The Dopaminergic Neuronal System Regulates the Inflammatory Status of Mouse Lacrimal Glands in Dry Eye Disease

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Purpose. Comparison of the parasympathetic and sympathetic neurons, including the dopaminergic neural system, in dry eye (DE)–induced pathophysiology has not been elucidated well. This study investigated the presence of dopamine receptors (DRs) and their functional roles in the lacrimal glands (LGs) of DE-induced mice.

Methods. After DE was induced in B6 mice for 2 weeks, the expression of tyrosine hydroxylase (TH), dopamine, and DRs (DR1, DR2, etc.) in the LGs and corneas were measured by quantitative RT-PCR, immunoblot, and ELISA. Using flow cytometry and ELISA, immune cell infiltration and inflammatory cytokine expression were determined in DE-induced LGs with or without DR blockers, SCH-23390 (DR1i), or melperone (DR2i). Corneal erosion scores were also investigated.

Results. The mRNA and protein levels of TH significantly increased in DE-induced LGs. The dopamine concentration of LGs was 9.51 pmol in DE (versus naive: 1.39 pmol; $P < 0.001$). Both DR1 and DR2 mRNA expression were significantly enhanced in desiccating stress compared with those in naive (3.7- and 2.1-fold, $P < 0.001$). Interestingly, DR1 and DR2 immunostaining patterns stained independently in DE-induced LGs. $CD3^+$ and $CD19^+$ cell infiltration was significantly increased by DR2i ($P < 0.001$) but not by DR1i. Furthermore, IFN-γ, IL-17, and TNF-α were significantly upregulated by DR2i compared with the blow-only condition. The severity of corneal erosion and inflammation was also aggravated by DR2i.

Conclusions. Upregulation of DR1 and DR2 was observed in DE-induced mouse LGs. As the inflammatory conditions are aggravated by the inhibition of DRs, especially DR2, their activity may be an important factor preserving ocular surface homeostasis.

Keywords: dry eye disease, dopamine, dopamine receptor, lacrimal gland

Neural integrity and reflex between the lacrimal gland (LG) and ocular surface are essential for ocular surface homeostasis. Damage to the corneal nerves, arising anywhere from cell bodies in the central nervous system (CNS) to the nerve endings embedded in the corneal epithelium, is thought to underlie several ocular surface diseases, such as neurotrophic keratitis, neuropathic pain, and dry eye (DE) disease.[1,2] The neural network involved in the neural regulation of tear production is extremely complicated, both anatomically and functionally. The muscarine M3 receptor, nicotinic receptors, vasoactive intestinal peptide,[3,4] substance P,[5,6] and sympathetic system contribute to tear production, in terms of regulating levels of aqueous fluid, electrolytes, and soluble peptide production[7,8]; therefore, the production of aqueous fluid and proteins is finely regulated by many neuronal peptides and neurotransmitters in addition to the cholinergic system.

Apart from the cholinergic system, the sympathetic and dopaminergic systems play important roles in the exocrine functions of the salivary glands[9] and LGs.[3,10,11] Dopamine increases the salivary flow rate and protein secretion by stimulating dopamine receptor 1 (DR1)–like receptors.[12] Additionally, the secretion of amylase, the most abundant protein in saliva, is mediated via specific DR1-like receptors.[13] Dopamine receptor 2 (DR2) has been found in a wide variety of ocular tissues, such as in keratocytes,[14] the retinal pigment epithelium (RPE),[15] and the corneal epithelium.[16] Within these tissues, DR2 has been reported to have functional roles in preventing RPE cell death,[15] reducing intraocular pressure,[17] and improving corneal wound healing and nerve density levels in a murine model.[18] Unfortunately, while there are a few previous studies reporting on dopaminergic receptors within the ocular system, the data on the influence of dopamine on the exocrine functions of

ocular glands are sparse and often inconsistent.[9] There have been few studies on identifying the role of the dopaminergic system in tear fluid secretion and dopamine subtype receptor expression levels or patterns in LGs. Moreover, dopamine has emerged as a fundamental regulator of inflammation, and dysregulation of the dopaminergic system affects both innate and adaptive immunity, contributing to the development of autoimmune and inflammatory diseases.[19]

Hence, this study has three aims. The first is to determine the existence of dopaminergic receptor subtypes in LGs and the ocular surface. The second is to determine the expression levels and functional role of the dopaminergic system in DE pathophysiology. The third is to investigate the interaction and reciprocal between the parasympathetic and dopaminergic systems in vivo using a DE murine model.

Materials and Methods

Animals and DE Induction

Six- to 8-week-old male C57BL/6 mice (Charles River Laboratory, Wilmington, MA, USA) were used according to the standards outlined in the AVO Statement for the Use of Animals in Ophthalmic and Vision Research. The research protocol was approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine. DE was induced by placing the mice in a controlled environment chamber (CEC). Mice in the CEC (with relative humidity below 13% and 3-m/s flow rate) were subcutaneously injected with 0.1 mL scopolamine hydrobromide (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) three times per day to achieve maximum ocular surface dryness. Additionally, to block the dopaminergic neurons, DR1 blocker (SCH-23390, 0.3 mg/kg; Cayman Chemical, Ann Arbor, MI, USA) and DR2 blocker (Melperone, 2 mg/kg; Tocris Bioscience, Abingdon, UK) were injected subcutaneously, following a previously published protocol.[20–22] All data were retrieved from two independent experiments ($n = 5$) in triplicate in each group.

Tissue Preparation

On each day of the experiment, mice were euthanized and the corneas and LGs were collected. Each tissue sample was halved and fixed in 3.7% paraformaldehyde for immunostaining or stored at −70°C for quantitative real-time polymerase chain reaction (qRT-PCR) or immunoblotting. The detailed methods have been described in our previous studies.[23,24]

Tissue RNA Extraction and qRT-PCR

RNA was isolated from mouse LGs using an RNeasy Micro Kit (QIAGEN, Valencia, CA, USA), and reverse transcription was performed using a Superscript III Kit (Invitrogen, Carlsbad, CA, USA). qRT-PCR was performed using SYBR Premix Ex Taq (TaKaRa Bio, Inc., Otsu, Japan) with a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Preformulated primers for DRs and other targets are described in the Table.

Immunofluorescent Staining

The method used for immunofluorescent (IF) staining has been described previously.[25] Briefly, the LGs and eyeballs were harvested and cryopreserved by embedding in optimal cutting temperature compound before staining. The histologic sections (5–7 µM) were collected on poly-L-lysine-coated slides and blocked with rabbit, goat, or rat serum followed by primary antibody conditioning for 40 minutes at room temperature and exposed to the following primary antibodies: FITC-conjugated DR1 (rat monoclonal anti-mouse, 2 µg/mL; Abcam, Cambridge, UK), DR2 (rabbit polyclonal anti-mouse, 1 µg/mL; Abcam), and mouse tyrosine hydroxylase (TH) (rat monoclonal anti-mouse, 2 µg/mL; ThermoFisher Scientific, Inc., Waltham, MA, USA). Antibodies were diluted 1:100 to 1:200, and samples were incubated overnight at 4°C in a dark room. After washing with Tris-buffered saline supplemented with Tween 20 (TBST), each section was exposed to secondary antibodies for 1 hour. After another wash with TBST, the sections were exposed to 4',6-diamidino-2-phenylindole (PureBlu; Bio-Rad, Hercules, CA, USA). Fluorescence microscopy and/or confocal microscopy (Axio Imager 2; Carl Zeiss, Oberkochen, Germany) were used to examine the samples.

Table. List of Designed mRNA Primers Used for qRT-PCR

Gene	Primer	Sequence (5′→3′)
DR1	Forward	GAG CAG GAC ATA CGC CAT TT
	Reverse	CCC TCT CCA AAG CTG AGA TG
DR2	Forward	GAT GTG CAC AGC AAG CAT CT
	Reverse	AGG ACA GGA CCC AGA CAA TG
DR3	Forward	CCC TCA GCA GTC TTC CTG TC
	Reverse	AAG AGA GGG TCC TGG CAT TT
DR4	Forward	GTG TGT TGG ACG CCT TTC TT
	Reverse	CGC GTT GAA GAT GGT GTA GA
DR5	Forward	CCA ATA CAA GCA GGG TGA G
	Reverse	AGG CAT GGG TAG CAT AGA
Tyrosine hydroxylase	Forward	CAG CTG GAG GAT GTG TCT CA
	Reverse	CAG CTG GAG GAT GTG TCT CA

Immunoblotting

Total protein concentrations of supernatant fractions were determined using a bicinchoninic acid protein assay (Bio-Rad). Equal amounts of protein aliquots were boiled in equal volumes of 2 × SDS Laemmli sample buffer and resolved on 8% or 10% (w/v) with the following primary antibodies: anti-TH (0.2 µg/mL; R&D Systems, Minneapolis, MN, USA) and anti–β-actin (2 µg/mL; Abcam).

ELISA

Commercialized ELISA was performed to measure the concentration of cytokines. Similarly, to measure the concentration of dopamine, a specific ELISA kit (Abnova, Taipei, Taiwan) was used. In addition, IFN-γ, IL-17, TNF-α (R&D Systems, Minneapolis, MN, USA), and IL-8 (ThermoFisher Scientific) levels were measured 14 days after DE induction, with or without DR blockers, in LGs. All experiments were repeated three times in at least triplicate.

Flow Cytometry

The flow cytometry strategy for LG cultures has been described previously.[25,26] Briefly, single-cell suspensions of four LGs from each condition were prepared by treating minced tissue fragments with 100 U/mL collagenase D and 15 µg/mL DNase (Sigma-Aldrich) for 40 minutes at

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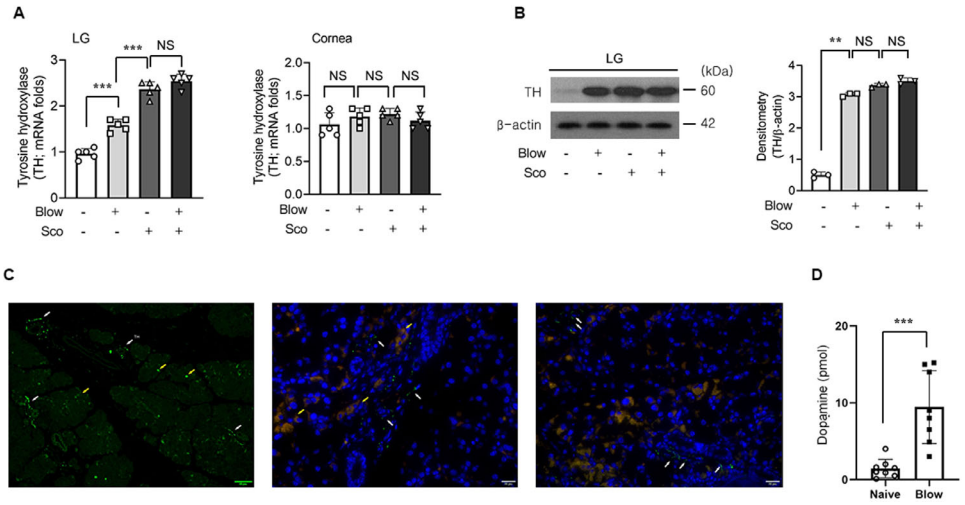

Figure 1. Expression of TH and dopamine in the LG and cornea in DE. (**A**) qRT-PCR analysis of TH mRNA expression levels in LGs and the cornea 14 days after air blowing only or scopolamine (Sco) injection ($n = 5$, one-way ANOVA and Bonferroni-corrected Dunnett test for post hoc analysis). *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. NS, not significant. (**B**, **C**) Representative immunoblotting assay (**B**) and immunofluorescence staining (**C**) for TH (*white arrows*: *green*) in DE-induced LGs. Autofluoresceins were marked with *brown* (*yellow arrows*). *Bar*: 50 µM. All data were retrieved from two independent experiments in triplicate in each group ($n = 5$, Mann–Whitney *U* test). *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. NS, not significant. (**D**) Using enzyme-linked immunosorbent assay, the concentration of dopamine was measured in LGs 7 days after DE induction ($n = 5$, Mann–Whitney *U* test). ***$P < 0.001$.

37°C. After blocking with 1 µg unlabeled anti-FcrR antibody (clone 2.4G2), cells (1×10^6) were washed with RPMI 1640 and surface-stained with PE-Cy5–conjugated anti-CD3, FITC-conjugated anti-CD4, and PE-conjugated anti-CD8 (eBioscience, San Diego, CA, USA), and the cells were analyzed using flow cytometry and fluorescence-activated cell sorting (FACS) (FACS Calibur; Becton-Dickinson, Mountain View, CA, USA).

Statistical Analysis

Continuous variables were examined for normality using the Kolmogorov–Smirnov test. Variables that conformed to normal distribution were presented as mean ± SD, and those that were not normally distributed were presented as median ± interquartile range. For two-sample analyses, the Mann–Whitney *U* test was used. The Kruskal–Wallis test was used to compare three or more groups. Bonferroni-corrected Dunnett procedure, as a post hoc analysis, was also used to compare each treated group and the control group. Statistical analysis was performed using SAS software (version 9.13; SAS Institute, Cary, NC, USA) and R (version 3.2.5; Statistics and Mathematics, Vienna, Austria). $P < 0.05$ was considered significant.

Results

Expression of TH and Dopamine in DE-Induced LGs and Cornea

First, the mRNA expression of the dopamine induction enzyme TH was measured in the LGs and cornea after DE induction. Interestingly, TH level significantly increased in LGs after DE induction by blowing air and increased even more on combining blowing air with the administration of scopolamine (Fig. 1A). However, TH expression was the same with or without scopolamine injection with air-blowing stimulus in the cornea. To determine the protein level of TH in LGs, immunoblotting for TH was performed, and upregulation of TH in the air-blowing condition was found compared to the naive status (Fig. 1B). However, we did not find a TH protein-level difference between scopolamine injection and blow-only conditions despite the difference in mRNA levels. Confocal microscopy showed that TH was only present along the nerve leashes located between the acinar cells or excretory ducts (Fig. 1C), which is in accordance with the results of a previous study.[10]

Then, we measured the concentration of dopamine in the LGs that was significantly elevated from 7 days after DE induction and continued until 14 days. There was no difference in the level of dopamine between days 7 (Fig. 1D) and 14 after DE induction (data not shown).

Enhanced Dopamine Receptor Expression in DE-Induced LGs

Although the expression of DR1 in the corneal epithelium has been previously reported in humans,[27] mice,[28] and rabbits,[29] there have been few reports on the dopaminergic receptor system in the LGs. Therefore, we measured the level of DRs using qRT-PCR in LGs as well as the cornea. Interestingly, expression of the subtypes of these receptors was quite different. In LGs, DR1 and DR2 expression was significantly upregulated under air-blowing conditions. In the cornea, DR1 significantly increased under air-blowing conditions (Fig. 2A); however, DR2 mRNA was significantly downregulated by air blowing. When the locomotive expression of DR1 and DR2 was compared, DR2 expression was found to be much higher in LGs than in the cornea. However, DR1 expression was dominant in the cornea compared to that in LGs. DR3 and DR4 expression did not change after DE induction in the cornea; however, a slight upregulation of DR3 expression was found in LGs ($P = 0.033$). DR5 mRNA expression was not enhanced in LGs under air-blowing conditions, despite the upregulation of DR1 expression (Fig. 2A).

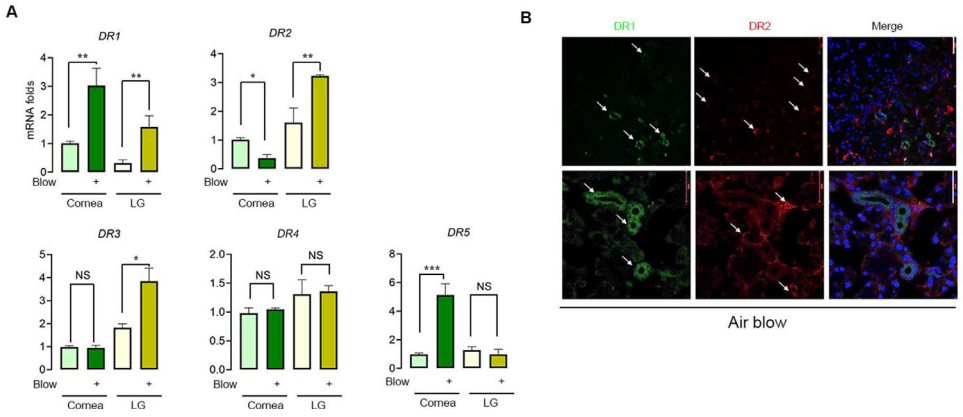

Figure 2. Expression of DR subtypes in DE-induced LGs. (**A**) qRT-PCR analysis of DR subtype mRNA expression levels in the cornea and LGs 14 days after air blowing. (**B**) Representative confocal microscopic findings for DR1 and DR2 (*white arrows*) immunostaining in LGs after 14 days of DE induction ($n = 5$, Mann–Whitney U test). $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$. NS, not significant. *Bar*: 50 µM.

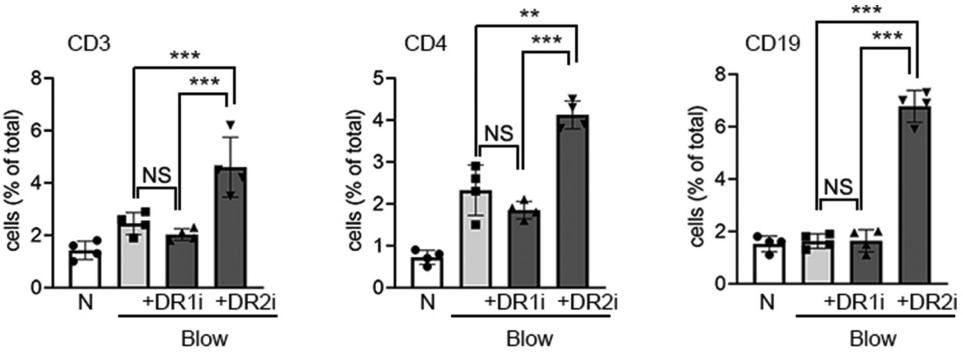

Figure 3. Determining leukocyte infiltrations in DE-induced lacrimal glands by neutralizing dopaminergic receptors DR1/DR2. Flow cytometry of CD3-, CD4-, and CD19-expressing cells 14 days after DE induction in LGs with or without DR1 (DR1i: SCH-23390, 0.3 mg/kg) or DR2 inhibitor (DR2i: melperone, 2 mg/kg) treatment ($n = 5$, one-way ANOVA and Bonferroni-corrected Dunnett test for post hoc analysis). $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$. N, naive condition.

With confocal microscopy examination of the receptor expression in LGs, we found that the expression pattern did not overlap between DR1 and DR2. Both receptors were expressed in different acinar cells. Under normal conditions, the receptors were not found in the LG acinar cells. However, with DE induction, the receptors were highly expressed in some acinar and tubular epithelial cells of the LGs (Fig. 2B).

Increased Leukocyte Infiltration in LGs by Neutralizing Dopaminergic Receptors DR1/DR2

The expression of LG inflammatory cytokines and number of cells were investigated under receptor neutralizing conditions to determine the functional role of DR subtypes in DE. T- and B-cell levels were significantly increased in LGs after DE induction, as we reported previously.[23–25] Surprisingly, dopaminergic receptor blockade significantly increased the percentage of infiltrating cells. Furthermore, all levels of three cell types that were investigated ($CD3^{+}$, $CD4^{+}$, and $CD19^{+}$) were significantly increased only via the inhibition of DR2 and not that of DR1 (Fig. 3). The mean percentage of $CD3^{+}$ and $CD4^{+}$ cells in DE increased by 2.4- and 1.9-fold after DR2 blocking, respectively. For $CD19^{+}$ cells, the cell percentage was increased by 3.2-fold in DE with DR2 inhibition (DR2i).

Next, we measured the levels of several DE-related key cytokines, such as IFN-γ, IL-17, IL-8, and TNF-α. All cytokines were significantly elevated after DE induction, which was accelerated by DR2i (Fig. 4). The expression of IFN-γ was similar between DR1 inhibition (DR1i) and air blowing conditions. However, IFN-γ expression was significantly upregulated after DR2i. Similar to IFN-γ, IL-17 and IL-8 levels were significantly higher only after DR2i under air-blowing conditions (Fig. 4). However, TNF-α expression after DR2i was similar to that after DR1i, despite the increased expression by air blowing. Besides the level of IL-17 ($P < 0.001$), when both receptors were inhibited simultaneously, no cytokine expression levels were synergistically upregulated, and these were similar to those observed after DR2i.

Enhanced Ocular Surface Inflammatory Conditions by Neutralizing Dopaminergic Receptor DR2

From the above results, we found that DRs had a role in the downregulation of the inflammatory status of LGs. Next,

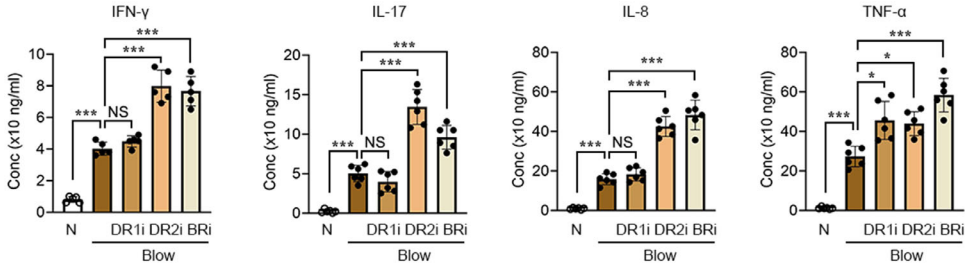

Figure 4. Measurement of inflammatory cytokine levels in DE-induced LGs using ELISA. IFN-γ, IL-17, IL-8, and TNF-α expression levels in LGs were measured 14 days after DE induction with or without dopamine receptor inhibitors. BRi, both receptors inhibited; DR1i, SCH-23390; DR2i, melperone; N, naive condition. All experiments were repeated three times in at least triplicate ($n = 5$, one-way ANOVA and Bonferroni-corrected Dunnett test for post hoc analysis). *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. NS, not significant.

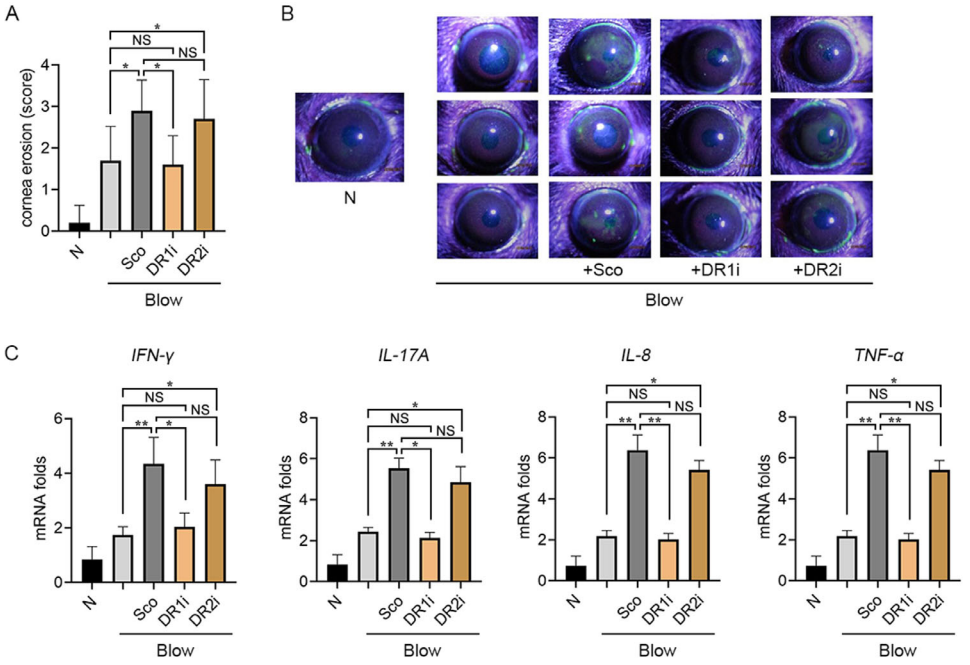

Figure 5. Ocular surface inflammatory conditions via dopaminergic receptor blocker treatment. (**A**, **B**) Mean corneal erosion scores (**A**) and representative corneal photo (**B**) of each treatment group 14 days after DE induction. (**C**) qRT-PCR analysis of each cytokine in the DE-induced corneas. BRi, both receptors inhibited; DR1i, DR1 blocker (SCH-23390); DR2i, DR2 blocker (melperone); N, naive condition; Sco, scopolamine injection. All experiments were repeated three times and analyzed in at least triplicate ($n = 5$, one-way ANOVA and Bonferroni-corrected Dunnett test for post hoc analysis). *$P < 0.05$. **$P < 0.01$. NS, not significant.

the inflammatory status of the ocular surface was evaluated by neutralizing the dopaminergic system. Surprisingly, the corneal erosion score was significantly increased after DR2i treatment for DE, similar to that of the scopolamine-treated condition (Figs. 5A, 5B). However, after DR1i treatment, it was similar to that under air-blowing-only conditions in DE. Next, the ocular surface cytokine levels were measured using qRT-PCR. As observed in LGs, IFN-γ and IL-17 levels were significantly elevated under air-blowing conditions and after DR2i. However, DR1i did not significantly increase the expression of inflammatory cytokines (Fig. 5C). Interestingly, the expression of IFN-γ and IL-17A was comparable between scopolamine- and DR2i-treated mice.

Discussion

In this in vivo study, both the mRNA and protein levels of TH were found to be upregulated in DE-induced LGs. Additionally, the dopamine concentration and DR2 expression in LGs were significantly upregulated. Systemic blocking of DR2 enhanced lymphocyte infiltration and upregulated cytokine expression in LGs, subsequently aggravating the ocular surface inflammatory conditions in DE disease. Therefore, not only the cholinergic system but also the dopaminergic receptor, especially DR2, may regulate the inflammatory status of LGs in DE disease.

Dopamine was first identified in human tears several decades ago.[30] In addition, the enzymes involved in

dopamine metabolism (e.g., TH and dopamine-β-hydroxylase) have been found in LGs and the ocular adnexa of mice,[31,32] rats,[11] rabbits,[33] monkeys,[34,35] and humans.[8] However, there are sparse data available regarding the pathophysiologic role of the dopaminergic system in DE disease. Moreover, most previous studies have focused on the sympathetic or other monoamine neural systems (e.g., substance P) to determine the anatomic location and functional role in the ocular system and DE disease. However, dopamine is not only a tyrosine metabolite, key to producing adrenaline, but also an active neurotransmitter, thus playing a significant role in several neurodegenerative diseases. Therefore, we investigated the role of dopamine in DE disease pathophysiology by focusing on the LGs.

As in Parkinson disease, a neurodegenerative disease of the CNS, DE disease also has a neuroinflammatory component explained by the dysfunctional neural regulation and enhanced inflammatory conditions on the ocular surface with aging. As TH activity is the "rate-limiting step" for dopamine and norepinephrine production, we first determined TH expression in LGs through mRNA expression levels, immunoblotting, and IF staining. Interestingly, TH mRNA expression was gradually enhanced under both air-blowing and scopolamine injection conditions. However, at the protein level, we could not determine the difference between air blowing and scopolamine injection despite the upregulation of TH compared to the naive condition. These results may indicate that the air-blowing stress is sufficient for maximum TH induction. Furthermore, cholinergic blocking by scopolamine did not affect TH expression (Figs. 1A, 1B), which indicated that there was no reciprocal or interfering neural regulation between the cholinergic and dopaminergic systems under the DE disease-mimicking air-blowing condition. As mentioned above, to find a common pathway for the neural regulation between neurodegenerative diseases and DE disease, we expected a reciprocal regulation between the cholinergic and dopaminergic systems in DE disease. However, at least in the murine model, TH expression levels were independently regulated and not affected by the muscarinic receptor blocker scopolamine.

Another interesting finding in the dopaminergic system in DE was dopaminergic receptor subtype expression. Under basal, non-DE stress conditions, DR1 and DR2 expression were not observed with confocal microscopy. However, after DE induction, the mRNA levels of both receptors were enhanced in the LGs (Fig. 2A). Interestingly, confocal microscopy revealed that DR1 was mainly expressed in the secretory ducts and the portal area; however, DR2 was mainly located in the acinar cells. Additionally, DR1 mRNA expression levels were much higher in the cornea than in the LGs and increased both on the ocular surface and in the LGs synchronously in response to DE stress. In contrast, DR2 expression was much higher in LGs than in the cornea and was upregulated only in LGs (Figs. 2A, 2B). Although further detailed studies are needed, these results imply that the upregulation of DR2 is more sensitive and responsible for neural regulation in DE-stressed LGs. In addition, considering the localization pattern of DR1 and DR2 in LGs, the latter located in the acinar, DR2 might have a more direct role in aqueous tear and protein production.

To determine the functional role of DR subtypes, we used melperone and SCH-23390 to block DR2 and DR1, respectively. The functional roles of DR1 and DR2 receptors are usually contradictory, and DR1 and DR5 are DR1-like subtypes, while the DR2-like subtypes include DR2, DR3, and DR4.[36,37] Therefore, the effect of SCH-23390 might mimic the DR1-like subtype blocking effect. Similarly, melperone might block all DR2-like subtype receptors as well as histamine receptors (e.g., 5-HA_{2A}).[38,39] As a result of a simple neural blocking in vivo study, DR2i showed a significant elevation of inflammatory cytokine expression and cell numbers. Although the levels of IL-17A, TNF-α, and IL-8 were significantly elevated compared with those of IFN-γ, dopaminergic and cholinergic systems might not be responsible for Th1 or Th17 responses, respectively, and could have specific functional roles in enhancing the inflammatory response. Nonetheless, these systems may regulate DE-induced inflammatory responses through their receptor-specific systems. This might be plausible, especially when previous studies have shown enhanced IFN-γ and IL-17 expression using a muscarinic blocking agent with or without air-blowing stress.[40]

The dopaminergic neurons regulate not only the LG inflammatory status but also ocular surface inflammation. After blocking DR1 or DR2, the erosion score was significantly elevated (Figs. 5A, 5B), although DR2 blocking increased the scores more significantly. Interestingly, the erosion score and inflammatory cytokine expression levels were not different between the scopolamine and DR2i groups. These results might indicate a close interaction between cholinergic and dopaminergic systems, suggesting a "common" and not an "independent" or "synergistic" neural regulation in DE disease pathophysiology. However, we did not identify a synergy between cholinergic and dopaminergic systems (data not shown) in the DE disease inflammatory response in either the LGs or the cornea. In addition, considering the topographic patterns and different levels of inflammation between DR1 and DR2 in the LGs (Fig. 2B), both receptors might be independently involved in DE-induced inflammatory responses.

This study has several limitations. For identifying the source of dopamine secretion, we relied on the staining pattern and concluded that TH only existed on the neural leashes in the LGs. However, TH is found in many types of inflammatory cells, especially T and dendritic cells.[41,42] Considering inflammatory T and myeloid cell infiltration in DE disease, the source of dopamine might be more diverse and complicated in DE-induced LGs. Moreover, we did not investigate dopamine metabolism by measuring levels of dopamine β-hydroxylase that converts dopamine to norepinephrine. Although the sympathetic system is an essential system for tear production,[10,31] we focused on dopamine induction and receptor expression in LGs in this study. The activity of dopamine β-hydroxylase and the sympathetic system should be investigated in a collaborative manner with the dopaminergic and cholinergic systems. Last, human dopaminergic receptors might be expressed differently from those of mice, whether in healthy or diseased states. Thus, a study using human LG samples is needed in the future and is essential to understand the specific neural regulation of DE disease.

In conclusion, we found that the dopaminergic neural system played a significant role in DE pathophysiology, similar to the cholinergic system. However, the neural regulation between both systems appears to be extremely complicated and does not involve collaborative work as a reciprocal role in DE disease. Further in vitro studies with human samples may provide more precise data for the role of the dopaminergic neural system in LG inflammation and the collaborative role of DRs with muscarinic receptors and thus

reveal a target to treat dysfunctional neural regulation in DE disease.

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