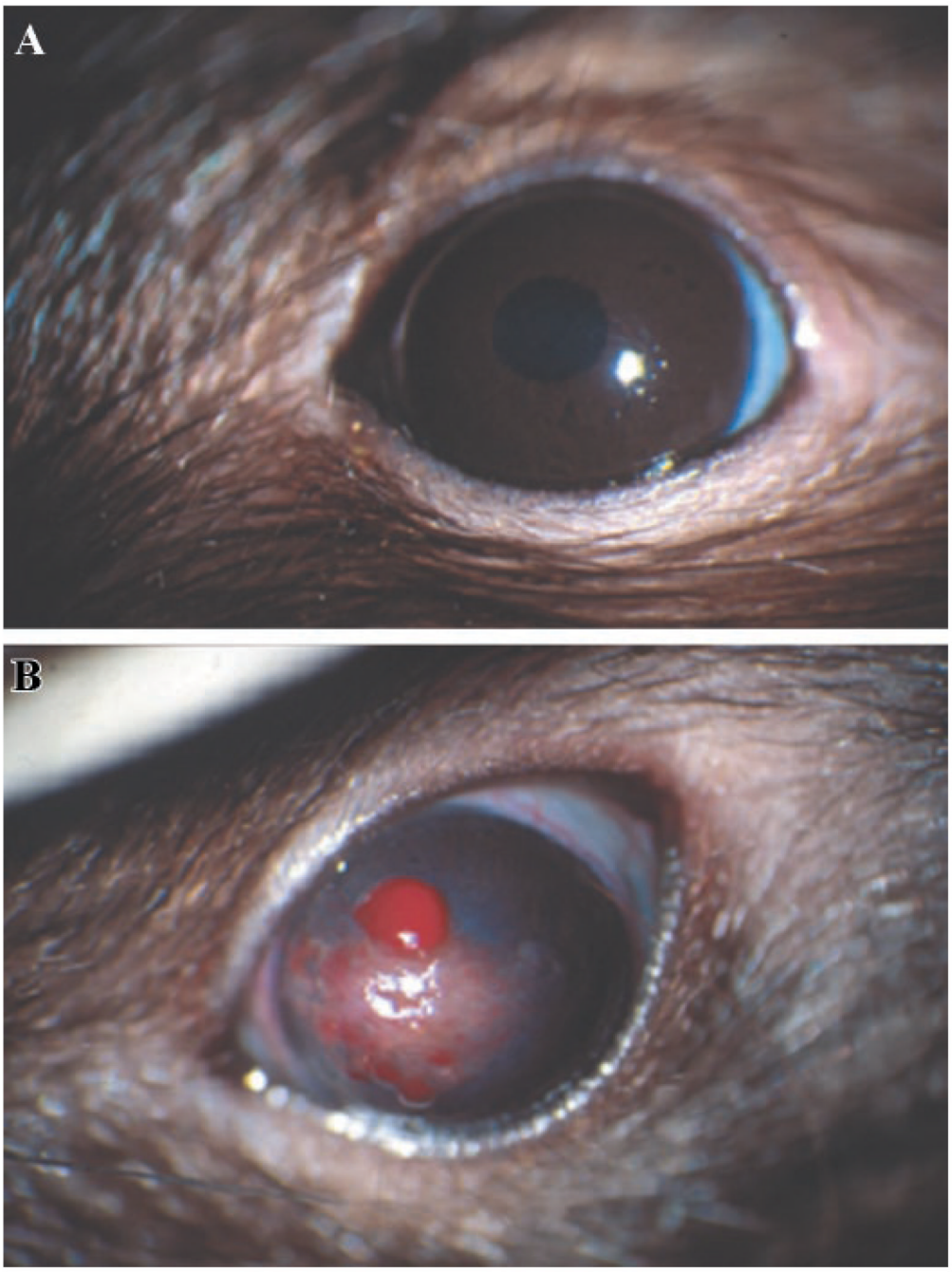

Fig. 1.
Slit-lamp microscopy of the rat ocular surface 7 days after parasympathectomy. Control (*A*) and parasympathectomy (*B*).

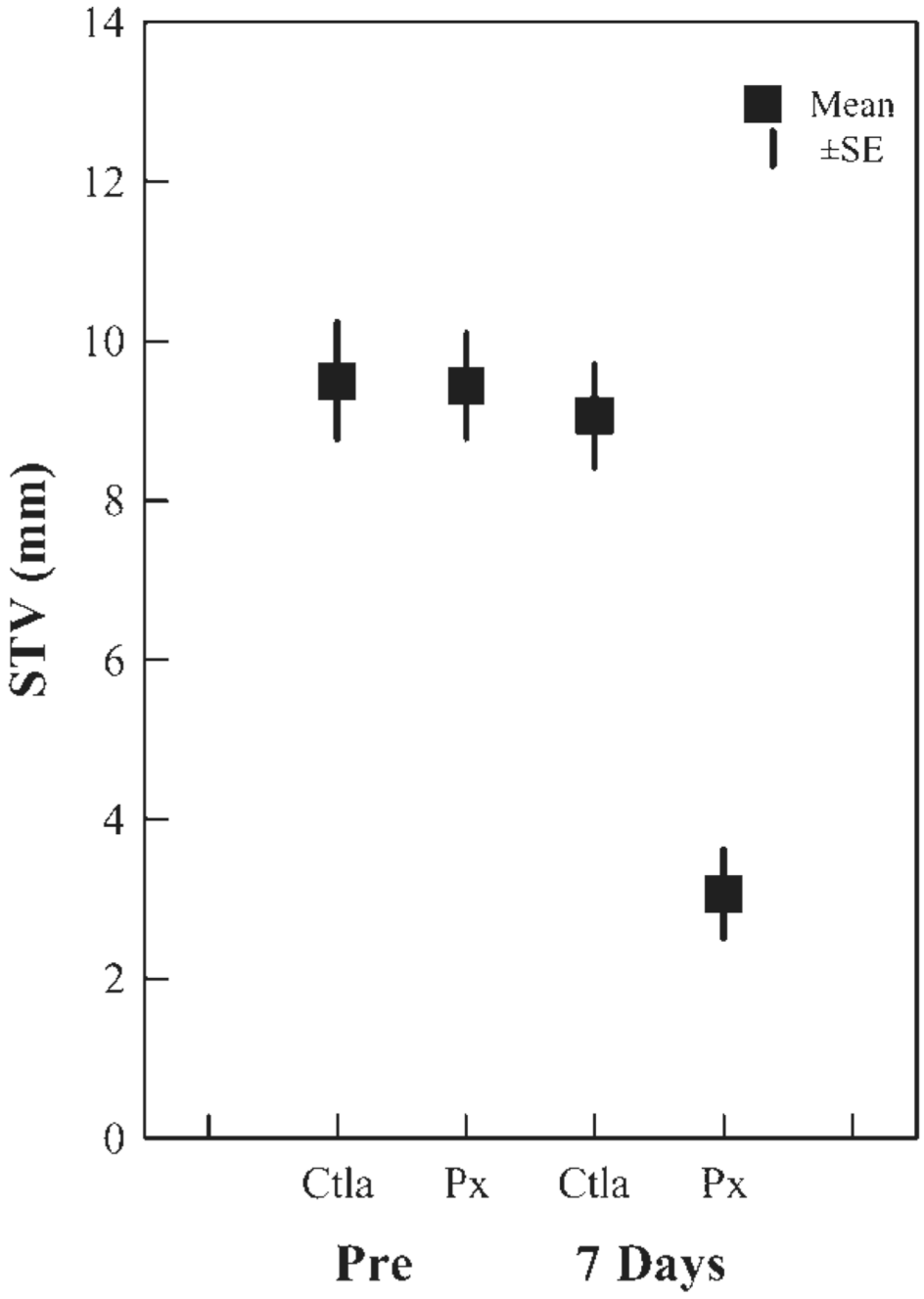

Fig. 2.
Tear output from the control (Ctla) and parasympathectomized (Px) eyes before (Pre) and 7 days after surgery. STV, Schirmer tear value.

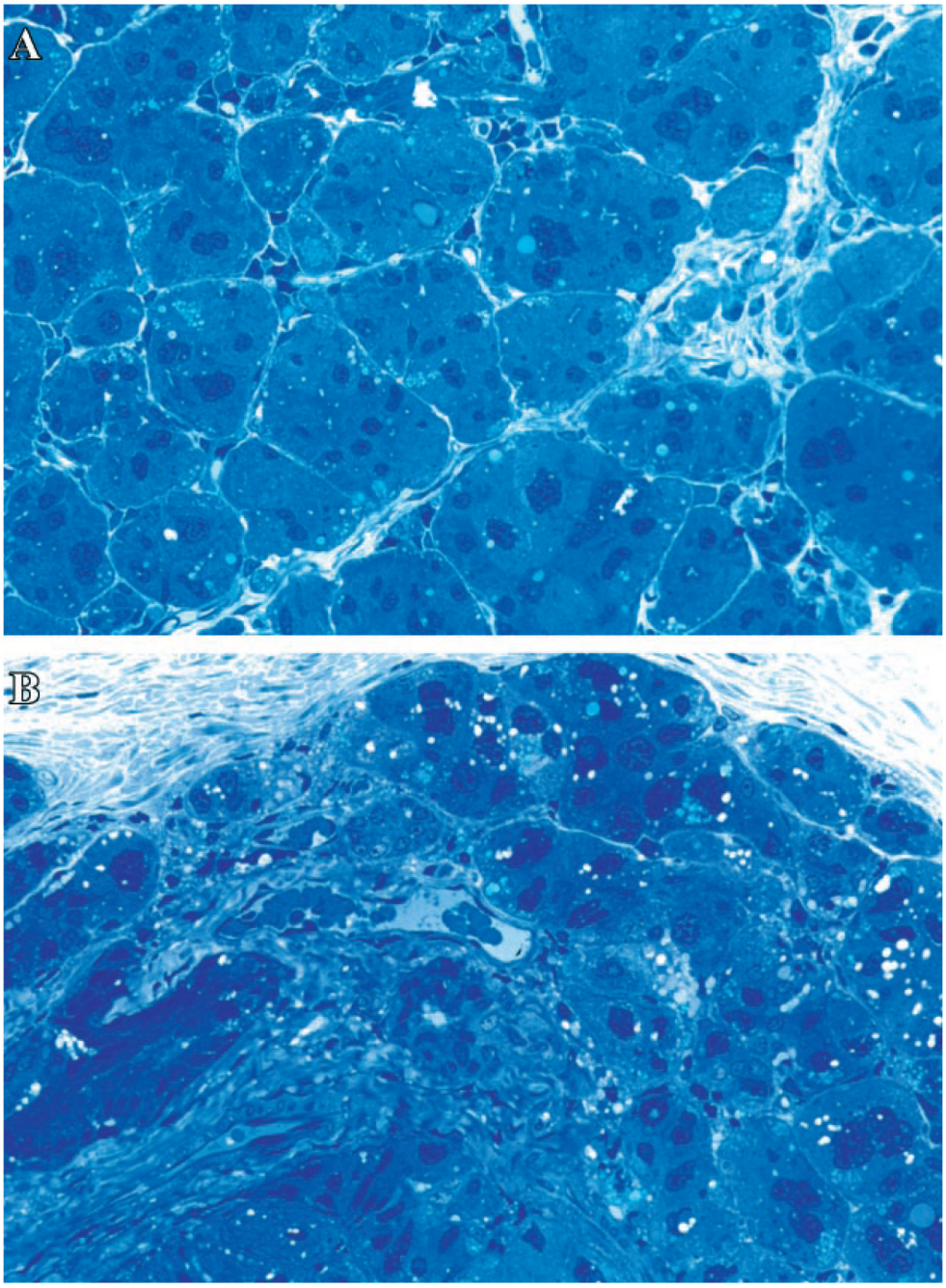

Fig. 3.
Light micrographs of the lacrimal gland 7 days after parasympathectomy. *A*: control side. *B*: parasympathectomized side. Toluidine blue; original magnification ×100.

Table 1

Transcripts present in the control lacrimal gland and absent in the parasympathectomized lacrimal gland with SLR less or equal to −1

Accession No.	Median SLR	Description
S48813	−1.88	β-Adrenergic receptor kinase
M74152*	−1.36	Prolactin receptor
E03344	−1.24	Peroxisome forming factor
U51898	−1.15	Ca^{2+}-independent phospholipase A_2
AJ005046	−1.13	Fructose-1,6-bisphosphatase

Expressed sequence tags (ESTs) were identified based on UniGene or LocusLink.

* Gene also represented in Table 3 SLR, signal log ratio.

Table 2

Transcripts absent in the control lacrimal gland and present in the parasympathectomized lacrimal gland with SLR ≥1

Accession No.	Median SLR	Description
U14647	2.46	Interleukin-1β converting enzyme (caspase 1)
U10894	2.41	mRNA expressed in carotid artery tissue
D12524	1.48	c-kit receptor tyrosine kinase
U77933	1.2	Nedd2/Ich-1 (caspase 2)
U17919	1.13	Allograft inflammatory factor-1
AF025670	1.08	Caspase 6
M64986	1.14	Amphoterin
AF086758*	1.74	Na-K-2Cl cotransporter (Nkcc1)
U49099	1.05	*cis*-Golgi p28
M18416	1.14	Nerve growth factor-induced (NGFI-A) gene
L07114	1.75	Apolipoprotein B mRNA editing protein
L22339	2.34	*N*-hydroxy-2-acetylaminofluorene (ST1C1)
S56937	1.38	UDP-glucuronosyltransferase
AA926129	1.08	SOD-2
AA800844	1	Protein-lysine 6-oxidase precursor
D00753	1.03	Contrapsin-like protease inhibitor related protein (CPi-26)
S72637	1.51	Tumor suppressive gene
X03347	1.99	Unknown
AA799507	1.96	Unknown
AA893663	1.3	Unknown

ESTs were identified based on UniGene or LocusLink.

* Gene also represented in Table 4.

Table 3

Significant genes with >50% decrease change call

Accession No.	Median SLR	Description
Translocation		
AA685175	−1.55	Ribosome binding protein 1 isoform mRRp61
AF100470	−1.28	Ribosome attached membrane protein 4 (RAMP4)
M96630	−0.73	sec61 homolog
X55298	−0.66	ribophorin II
AA819338	−0.65	TRAP-delta
D13623	−0.58	p34 protein
Fold/process		
S63521*	−1.31	Glucose-regulated protein GRP78
02918	−1.27	Protein disulphide isomerase
D78308	−1.11	Calreticulin
X55660	−1.1	Furin
M14050*	−1.08	Immunoglobulin heavy chain binding protein (BiP)
AA875098	−1.0	FK506 binding protein precursor
AA891161	−0.79	Probable ER oxidoreductin 1-beta homolog
AF071225	−0.47	Cyclophilin B
Glycosylation		
D87991	−1.22	UDP-galactose transporter related isozyme 1
AF087431	−0.81	Glycoprotein processing glucosidase I
AA891937	−0.67	Dolichyl-di-phosphooligosaccharide-protein glycotransferase
Vesicle transport/ transporter		
AJ004912	−0.73	Integral membrane protein Tmp21-I (p23)
U18771	−0.56	rab26
Protein synthesis		
AB015432	−2.0	LAT1 (L-type amino acid transporter 1)
X89225	−0.9	4F2hc surface antigen
U05014	−0.73	PHAS-I
Signaling		
J02705	− 1.66	Oncomodulin
L27081	− 1.52	Melanocortin 5 receptor (MC5R)
M74152	− 1.36	Prolactin receptor
AB007690	−1.14	Vesl-2 (delta 11)
Y08355	−0.73	PKC-zeta-interacting protein
AA892649	−0.74	γ-Aminobutyric acid receptor associated Protein
AA957510	−0.73	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2)

Accession No.	Median SLR	Description
AA891209	−0.71	Interleukin 25
X13933	−0.55	Calmodulin
Metabolism-mitochondria		
AI012802	−1.24	Hydroxyacyl glutathione hydrolase; glyoxalase II
M89945	−1.08	Farnesyl diphosphate synthase gene
J04791	−0.94	Ornithine decarboxylase (ODC)
J03914	−0.75	Glutathione *S*-transferase Yb subunit gene
D13123	−0.65	P1 mRNA for ATP synthase subunit c
Protein metabolism		
J04206	−1.56	Cystatin S
X16273	−1.42	Serine proteinase inhibitor-like protein
AI010453	−0.83	α1-antitrypsin
Unassigned		
Y13336	−1.09	Defender against cell death 1 (DAD-1)
L20900	−0.94	Autoantigen p69
AF008554	−0.76	Implantation-associated protein (IAG2)
S75019	−0.66	Antiquitin
AI639365	−0.9	Unknown
AA799822	−0.69	Unknown
AA800034	−0.52	Unknown

The transcripts from these genes were decreased in the parasympathectomized lacrimal gland with a >50% change call (decrease, marginal decrease).

ESTs were identified based on Unigene or LocusLink.

*Same gene with different accession numbers.

Table 4

Significant genes with >50% increase change call

Accession No.	Median SLR	Description
Extracellular		
U00964	3.51	Common salivary protein 1
AA891204	2.11	Secreted acidic cysteine-rich glycoprotein (SPARC)
X07648	1.9	Amyloidogenic glycoprotein (rAG)
AA891054	1.52	Mucin 2 precursor
X05834	1.37	Fibronectin
S72594	1.35	Tissue inhibitor of metalloproteinase type 2 (TIMP-2)
U32681	1.28	Ebnerin
Y13275	1.68	D6.1A protein
AF065438	1.19	Mama
U07619	1.18	Tissue factor protein
AA800318	1.16	Plasma protease C1 inhibitor precursor
X59859	1.03	Decorin
AI231292	0.95	Cystatin C
M81231	0.92	Pulmonary surfactant protein (SP-D)
AI012030	0.88	Matrix Gla protein
M31322	0.69	Sperm membrane protein (YWK-II)
Cell structure		
S77900	2.13	Myosin regulatory light chain isoform C
AA892506	2.08	Coronin, actin binding protein 1A
M83107	2.02	SM22
X81449	1.6	Keratin 19
AF002281	1.37	α-actinin-2 associated LIM protein
L00382	1.07	β-tropomyosin and fibroblast tropomyosin 1
M15474	0.76	α-tropomyosin
M60666	0.67	α-tropomyosin 2
X52815	0.62	Cytoplasmic-γ isoform of actin
AI072634*	0.71	Keratin complex 1, acidic, gene 18
X81448*	0.44	Keratin 18
AA860030	0.39	β-tubulin (isotype Mbeta 5)
Immunity and inflammation		
Y08358	2.1	Eotaxin
X52477	1.89	Pre-pro-complement C3
AA799803	1.82	Complement C1r component precursor
M15562	1.34	MHC class II RT1.u-D-alpha chain
X14323	1.21	IgG receptor FcRn large subunit p51
X14254	1	MHC class II-associated invariant chain

Accession No.	Median SLR	Description
M57276	0.99	Leukocyte antigen MRC-OX44
L26267	0.76	Nuclear factor kappa B p105 subunit
M28671	0.73	Rearranged IgG-2b gene
AF029240	0.72	MHC class Ib RT1.S3 (RT1.S3)
K02815	0.7	MHC class II RT1-β alpha chain)
M36151	0.67	MHC class II A-beta RT1.B-b-beta gene
Protein metabolism		
S81497	2.57	Lysosomal acid lipase
AI177256	1.38	Lysosomal membrane glycoprotein 1
X98517	1.27	Macrophage metalloelastase
D10729	0.95	Proteasome subunit RC1
AI172162	0.35	Proteasome (prosome, macropain), beta type 4
Signaling		
AI171962	2.23	Annexin 1
L13039	1.57	Annexin II
M60753	1.29	Catechol-*O*-methyltransferase
AA858617	1.03	TC10-like Rho GTPase
X67788	0.85	Ezrin p81
X60769	0.7	Silencer factor B
D85183	0.69	SHPS-1
U18314	0.67	Lamina associated polypeptide 2 (LAP2)
J05122	0.65	Peripheral-type benzodiazepine receptor (PKBS)
AA899253	0.64	Myristoylated alanine-rich C-kinase substrate (MARCKS)
AI234604	0.55	Similar to 70-kDa heat-shock-like protein
M11942	0.42	70-kDa Heat-shock-like protein
Metabolism-mitochondria		
U18729	2.07	Cytochrome b558 α-subunit
D13122	1.13	ATPase inhibitor protein
AA892314	0.81	Isocitrate dehydrogenase 1
AI172411	0.7	Glutathione peroxidase 3
M26125	0.48	Epoxide hydrolase
M84716	0.34	Fte-1
AI169802	0.67	Ferritin, heavy polypeptide 1
M34043	0.66	Thymosin β-4
Ribosome		
J03969	1.06	Nucleolar protein B23
M12156	0.8	hnRNP A1
X15216	0.65	Ribosomal protein L21
AI008641	0.62	Ribosomal protein L22
X51707	0.6	Ribosomal protein S19

Accession No.	Median SLR	Description
AA850940	0.53	Ribosomal protein L4
AI177683	0.52	hnRNP protein
AI104544	0.51	Ribosomal protein S17
M55015	0.51	Nucleolin gene
M29358	0.5	Ribosomal protein S6
AA875102	0.5	Small nuclear ribonucleoprotein E
X57432	0.48	Ribosomal protein S2
53504	0.47	Ribosomal protein L12
AA799899	0.46	Ribosomal protein L18a
X62166	0.42	Ribosomal protein L3
58465	0.41	Ribosomal protein S5
AA817997	0.4	Ribosomal protein L24
M34331	0.39	Ribosomal subunit protein L35
X52733	0.38	Ribosomal protein L27a
M17419	0.38	Ribosomal protein L5
X14671	0.37	Ribosomal protein L26
AA849038	0.3	Ribosomal protein L31
AA891729	0.25	Ribosomal protein S27a
Transport		
X04979	1.85	Apolipoprotein E
AF051561	0.67	Na-K-Cl cotransporter (Nkcc1)
M15882	0.45	Clathrin light chain (LCA1)
Unassigned		
X0726	2.32	Gene 33 polypeptide
AI639107	2.04	Unknown
AA893088	1.14	Unknown
AA892986	1.13	Unknown
AI176460	0.96	Unknown
U77829	0.94	gas-5 growth arrest homolog
AA874803	0.74	Unknown

The transcripts from these genes were increased in the parasympathectomized lacrimal gland, with >50% change call.

ESTs were identified based on UniGene or LocusLink.

* Same gene with different accession numbers.